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Intrahost evolution of HIV-1 phenotypes

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ACADEMIC THESIS

which by due permission of the Faculty of Medicine at Lund University,
will be publicly defended in Segerfalksalen, Wallenberg Neurocentrum, BMC, Sölvegatan 17, Lund,
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Intrahost evolution of HIV-1 phenotypes

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Lund 2012

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-IV):

I **Marie Borggren**, Johanna Repits, Carlotta Kuylenstierna, Jasminka Sterjovski, Melissa J Churchill, Damian FJ Purcell, Anders Karlsson, Jan Albert, Paul R Gorry, Marianne Jansson. *Evolution of DC-SIGN use revealed by fitness studies of R5 HIV-1 variants emerging during AIDS progression* Retrovirology, 5:28, 2008

II **Marie Borggren**[*], Johanna Repits[*], Jasminka Sterjovski, Hannes Uchtenhagen, Melissa J Churchill, Anders Karlsson, Jan Albert, Adnane Achour, Paul R Gorry, Eva Maria Fenyö and Marianne Jansson. *Increased Sensitivity to Broadly Neutralizing Antibodies of End-stage Disease R5 HIV-1 Correlates with Evolution in Env Glycosylation and Charge* PLoS One, 6(6):e20135, 2011 *These authors contributed equally to this work.

III **Marie Borggren,** Mia Eriksson, Joakim Esbjörnsson, Anders Karlsson, Jan Albert, Eva Maria Fenyö, Patrik Medstrand, Marianne Jansson. *CXCR4-using HIV-1 emerging after coreceptor switch further evolves toward increased infectivity* Manuscript

IV **Marie Borggren,** Lars Navér, Charlotte Casper, Anneka Ehrnst, Marianne Jansson. *HIV-1 of R5 phenotype detected early after birth in vertically infected children displays reduced DC-SIGN use* Manuscript

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Abbreviations

aa	Amino acid
AIDS	Acquired immunodeficiency syndrome
AZT	Zidovudine
C1-C5	Constant region 1 to 5 in gp120
CD4	Cluster of differentiation 4
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
DC	Dendritic cell
DC-SIGN	Dendritic cell specific ICAM-3 grabbing non-integrin
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immuno sorbent assay
env	Envelope gene
Env	Envelope glycoprotein gp120/gp41 trimer
gag	Group antigen gene
GALT	Gut-associated lymphoid tissue
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus type 1
HR	Heptad repeat
HTLV	Human T-cell leukemia virus
IC50	Inhibitory concentration 50%
ICAM	Intercellular adhesion molecule
kb	Kilobases
LTR	Long terminal repeat
mAb	Monoclonal antibody
MHC	Major histocompability complex
MPER	Membrane proximal external region
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PNGS	Potential N-linked glycosylation site

pol	Polymerase gene
R5 HIV-1	Exclusively CCR5-using HIV-1
R5X4 HIV-1	CCR5 and CXCR4-using HIV-1
RER	Rough endoplasmic reticulum
RANTES	Regulated on activation, normal T-cell expressed, and secreted
RNA	Ribonucleic acid
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
V1-V5	variable region 1 to 5 in gp120
X4 HIV-1	Exclusively CXCR4-using HIV-1

Aims of this thesis

The overall aim of this thesis was to study how HIV-1 phenotype evolves and changes within the patient along with disease progression. Both biological changes and Env molecular modifications were examined.

Paper I: To study how the R5 HIV-1 evolves in regard to DC-SIGN binding and use, and investigate molecular mechanisms to explain these changes.

Paper II: To investigate if R5 HIV-1 sensitivity to broadly neutralizing antibodies evolves and correlate this to molecular Env modifications.

Paper III: To analyse how CXCR4-using HIV-1, emerging after coreceptor switch, evolves late in disease and relate the phenotypic evolution to molecular alterations of Env.

Paper IV: To examine DC-SIGN use of R5 HIV-1 during vertical transmission, comparing maternal virus with virus outgrowing in the newly infected child and the development of DC-SIGN use during disease progression in the child.

Summary

HIV-1 evolves constantly within an infected individual, due to its mutation-prone viral enzyme, high viral turnover and pressure from the host immune system. Therefore, viruses isolated at different time points from the same individual are never exactly the same and, accordingly, rarely function the same way. However, if we can understand how HIV-1 phenotypically evolves in the newly infected host and during disease progression, we may develop better therapeutics and perhaps halt the spread of the virus.

This thesis is based on studies in which we have investigated how HIV-1 phenotypically evolves within infected individuals. We studied viruses emerging in infected adults, during late stage disease, and in vertically infected children, from shortly after birth until immunodeficiency. Some patients maintained viruses that exclusively used CC chemokine receptor 5 (CCR5) as coreceptor, R5 HIV-1, throughout the infection. Others had viruses whose coreceptor use was altered to include CXC chemokine receptor 4 (CXCR4). We analyzed sequentially obtained viruses from both groups of patients and studied phenotypic features in relation to molecular alterations in the viral envelope glycoproteins (Env).

We found that the virus evolution at late stage disease toward increased infectivity and replicative capacity was fairly similar within patients harboring R5 or CXCR4-using HIV-1. The R5 HIV-1 also showed a decrease in *trans*-infection ability, mediated by the C-type lectin DC-SIGN, at end-stage disease. In addition, end-stage R5 HIV-1 were more sensitivity to certain broadly neutralizing antibodies. Furthermore, phenotypic alterations correlated with the decline in CD4+ T cell count during development of immunodeficiency. The observed evolution in phenotypic features also correlated with molecular alterations of the viral envelope glycoprotein gp120, with an increase in net positive charge and a loss of potential N-linked glycosylation sites (PNGS) at the end-stage of the disease. In addition, the efficiency of HIV-1 DC-SIGN use correlated with the presence of a specific glycan site in gp120.

Studies on R5 HIV-1 from vertically infected children and their mothers demonstrated that efficient use of DC-SIGN for *trans*-infection do not appear to be a benefit for newly transmitted virus variants. Instead, the efficiency of virus DC-SIGN use increased during disease progression, from early after birth until immunodeficiency.

These studies reveal that the phenotypes of R5 and CXCR4-using HIV-1 may evolve in an adaptive manner during disease progression and transmission.

Sammanfattning på svenska

Det är snart 30 år sedan HIV, humant immunbristvirus, identifierades som orsaken till AIDS och det finns fortfarande ingen botande medicin eller ett profylaktiskt vaccin. Tidigt efter virusets upptäckt fanns höga förhoppningar om att ett vaccin eller ett botemedel snart skulle vara utvecklat. Idag vet vi att det är långt kvar tills detta är verklighet. För att komma dit behöver vi veta mer om viruset, hur det fungerar och hur det utvecklas.

I en infekterad patient pågår en konstant kamp mellan kroppens immunförsvar och viruset. Från det att en individ infekteras och nya viruspartiklar börjar sprida sig i kroppen attackerar immunförsvaret viruset, som i sin tur hela tiden smiter undan genom att gömma, förändra och snabbt föröka sig. Då HIV infekterar viktiga immunceller, kommer immunsystemet till slut att utarmas, vilket leder till en kollaps av immunförsvaret. Viruset får då fritt spelrum, samtidigt som kroppen inte kan försvara sig mot, i normala fall, ofarliga infektioner, så kallade opportunistiska infektioner. Vid det stadiet i sjukdomen har AIDS utvecklats.

För att HIV ska kunna infektera en cell krävs två molekyler, så kallade receptorer, på cellytan. Den primära receptorn är CD4 och den andra receptorn, coreceptorn, är antingen CCR5 eller CXCR4. Dessa receptorer är i vanliga fall involverade i immunsystemet som känner igen smittämnen och eliminerar dessa från vår kropp. När HIV binder till dessa receptorer så tar sig viruset in i värdcellen och inkorporerar sin arvsmassa i värdcellens arvsmassa. Där kan viruset sitta under längre eller kortare tid för att sen producera mängder med nya partiklar när värdcellen aktiveras. Virus som använder CD4 och CCR5 är vanligast i början av infektionen och finns ofta kvar under hela sjukdomen. Virus som använder CXCR4 istället för, eller samtidigt som, CCR5 utvecklas hos en del patienter under senare delen av sjukdomsförloppet.

Vi har studerat hur virus utvecklas under den senare delen av sjukdomsförloppet, antingen hos patienter med virus som bara använder CCR5 eller hos patienter med virus som har utvecklats att också använda CXCR4. Genom att isolera virus vid olika tidpunkter från enskilda patienter, har vi studerat hur viruset förändrar sig funktionellt med avseende på olika typer av infektioner i cellkulturer. Våra resultat visade att virus som har isolerats från patienter i sent AIDS skede är mer infektiösa och växer snabbare vid direkt infektion av värdceller än virus från den kroniska fasen hos samma patient. Vi fann också att CCR5-beroende virus isolerade i AIDS-stadiet var mer känsliga för vissa typer av neutraliserande, det vill säga infektionsblockerande, antikroppar.

Dessa biologiska förändringar hos virus, det vill säga ökande infektivitet och känslighet för antikroppar, fann vi uppkom parallellt med förändringar i ett av virusets höljeproteiner, gp120. Vi fann att ju mer infektiöst och känsligt för neutralisation virus var, desto mindre sockermolekyler fanns det på gp120 och laddningen på gp120 var mer positiv.

Vi undersökte också ifall virus använde DC-SIGN receptorn för effektivare infektion av värdceller. DC-SIGN är en receptor på antigen-presenterande celler, som i vanliga fall bidrar till att immunförsvaret känner igen främmande mikrober. HIV verkar dock ha utvecklat sätt att utnyttja DC-SIGN, genom att binda till receptorn utan att inaktiveras. Istället ackumuleras infektiösa HIV partiklar på den antigenpresenterande cellens yta som effektivt kan sprida sig till värdceller som uttrycker CD4 och CCR5/CXCR4, i en så kallad *trans*-infektion. När vi studerade denna typ av *trans*-infektion såg vi att CCR5-beroende virus från AIDS-stadiet var sämre på att använda DC-SIGN. Virus med effektiv DC-SIGN-användning hade i större utsträckning gp120 med en specifik sockermolekyl, jämfört med virus som inte lika effektivt kunde utnyttja DC-SIGN.

DC-SIGN har föreslagits vara en inkörsport för virus vid infektion av en ny individ, eftersom denna receptor uttrycks i vävnader där den primära HIV kontakten sker. Vi undersökte även hur DC-SIGN används av virus som smittar över från mor till barn under graviditet eller vid födelsen. Vi noterade att effektiv DC-SIGN-användning inte verkade vara någon fördel för virus som smittar mellan mor och barn. Istället utvecklades virus under barnets senare sjukdomsförlopp med bättre DC-SIGN användning.

Våra resultat visar att HIV-1 förändras och selekteras under sjukdomsförloppet, vilket troligtvis beror på immunförsvarets förmåga att attackera viruspopulationen. Virus från den kroniska fasen av sjukdomen, när immunförsvaret fortfarande är relativt funktionellt, är bra på att gömma sig från neutraliserande antikroppar, till exempel genom att bygga på höljeproteinets skyddande sockerlager. Dessa virus kan dessutom använda alternativa infektionsvägar, så som *trans*-infektion via DC-SIGN. När sen immunförsvaret försvagas kan virus fritt utvecklas till att bli mer infektiöst samtidigt som det inte på samma sätt behöver gömma sig för immunsystemet.

Vi hoppas att våra resultat och slutsatser kan hjälpa till att bättre förstå hur virus utvecklas inom patienten vid olika sjukdomsstadier. Denna kunskap kan förhoppningsvis också leda till bättre behandlingsmetoder och framtida utveckling av HIV-förebyggande strategier.

Introduction

The HIV-1 pandemic

The first cases of acquired immune deficiency syndrome (AIDS) were reported in 1981, when a few young men in New York and California suddenly displayed rare diseases typical of immunodeficiency, such as an aggressive form of Kaposi's Sarcoma and a rare lung infection, Pneumocystis carinii pneumonia[1, 2]. At first, it was thought that this disease only affected the homosexual community, but it was soon clear that other groups were also affected[3]. By the end of 1981, there were also reports of cases in Europe[4]. In 1982, the disease was denoted as AIDS, as the previous name of GRID, gay-related immune deficiency, was no longer appropriate. More people began taking notice of this new disease because it was then clear that a much wider group of people could be affected. Public anxiety grew because very little was known about transmission. There were many theories of what caused AIDS, such as fungi, chemicals or autoimmunity to leukocytes. Two different laboratories in the United States (U.S.) and France had the same principal idea, believing that a retrovirus caused AIDS. This idea was based on previous findings that a retrovirus called HTLV, which causes an unusual T-cell leukemia, seemed similar in many aspects to the agent causing the new disease[5]. The search for a retrovirus in AIDS patients started, and in May 1983, Luc Montagnier and Francoise Barré-Sinoussi of the Pasteur Institute in Paris reported that they had isolated a new virus that they suggested to be the cause of AIDS[6]. Soon there after, reports from the U.S. confirmed the finding[7, 8], and AIDS was established to be the consequence of a new retrovirus that, in 1986, was given the name human immunodeficiency virus, HIV[9]. After the initial discovery of HIV, successful research on the virus and the disease followed very rapidly[10]. In just two years, between 1984 and 1985, the viral genome was sequenced, genes and proteins defined, target cells revealed and the major transmission routes revealed. A similar virus causing AIDS in nonhuman primates of Asian origin, simian immunodeficiency virus (SIV) was isolated and could be used in animal models. A blood test for the detection of viral antibodies became available in 1985, and the development of the first therapy based on zidovudine (AZT), began soon after[5]. Despite extensive research, the pandemic grew rapidly and soon became a huge global disease, especially in sub-Saharan Africa. In 1986, a second virus with a close relationship to HIV was identified in West African individuals[11]. The two types of virus were closely related but distinct and were thus called HIV-1 and HIV-2, where HIV-1 is responsible for the pandemic, and HIV-2 is mainly found

in West Africa. HIV-2 proved to be a less pathogenic virus than HIV-1, with a lower transmission rate.

Today, more than 30 million people are living with HIV, and more than 30 million have died from AIDS-related causes[12]. As for some more positive news, the overall incidence of new infections has decreased by approximately 20% over the last ten years, perhaps due to the introduction of therapy and prevention efforts. This trend is seen in Africa, where most HIV-infected individuals still live, and eastern Asia. However, the infection rate has instead continued to increase in Eastern Europe and central Asia, but now seems to have stabilized[12].

Origin of HIV-1

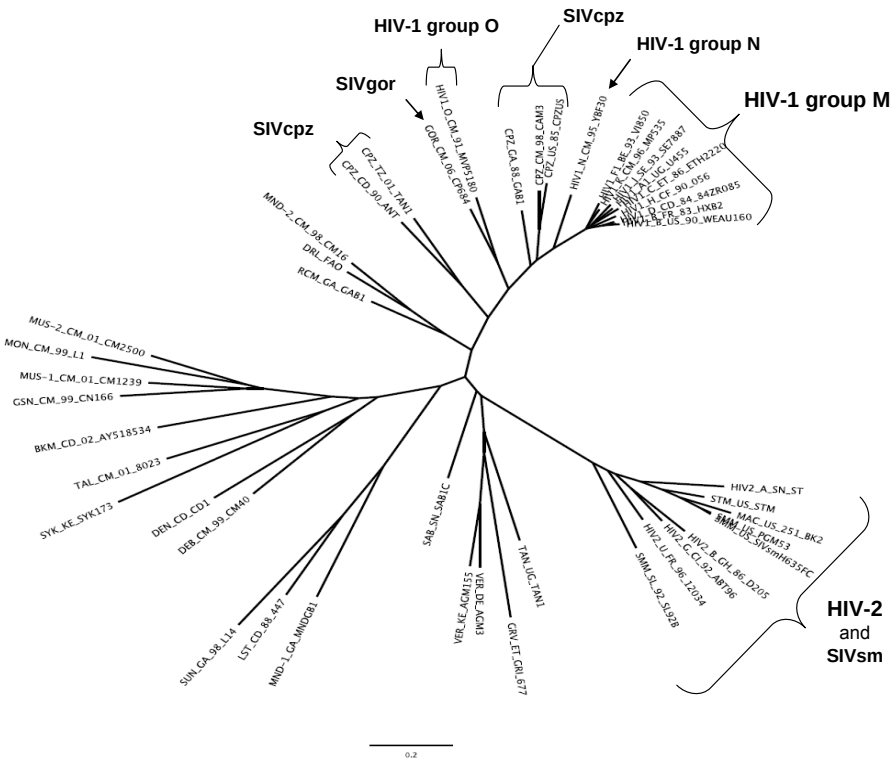

Figure 1. Evolutionary relationship between HIV and SIV. Phylogenetic tree based on the *pol* gene, demonstrating how HIV-1 is closest related to SIV found in chimpanzees and gorillas, whereas HIV-2 is related to SIV found in sooty mangabey. Kindly provided by Helena Skar and Salma Nowroozalizadeh.

Even though the HIV-1 pandemic was identified as late as in the 1980s, there are reports of earlier cases[13-15] that can help us to trace the origin of HIV to between end of the 19th century and beginning of the 20th century[14]. During this time period, HIV emerged from its ancestor SIV, of which different types are spread among African monkeys and which is believed to be at least 32,000 years old[16]. HIV-1 has its origin from SIV found in chimpanzees and gorillas, and HIV-2 originated from SIV found in sooty mangabey monkeys (Figure 1). Zoonosis of the viruses from monkeys to humans has likely occurred through the killing and eating of monkeys. The crossover of SIV to humans has occurred several times and resulted in different groups of HIV-1 (groups M, N and O), where group M, further divided into several subtypes, has caused the pandemic spread. Research on wild chimpanzees has shown that the most likely first transfer to humans occurred in Southern Cameroon[17], but the establishment of the infection was identified in Kinshasa, in the Democratic Republic of Congo. This geographic difference may be due to the travel of infected individuals between the two locations. Travel, domestic and international, is probably the major cause of the widespread pandemic we see today. There are reports tracing the infection from Africa to Haiti around 1966, and from there it was brought into the U.S. around 1969[18].

The HIV-1 genome

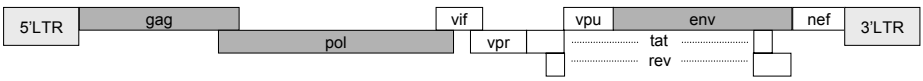

Figure 2. Genome organization of HIV-1. The HIV-1 genome is composed of two identical single strands of RNA, of approximately 10 kb.

HIV-1 is a lentivirus that belongs to the family of retroviruses. The term "lentivirus" means "slow virus," which refers to a long incubation time in the host. Lentiviruses have been found in many different animals, such as cats (feline immunodeficiency virus), sheep (visna virus), goats (caprine arthritis encephalitis virus) and horses (equine infectious anemia virus). All retroviruses have their genetic material in the form of RNA and they posses the ability to perform retrograde flow of information, meaning RNA → DNA mediated by the viral enzyme reverse transcriptase (RT). The HIV-1 genome is composed of three genes coding for structural proteins (existing in all replication competent retroviruses) and six genes encoding auxiliary proteins (extra genes in lentiviruses) (Figure 2). The long terminal repeats, LTRs, flanking both sides of the genome, work as promoters for cell-specific transcription activators. When no activator is bound,

the transcription level is very low. However, when the host cell is activated, as during T-cell stimulation, the transcription of the viral genome is initiated by cellular transcription factors[19, 20].

Table 1. HIV-1 genes and gene products[19, 20].

Gene	Protein	Function
Structural		
gag	p17	Matrix protein, interacts with gp41
	p24	Core protein
	p6	Core protein, bind Vpr
	p7	Nucleocapsid, bind to RNA
pol	Protease	Proteolytic cleavage of Gag and Pol
	RT	Polymerase and RNase H activity
	IN	DNA provirus integration into host genome
env	Gp120	Surface envelope protein, receptor binding
	Gp41	Transmembrane protein, cell fusion
Regulatory		
tat	Trans-activator of transcription	Positive regulator of LTR-driven transcription
rev	Regulator of expression of virion protein	Allows export of unspliced and partly spliced mRNA from nucleus
Accessory		
vif	Virion infectivity protein	Disrupts antiviral activity by cellular APOBEC
vpr	Viral protein R	Transport of DNA to nucleus, cell cycle arrest, enhance viral replication
vpu	Viral protein U	Downregulates CD4 surface expression, enhance virion release from cell membrane
nef	Negative regulatory factor	Decrease CD4 and MHC class I expression, alters viral replication

The structural genes *gag*, *po*l and *env* are all translated into precursor proteins, which are cleaved into several products. The *gag* gene will give rise to the matrix proteins, capsid proteins and nucleoproteins. The *pol* gene codes for three enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene encodes the precursor envelope glycoprotein gp160, which is processed by cellular enzymes to gp120, the outer envelope glycoprotein, and gp41, a transmembrane envelope glycoprotein that noncovalently attaches gp120 to the virus envelope. Of the six HIV-1 auxiliary genes, two give rise to regulatory proteins, Tat and Rev, which are crucial for viral replication. The remaining four HIV-1 auxiliary genes produce accessory proteins, Nef, Vif, Vpr and Vpu, which enhance viral replication and help the virus to evade immune defense. The HIV-1 genes and the functions of their products are summarized in Table 1[19, 20].

HIV-1 structure

The virus particle is composed of two identical single positive RNA strands (Figure 3). Within the viral core, in close association with the genome, the viral enzymes integrase and reverse transcriptase are found. The p7 nucleoprotein binds tightly to the RNA genome and attaches the genome to the capsid protein p24. The p24 capsid proteins make up the viral core, which also includes viral protease and the remaining accessory viral proteins. Detection of p24 and RT are used in *in vitro* assays to determine virus quantity (see *Materials and methods* section). A layer of matrix proteins consisting of p17 is found surrounding the capsid. p17 anchors to one of the virus envelope glycoproteins, namely gp41. Gp41 binds to the second glycoprotein, gp120, which is located on the outside of the virus particle. Gp41 and gp120 are assembled into trimers[19, 20]. HIV-1 is an enveloped virus, surrounded by a membrane that forms around the capsid during budding from the infected cell membrane. Thus, from the outside, the virus looks like any host object, except for the viral envelope glycoproteins that are embedded in the membrane. The viral envelope glycoprotein trimers, also known as Env, are often described as spikes protruding from the surface and published studies show a range of four to 35 spikes per virus particle[21-25]. Increasing numbers of spikes per HIV-1 particle have been shown to correlate with enhanced infectivity of the virus[26].

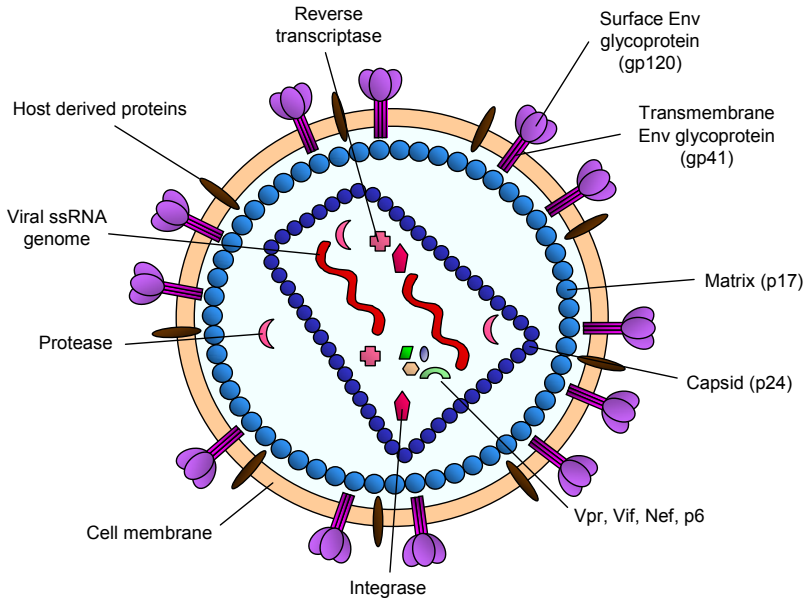

Figure 3. Structure of HIV-1. Kindly provided by Salma Nowroozalizadeh with modifications.

Env structure

Gp120

The envelope glycoproteins are crucial in the virus replication cycle and, at the same time, a vulnerable site for the host immune system to recognize and attack the virus.

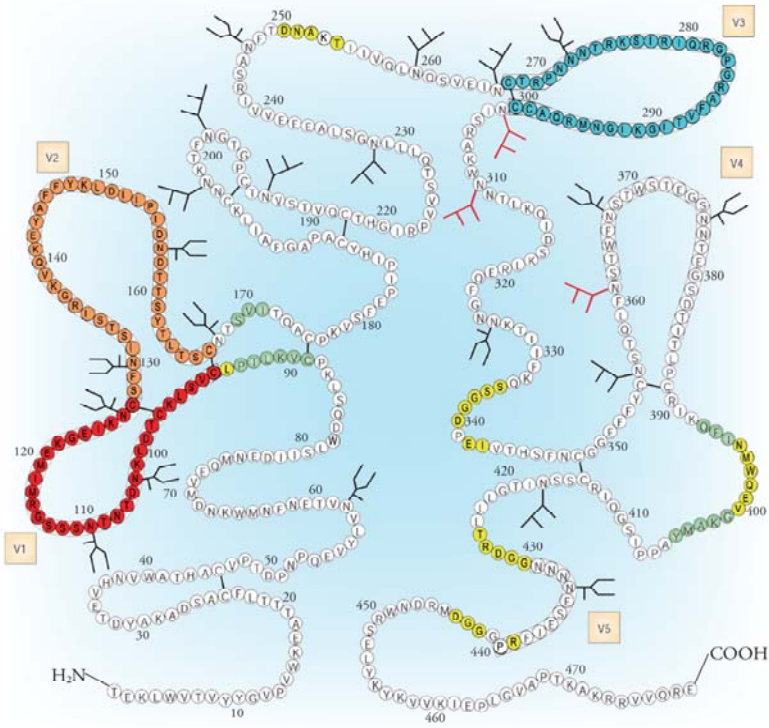

Figure 4. HIV-1 gp120. Schematic figure of the gp120 molecule including the variable loops, V1-V5. The CD4-binding domain is highlighted in yellow and the CD4-induced epitope is marked in green. The glycosylation sites are indicated by branches and the glycans important for mAb 2G12 is marked in red. The figure was adopted with permission from[27].

Gp120 of HIV-1 can be divided into five variable regions (V1-V5) and five constant regions (C1-C5) (Figure 4)[28, 29]. The term "variable" refers to a high degree of variability within the sequence, and "constant" refers to a relatively more conserved sequence. A set of 18 conserved cysteine residues is often found throughout gp120, which forms nine disulfide bonds and orders the tertiary structure of gp120[30]. The variability of gp120 is a result of recombination, point mutations, insertions and deletions. The V1V2 region is the most variable domain,

with both length variation and sequence variability[31-33], which has had consequences in the search for a crystal structure of gp120. The pursuit of quaternary structure of the Env trimer, i.e., what the protein complex actually looks like, has drawn considerable attention. This knowledge would be very valuable when trying to find suitable therapy and vaccine targets. The first attempt to visualize the gp120 structure was published in 1998, when the crystal structure was determined by using a truncated gp120 core[34]. The gp120 core had the V1V2 and V3 regions deleted and all the sugar groups removed, and was in complex with the CD4 receptor and a neutralizing human antibody. Gp120 consists of an inner domain and an outer domain. The inner domain faces the inside of the envelope trimer and comprises the N-terminal of the C1 region and the C-terminal of the C5 region, which are believed to interact with gp41. The inner domain is linked to the outer domain via a four-stranded bridging sheet, which is important for coreceptor binding, and the remainder of gp120 makes up the outer domain. Extensive research has resulted in better crystal structures of gp120, either in its unligated form or in complex with CD4 or with certain antibodies, but these crystal structures still lack the V1V2 domain (Figure 5)[23, 35-37]. Recently, the crystal structure of only the V1V2 region in complex with an antibody was published, which demonstrated that certain glycans in this region are good targets for broadly neutralizing antibodies (see section *Result and Discussion: Efficiency of DC-SIGN use coupled to the gp120 sequence*)[38]. However, the structure of the entire gp120 trimer has still not been completely resolved. Studies have also shown that gp120 is a very flexible protein which upon binding to the primary receptor, CD4, has a dramatic shift in its folding[39-41]. The CD4 binding site on gp120 is not a continuous sequence; instead, conserved residues found in the constant regions are folded into close proximity in the tertiary structure of gp120[34, 42-45]. The variable regions are not involved in CD4 binding, but the V3 region is important for coreceptor specificity. Instead, because the variable regions are exposed on the gp120 surface, they function as protection from the host immune response since these regions to a high degree can be mutated without alteration of the function of gp120[46, 47]. Thus, it seems that gp120 of HIV-1 has evolved to successfully hide key functions from antibody recognition and at the same time to have a high variability to escape from the host immune response.

Gp41

Gp41 is the viral envelope glycoprotein that anchors gp120 to the viral membrane. Its main function in infectivity is to mediate fusion between the virus and the target cell membranes. Much like gp120, gp41 is divided into different regions. The extracellular domain is in contact with gp120 and contains the critical fusion peptide and the heptad repeat regions HR1 and HR2. In addition, the extracellular domain includes the membrane proximal external region (MPER), a highly conserved sequence of 24 amino acids, which has been shown to be targeted by broadly neutralizing antibodies. The transmembrane domain of gp41 is a

conserved region that penetrates the viral membrane. Protruding inside the virus particle is the cytoplasmic tail of gp41[48].

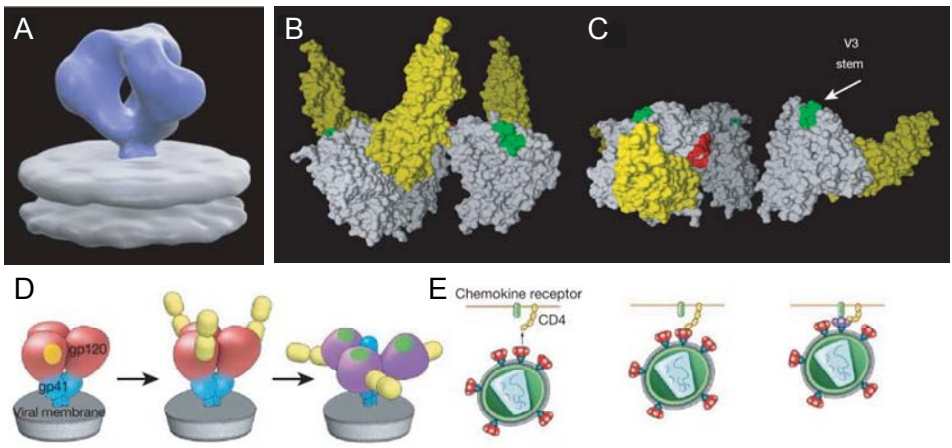

Figure 5. HIV-1 gp120 trimer. A) A cryo-electron tomography image of the unligated trimeric glycoprotein spike embedded in the viral membrane. B) Model of the gp120 trimer (white) conformation when CD4 (yellow) is binding. C) The subsequent conformational changes of gp120 when CD4 has bound. The V1V2 stem is indicated in red and the V3 stem in seen in green. D) and E) demonstrates a schematic view of the CD4-induced conformational changes in gp41 (blue) and gp120 (red). The CD4 binding site is marked as an orange patch and CD4 is seen in yellow. Upon binding, the coreceptor binding site is exposed (green). The figure was adopted with permission from[23].

Env glycosylation

An additional approach that HIV-1 has developed to evade the immune response, including neutralizing antibodies, is the extensive shield of carbohydrates that covers the surface of the viral envelope glycoproteins (Figure 6). These carbohydrates are synthesized entirely by the infected host cell and are therefore antigenically mainly recognized as "self". The glycans contribute to approximately half of the molecular mass of gp120 and cover most of the surface, making it immunologically rather silent, i.e., the virus hides from the immune system[49]. The glycosylation occurs during translation, after the envelope glycoproteins have been transcribed together as a polyprotein precursor, on the rough endoplasmic reticulum (RER). During translation, gp160 is glycosylated with N-linked (and some O-linked) oligosaccharide chains[30, 50]. N-linked sugars mean that oligosaccharide chains are linked to asparagines, in the sequences Asn-X-Ser or Asn-X-Thr (where X is any amino acid except proline). Such sites in the amino acid sequence are easily distinguished as potential N-linked glycosylation sites (PNGS), and there are 20-35 PNGS in gp120 and three to five in gp41. The gp160 monomers will form trimers in the RER and then continue to the Golgi apparatus[48], where further modification of the oligosaccharides will complete the final configurations of complex oligosaccharides or high-mannose

oligosaccharides. The difference between the two types is that high-mannose oligosaccharides contain just two N-acetylglycosamines and many mannose residues, whereas complex oligosaccharides can have additional N-acetylglycosamines as well as galactose, sialic acid and/or fucose residues. What determines the type of oligosaccharide is the glycan position in the precursor protein when it enters the Golgi apparatus. If the oligosaccharide is more accessible for processing enzymes in the Golgi apparatus, it is more likely to be converted into the complex type, and vice versa[51]. Previously, it was assumed that the glycans on gp120 consisted of both complex type and high-mannose oligosaccharides. More recently, it has been demonstrated that the majority of glycans on the envelope spikes from infectious virus particles consist of high-mannose type oligosaccharides[52-54].

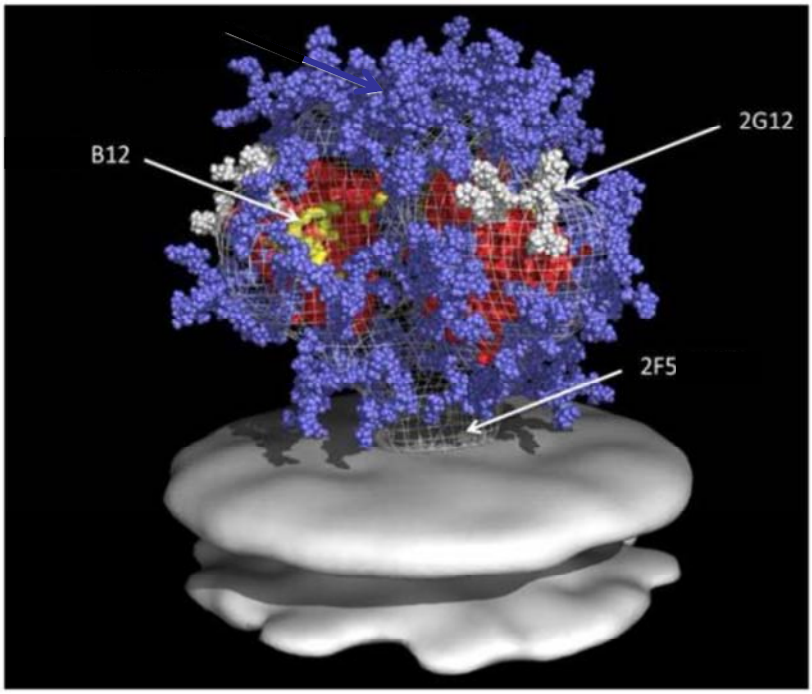

Figure 6. HIV-1 gp120 trimer covered by glycans. An unligated model of HIV-1 env trimer where all Env glycans are demonstrated in blue and white. Glycans of V1V2, V3 and gp41 region were manually added to obtain an approximate model of the full glycan shield. Gp120 is shown in red and included in the figure are also epitopes of the mAbs used in neutralization assays in this thesis (see *Material and method* section). The glycan epitope specific for mAb 2G12 is illustrated by white glycans and the epitope for mAb b12 is shown in yellow. The figure was adopted with permission from[55].

HIV-1 replication cycle

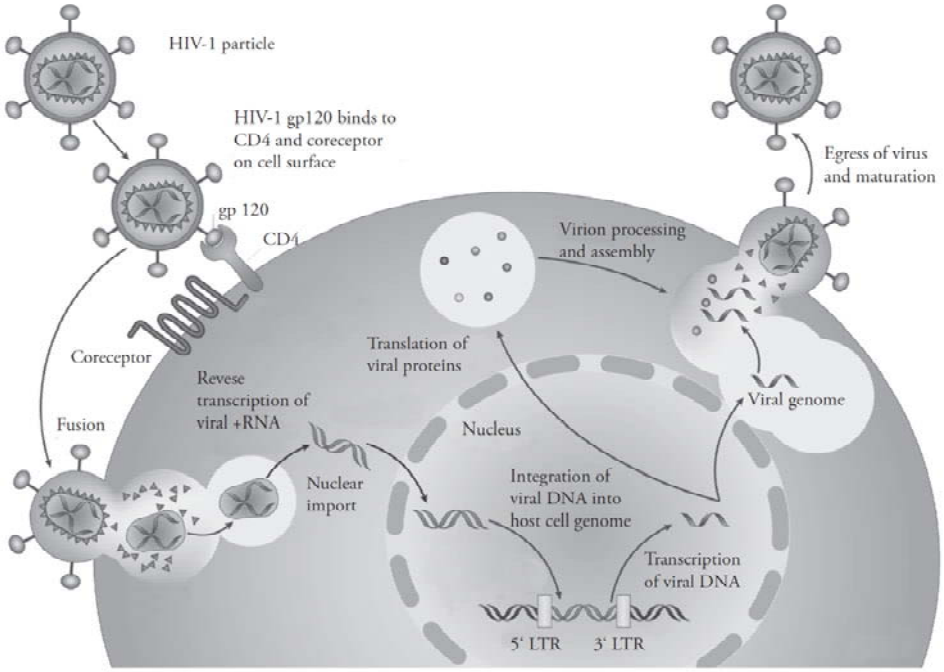

Figure 7. HIV-1 replication cycle. Schematic representation of HIV-1 replication. The figure was adopted with permission from[56] and modified by Joakim Esbjörnsson.

The replication cycle of HIV-1 is illustrated in Figure 7. Infection is initialized with the binding of gp120 to the primary receptor CD4 (Figure 8). The binding to CD4 triggers a conformational change in gp120 that allows the binding to a secondary receptor, the coreceptor. The two most physiologically relevant coreceptors are CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 4 (CXCR4)[57-61]. After the binding of gp120 to the coreceptor, additional conformational changes of both gp120 and gp41 expose the gp41 fusion peptide. Once the fusion peptide is inserted into the cell membrane, the HR1 and HR2 regions of gp41 interact with each other to form the six-helix bundle in a hairpin structure. This hairpin brings the viral membrane in such close proximity to the cellular membrane that a fusion pore is formed, resulting in the delivery of the viral core into the cytoplasm[19, 20].

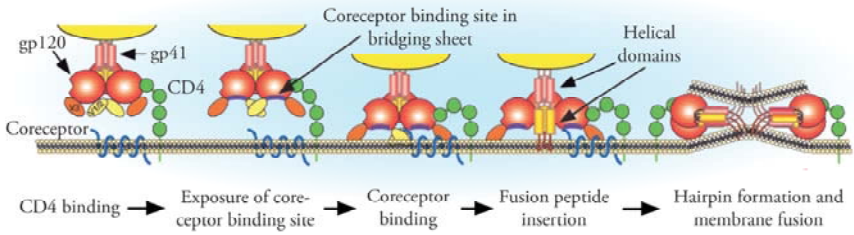

Figure 8. HIV-1 entry process. The entry is initiated with the binding of cellular CD4 to gp120, which induces a conformational change resulting in the exposure of coreceptor binding site. Subsequent binding to coreceptor by gp120 induces additional changes, leading to the insertion of the fusion peptide of gp41 into the cellular membrane. Fusion of viral and cellular membrane follows. The figure was adopted with permission from[62] and modified by Joakim Esbjörnsson.

HIV-1 can enter target cells as free virus particles, fusing directly with the cell membrane, or via endocytosis followed by fusion in an endosome[63]. However, HIV-1 can also infect via cell-to-cell contact, and this pathway has been shown to be very efficient[64]. The virus is then protected from the surrounding environment in specialized junctions referred to as synapses. HIV-1 can use existing cell-to-cell contacts, such as the immunological synapse between antigen-presenting cells and T cells. In addition, HIV-1 can establish cell-to-cell contact between infected and uninfected T cells, which normally do not form synapses with each other[65]. Such synapses are called infectious or virological synapses. Cellular adhesion molecules and receptors will accumulate into these synapses, and HIV-1 can efficiently spread into new cells.

Once inside the cytoplasm, viral RT begins the synthesis of double-stranded DNA from the viral single stranded RNA genomic 5' LTR. RT is a unique polymerase enzyme found in retroviruses[66, 67], and due to the features of this enzyme, HIV-1 has very high variability in the viral genome. Unlike DNA polymerases, RT has no proofreading ability. Compared to the mutation rate for cellular polymerases (one mutation per 10^8 amplified base pairs[68]), RT incorporates, on average, one point mutation per 10^4 amplified base pairs, i.e., one mutation for every replication cycle[69]. Another important feature of RT is the ability to switch templates during replication, resulting in recombination, if the cell is infected with several virus variants[70-73]. However, even though recombination events may occur frequently, we can only detect them if the virus variants differ enough from each other. Both of these features influence the variability of the viral amino acid sequences that make up the viral proteins. Many, or probably most, mutations will result in a non-functional virus particle, but some will result in a virus with unique and improved abilities to survive in the host. The impact of the sequence variability will be discussed more in the *HIV-1 variation and evolution* section.

While double-stranded DNA is formed, the pre-integration complex, consisting of viral and host cell proteins surrounding the viral genetic material, is translocated to

the nucleus membrane and imported into the cell nucleus. Unlike many retroviruses, HIV-1 DNA can be imported into the nucleus and integrated into the host genome of a non-dividing cell[74]. Integration of the viral genome into the host genome is mediated by the viral integrase (IN). The integration location is preferentially in or near activated genes[75], and once in the genome, the virus is referred to as a provirus. In this form, the virus can stay latent in the cell for a long time, and the virus replication is initiated when the host cell is activated. HIV-1 uses the cell machinery for replication, but the synthesis of viral RNA and proteins is highly regulated by viral regulatory proteins. The early viral proteins, Tat and Rev, regulate the expression of the late viral proteins, the structural and accessory proteins, in a complex process. Newly produced viral proteins and the RNA genome assemble in the cytoplasm at the cell membrane, where processed Env is expressed and new virus particles will form. The final step of the virus life cycle includes budding from infected cells, followed by viral protease processing of Gag and Gag-Pol precursors to form mature infectious particles[19, 20].

HIV-1 cellular receptors

Soon after the first isolation of HIV-1, CD4 was described as the main virus receptor for target cell entry[39-41]. CD4 is an immunologically important receptor, which binds to MHC class II molecules on antigen-presenting cells. Such interactions facilitate signal transduction and activation if the cell recognizes the MHC class II–peptide complex. CD4 is expressed by the T helper cells, monocytes, macrophages and dendritic cells (DC). Soon after the discovery of CD4 as a receptor for HIV-1, it became evident that one or more factors or receptors were essential for HIV-1 infection. However, it was not until 1996 that a coreceptor, CCR5 or CXCR4, were identified as the missing factor necessary for infection[57-61].

In addition to the major above-mentioned receptors for HIV-1, the virus is able to bind to a number of other receptors expressed on various cells, with different outcomes. For example, HIV-1 has the ability to bind the gut-homing integrin α4β7 expressed on CD4+CCR5+ T cells[76], and this interaction has been suggested to contribute to the early viral replication in the gut-associated lymphoid tissue (GALT) (see section *Pathogenesis*)[77]. Other alternative receptors for HIV-1 attachment to cells are the syndecans, which are highly expressed by macrophages and have the potential to modulate the infection[78]. The syndecans also have the ability to transmit the virus to target cells, a feature they share with the C-type lectins, which will be discussed below.

HIV-1 phenotypes related to coreceptor use

Before 1996, different variants of HIV-1 were identified based on their replicative capacity and cytopathic effects in primary cells and cell lines. Viruses isolated from AIDS patients were demonstrated to replicate rapidly and to high titers in cell lines and also induced syncytia in peripheral mononuclear cells (PBMC) and were thus designated rapid/high or syncytia inducing (SI)[79-81]. Viruses from non-AIDS patients demonstrated, in general, different characteristics in PBMC, with slow replication and low titers, and were not capable of inducing syncytia, thus termed slow/low or NSI[79-82]. When the coreceptors were discovered, the observed differences in the phenotypes of HIV-1 could be correlated with coreceptor use. The viruses dependent on CCR5 for cell entry were homologous with slow/low and NSI viruses, and the viruses either using CXCR4 exclusively or able to use both CCR5 and CXCR4 were homologous with rapid/high and SI viruses[83, 84]. Thus, a new virus nomenclature was introduced, where monotropic CCR5-using viruses were termed R5, monotropic CXCR4-using viruses were termed X4, and dualtropic viruses using both CCR5 and CXCR4 were termed R5X4[85] (Figure 9).

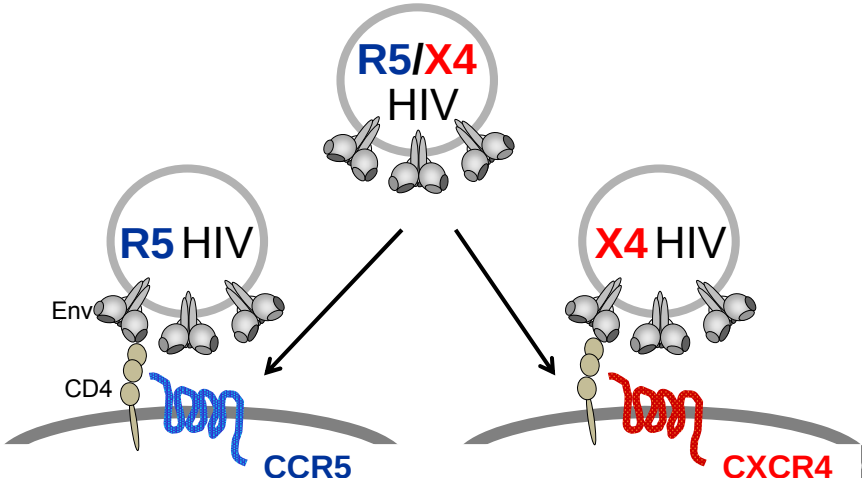

Figure 9. Classification of HIV-1 based on coreceptor tropism. Virus using CD4 and CCR5 for entry are called R5 HIV-1 and virus using CD4 and CXCR4 for entry are called X4 HIV-1. Virus able to use both CCR5 and CXCR4 in addition to CD4 for entry are called R5X4 virus.

CCR5 and CXCR4 are chemokine receptors located in the plasma membrane as a 7-transmembrane G-protein coupled receptor. Of CD4 expressing cells, CCR5 is found on macrophages, monocytes, DC, microglia and T cells (especially activated and memory), whereas CXCR4 is distributed on DC and T cells (especially naïve T cells)[86, 87]. The natural ligands for the receptors are small peptides called chemokines, which are important regulators of leukocyte trafficking. The ligands for CCR5, RANTES, MIP-1α and MIP-1β (also known as CCL5, CCL3 and

CCL4) were actually discovered to inhibit the replication of some HIV-1 variants, being T cell line adapted, before CCR5 was discovered to be a coreceptor[88]. The natural ligand for CXCR4 is SDF-1α[89] (CXCL12). CXCR4 is an essential housekeeping receptor, meaning it is constitutively expressed and is involved in maintaining the homeostatic conditions in the body. CCR5 is instead an inducible pro-inflammatory receptor, which shows redundancy with other inflammatory chemokine receptors. Strong evidence for the importance of CCR5 as an HIV-1 entry receptor was demonstrated by the link between resistance to infection and the lack of a functional CCR5, as a result of a 32 base pair deletion in the *CCR5* gene[90, 91]. Other chemokine receptors have also been demonstrated to work as coreceptors for HIV-1 infection *in vitro*, but the importance of these receptors *in vivo* is not well supported[77]. However, CCR3 has been shown to work as coreceptor for HIV-1 circulating during the primary infection, detected by direct Env cloning from patient blood samples, suggesting that virus isolation via PBMC *in vitro* cultures select for CCR5 use and not CCR3[92]. Although CD4 is considered the primary receptor for HIV-1, the coreceptor binding seems to be more essential for entry. It has been demonstrated that some HIV-2 and a few HIV-1 viruses are able to infect cells independently of CD4 and only using a coreceptor[93-95]. These viruses have been suggested to have Env with a more exposed coreceptor binding site, i.e., a pre-triggered conformation[96].

Gp120 determinants of coreceptor usage

The main determinant for coreceptor use is harbored within the gp120 V3 region. In particular, positions 11 and 25 of the V3 loop are of importance for coreceptor use. A positively charged amino acid in either or both of these positions is linked to usage of CXCR4[97, 98]. However, other alterations of charge and PNGS within V3 and in other regions of gp120 (especially V2) have also been reported to affect coreceptor use[99-104]. With the use of known sequence differences for CCR5- and CXCR4-using HIV-1 variants, methods of sequence-based algorithms to predict coreceptor use have been developed[105, 106]. Often, these methods have been based on the V3 region of HIV-1 subtype B sequences, and they are not always consistent (see **paper III**).

Coreceptor evolution and switch

During disease progression, HIV-1 can evolve with respect to coreceptor use. R5 viruses dominate in the acute phase of the infection, after transmission, even when the donor (transmitting individual) harbored both R5 and CXCR4-using viruses[107-110]. The reason for this dominance is not fully understood, however, CCR5 is highly expressed on the cells initially infected in the new host and it has also been suggested that R5 viruses have better fitness early in the infection[77]. Despite the high number of different virus variants in the donor, only a few virus particles initiate the infection in the new host, i.e., the virus goes through a bottleneck when

infecting a new host[77]. However, during disease progression, HIV-1 coreceptor use can evolve in different directions (Figure 10). One direction includes the emergence of viruses able to use CXCR4, which occurs in so called "switch virus patients". The development of virus variants using CXCR4 is often associated with an accelerated disease progression and a poor prognosis for survival, while not true for all individuals harboring CXCR4 using viruses[83, 84, 111, 112]. The other direction, observed in the "non-switch virus patients", involves alteration of the virus while exclusively maintaining the R5 phenotype throughout the entire disease course[113-123]. The frequency of infected individuals with a switch in virus coreceptor use, to include CXCR4-using viruses is different for different subtypes of HIV-1. For subtype B, approximately 70% of infected individuals have a switch in virus phenotype, whereas for subtype C, the switch level is very low, and the opposite is true for subtype D[124].

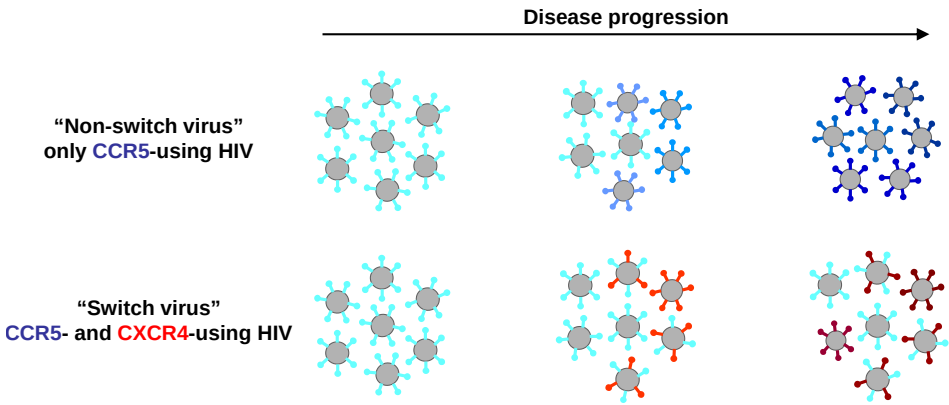

Figure 10. Two pathways of HIV-1 coreceptor use during disease progression. Early in infection R5 HIV-1 (blue) is dominating. In "non-switch virus patients" the R5 phenotype is maintained through the whole disease course while in "switch virus patients", HIV-1 with the ability to use both CCR5 and CXCR4 (blue/red) or viruses exclusively using CXCR4-using virus (red) will develop.

It is not known why some patients develop HIV-1 that switches coreceptor use to include CXCR4. However, three different hypotheses have been considered to explain the phenomena (reviewed in[125]). First, the transmission-mutation hypothesis suggests that R5 HIV-1 is selectively transmitted and evolves into X4 HIV-1 as a result of random mutations once infection has been established. It has been reported that children infected by their mothers developed their own X4 HIV-1 from their existing R5 population early in infection, and their X4 viruses were not related to the maternal X4 virus population[126]. Such evidence would support this hypothesis, but at the same time, the transmission-mutation hypothesis seems too simple. With our knowledge of the high mutation rate and variability of HIV-1, the switch to the X4 virus would occur more often then what is observed *in*

vivo. The second hypothesis is the immune-based hypothesis, suggesting that X4 viruses are more vulnerable to the host immune responses. Indeed, X4 viruses that emerge soon after the switch are more sensitive to neutralizing antibodies[127]. Thus, as the pressure of the immune response wanes, CXCR4-using viruses are allowed to emerge. In agreement with this hypothesis, coreceptor switch was detected in infected rhesus macaques with low antiviral antibody response[128]. Furthermore, in the macaque model, CXCR4-using viruses have been shown to preferentially replicate in the absence of CD8+ T cells[129]. However, this hypothesis does not explain the lack of X4 virus in the acute phase, when no virus-specific immune response has been built. On the other hand, the selection of R5 HIV-1 at transmission could hypothetically explain the absence of the X4 virus. Finally, the target-based hypothesis suggests that the pool of target cells at different stages of the disease will affect whether the R5 or X4 viruses can replicate, as the coreceptor expression differs on memory and naïve CD4+ T cells. In addition to these three hypotheses, other explanations for why some patients switch and other do not have been postulated. A recent study showed that recombination between R5 and X4 HIV-1 co-existing in an individual can occur. In addition to a switch in coreceptor use, the recombinant virus might harbor the benefits of both the original R5 and X4 viruses[130]. Alternatively, perhaps it is not true that X4 viruses will only develop in a certain proportion of the infected patient, as generally thought. Instead, perhaps X4 viruses emerge continuously over time, but some infected individuals die before they develop these viruses[106].

DC-SIGN use for HIV-1 *trans*-infection

HIV-1 may also bind several C-type lectin receptors, including dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN), mannose receptor, langerin and DC-SIGN homologs, expressed by DC, macrophages and endothelial cells[131]. These receptors all bind to carbohydrate domains, which are present on pathogens or in host tissue. DC-SIGN is mainly expressed on the myeloid subsets of DC present in blood and in tissues. During the trafficking of DC, DC-SIGN can bind to ICAM-2 on endothelial cells. When DC interacts with T cells, DC-SIGN binds to ICAM-3 to mediate adhesion[132]. DC-SIGN specifically recognizes high-mannose and fucose oligosaccharides, and, upon binding to pathogens, these oligosaccharides are internalized and degraded, and the antigens are loaded onto MHC molecules[133]. However, several pathogens such as *Mycobacteria tuberculosis*, Ebola virus, hepatitis C virus and including HIV-1, have developed the ability to bind to DC-SIGN and utilize the receptor for enhanced infectivity of target cells[132]. Through gp120, HIV-1 attaches to DC-SIGN and is subsequently transferred to T cells via an infectious synapse, a process known as *trans*-infection[134]. Contrasting reports suggest that *trans*-infections occur without DC-SIGN or that DC-SIGN increases *cis*-infection[135-137]. Thus, the complete role of DC-SIGN has not been clarified, but the receptor seems to serve as one option for DC to efficiently spread HIV-1 to T cells. Exactly what occurs after HIV-1 has

bound to DC-SIGN is not clear. Initially, it was thought that HIV-1 transfer to T cells was mediated through internalized compartments[134, 138]. However, as cell lines expressing DC-SIGN are also capable of *trans*-infection, but not via internalization, the former statement was questioned[139]. Instead, it has been suggested that HIV-1 is transferred to T cells on the surface of DC-SIGN-expressing cells[140] or in surface-accessible compartments[141]. Several studies have also demonstrated that the enhanced infection of T cells is a result of a productive infection in the DC-SIGN-expressing cells, followed by a transfer of *de novo* virus particles to the T cells[139, 142-144]. Another option following HIV-1 binding to DC-SIGN and other C-type lectins is conventional degradation and MHC presentation[145]. The different models of *trans*-infection are shown in Figure 11. The function of DC-SIGN use *in vivo* has also been suggested as an escape from neutralizing antibodies[146, 147]. In addition, DC-SIGN might play a role during transmission, as the receptor is expressed by interstitial DCs and macrophages in the submucosa[148-151] and by maternal and fetal macrophages in the placenta[152]. Whether the virus uses DC-SIGN for transmission is not known, but an alternative role for virus DC-SIGN use in vertical transmission is discussed in **paper IV**.

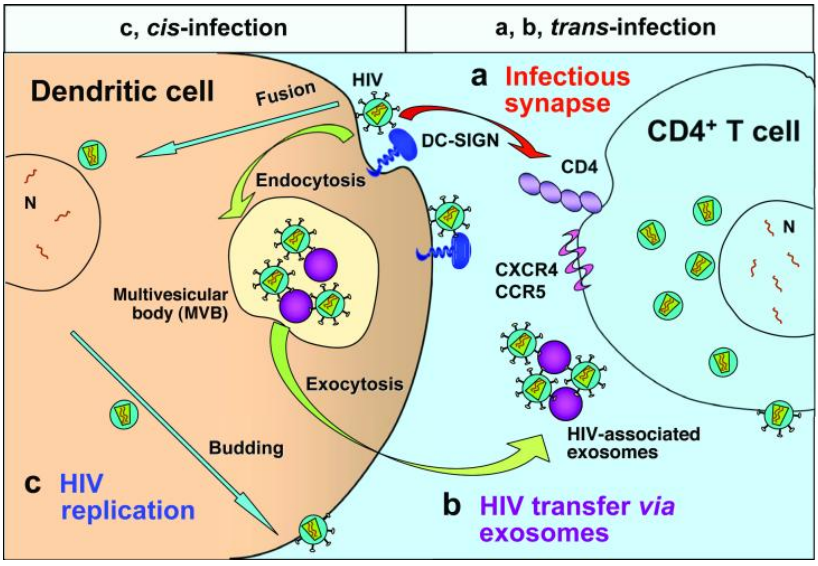

Figure 11. A schematic illustration of the potential outcomes of HIV-1 interaction with DC-SIGN. A) HIV-1 interacting with DC-SIGN is surface bound and released to target cells via the infectious synapse. B) HIV-1 binding to DC-SIGN leads to endocytosis of intact virions, which will be released to target cells via exocytosis in the infectious synapse. C) *Cis*-infection mediated by infected DCs and replication of *de novo* viruses. The figure was adopted with permission from[131].

HIV-1 variation and selection forces

Interhost variation

The high variability of HIV-1 is manifested on several levels in the infected population. Based on phylogenetic analysis, HIV-1 can be separated into three major groups, M (main), O (outlier) and N (non-M, non-O), where the M group includes the majority of the global virus isolates (Figure 1). Within the M group, the isolates are further divided into subgroups (or clades) A-D, F-H and J-K, including many circulating recombinant forms. The different subtypes are distributed in distinct geographical areas. Subtype C is the globally dominant subtype and is found where the HIV-1 prevalence is the highest, in southern and eastern Africa and in India. Subtype B is, however, the most intensively studied subtype, because it was the first one to be discovered and is most prevalent in Europe and North America.

A consequence of the HIV-1 variability is that the virus adapts over time in the population. There is evidence for HIV-1 adapting to the cellular immune responses by losing the epitopes for the most common HLA types in the population[153]. In a similar manner, HIV-1 seem to adapt to the humoral immune response, since virus recently isolated were shown to be more resistant to neutralization then virus isolated during the 1980s[154]. The same study showed how HIV-1 Env has evolved over time in the infected population, with longer variable regions and more PNGS over time.

Intrahost variation

At first, it was thought that HIV-1 would be genetically homogeneous, based on the knowledge of other known retroviruses. However, when sequencing was initiated, it became obvious that no two HIV-1 isolates were identical, even when isolated from a single individual[155]. Nucleotide changes were found throughout the genome, but the greatest variability was found in the *env* gene coding for the envelope glycoproteins. The term "quasispecies" was introduced to describe the pool of diverse viruses present in an infected individual. Thus, even though infection is established by a single or a few virus particles, within just a few days after infection, different virus variants can be detected in the host[156]. Reverse transcriptase plays a major role in the high variability of HIV-1 (see section *HIV-1 replication cycle*). In addition, the high level of virus production in the host, 10^{10} particles/day, adds to the variability[157, 158]. A major driving force for the variability is the pressure on the virus from the immune response. The viral envelope glycoproteins are the most prone to vary, particularly the most exposed variable regions on the envelope spikes. Insertions, deletions and changes in numbers of PNGS of the *env* gene are responses to the immune pressure[28]. The virus initiating the infection in a new host has been shown to have a reduced glycan shield, which

gradually builds up during disease progression, in parallel with the mounting immune response[159, 160]. Another source for genetic variation is the cellular protein APOBEC, which plays a role in the innate anti-viral immunity[161]. This enzyme mediates deamination of HIV-1 DNA, resulting in G-to-A substitutions in the genetic code, which often has deleterious effects on virus replication. HIV-1 counteracts this effect via the viral protein Vif. However, low levels of APOBEC activity that overcomes Vif inhibition, induces mutations that are not lethal for virus and instead a source for variability[162-164].

Once an infected individual begins antiretroviral treatment, pressure on relevant drug targets, such as reverse transcriptase and protease, is also apparent, and the risk of the development of resistant virus variants increases[157, 165].

Transmission

Routes of HIV-1 infection are via blood or body fluids. Globally, the main route of transmission is via sexual intercourse, where rectal intercourse has the highest probability of infection (1/20-1/300). Via vaginal intercourse, the probability is 1/200-1/2000, and the lowest risk is via the oral route, with a probability of 1/2500[166]. The risk of transmission is also related to virus levels, viremia, in the transmitting donor, where the risk of transmission is highest during acute infection and the AIDS phase when viremia is very high. HIV-1 has several possible target cells in the genital and oral mucosa, including CD4-expressing T cells, Langerhans cells (LCs) and DCs, which can capture the virus and transfer it to target cells. In fact, it has been demonstrated that this *trans*-infection in *ex vivo* human cervical tissue samples can be partially blocked by C-type lectin antibodies[167]. The virus can be actively transported through mucosa via host cells or transcytosed through the epithelium. Breaches and inflammation in the mucosa due to genital infections or sexual intercourse are obviously also an entrance for the virus[166].

However, the virus has to overcome several barriers, which may reflect the differences in probability of infection via different routes. A C-type lectin, langerin, expressed by LC has been shown to degrade HIV-1 instead of disseminating the virus to target cells[168]. Antimicrobial peptides, such as defensins and cathelicidins, are present in mucosal sites and have the potential to inhibit the virus[169, 170]. Moreover, mucin present in seminal plasma can potentially block the virus dissemination via DC[171].

Mother-to-child transmission

One mode of virus transmission is mother-to-child transmission (MTCT). Without treatment, approximately 30-45% of children will be infected, where 15-20% occurs during pregnancy and delivery, and 10-20% occurs through breast

feeding[172]. However, with proper antiretroviral prophylaxis used during pregnancy and delivery, and with alternative feeding, the percentage can be reduced to less than 1%[173]. Transmission during pregnancy, *in utero*, is thought to occur when the virus crosses the placenta. Trophoblast cells form the outer layer of the placenta and serve as an efficient barrier for passage of HIV-1. Thus, HIV-1 must pass these cells via breaches, perhaps due to bacterial infections, or via transcytosis[174]. Transmission during delivery, *intrapartum*, occurs when the infant is exposed to maternal blood and genital secretions. The virus can enter the mucosal surfaces of the infant but also via the placenta because insults, such as microtransfusions, during delivery permit the virus to cross over to the infant[175]. Breast milk contains lower amounts of virus than plasma, but as the child is continuously exposed, the transmission risk could be higher. However, other factors in breast milk probably reduce the risks of transmission. Breast milk helps to develop a healthy and protective gut epithelial in the child. In addition, several components of breast milk have been demonstrated to inhibit HIV-1 infection and binding to C-type lectins *in vitro*[176-182].

Pathogenesis

Disease progression in an HIV-1 infected individual is routinely monitored by clinical symptoms and measurements of plasma viral load and CD4+ T cell counts (Figure 12). The disease can be divided into three phases: the acute phase following transmission, the chronic phase when the patient is clinically asymptomatic and the AIDS phase.

Acute phase

Following transmission, HIV-1 will rapidly spread to lymph nodes and other lymphocyte-rich compartments throughout the body, such as the GALT. There, the virus will encounter high densities of CD4+ target cells, resulting in massive viral replication. The consequence of this viral replication burst is that a great majority of the CD4+ T cells in the GALT are irreversibly depleted within the first week of infection[184-186]. This is likely because the GALT contains high levels of HIV-1 primary target cells, i.e., CCR5+CD4+ T cells. The primary infection can be manifested in the individual by flu-like clinical symptoms, including fever, body ache and swollen lymph glands. Shortly after transmission, an antiviral immune response can be detected, which reduces the virus levels in the plasma down to the so-called “viral set point”. This level of plasma viral load correlates with subsequent disease progression, i.e., a lower viral set point is a predictor of a slower disease progression[187].

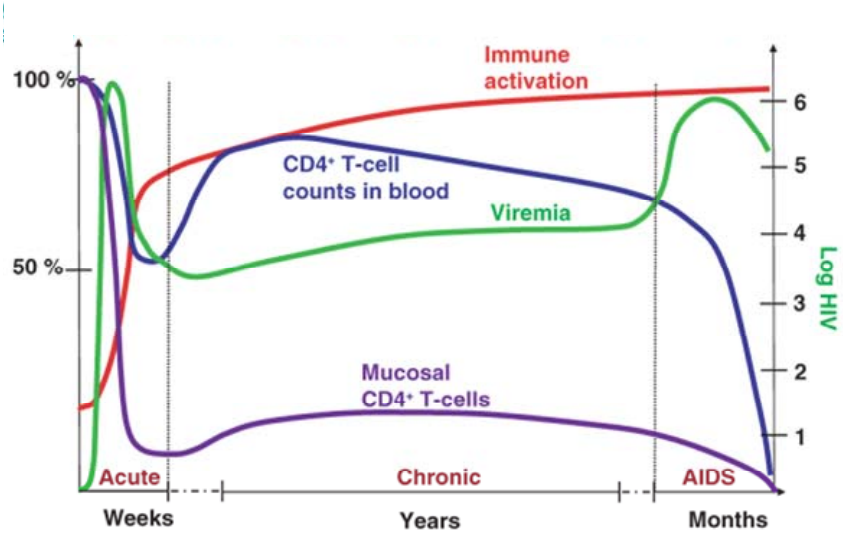

Figure 12. HIV-1 disease progression. Changes in numbers of mucosal and blood CD4+ T cells and viremia are shown in relation to level of immune activation over the course of HIV-1 infection. Mucosal T cells (purple) are rapidly lost during the acute phase and at the same time there is a rapid increase in plasma viral load of HIV-1 (green). CD4+ T cells in blood (blue) will decline during the acute phase but increase again. The immune system (red) is rapidly activated with a steadily increase of activation during the chronic phase. The mucosal CD4+ T cells remain low during the chronic infection and the CD4+ T cells in blood will gradually decrease. At the same time viremia slowly increases and when AIDS develops the gradual changes seen in the chronic phase will accelerate. The figure was adopted with permission from[183].

Chronic phase

During the chronic phase, the individual experiences minimal clinical symptoms, and the virus levels are partially controlled by both cellular and humoral immune responses. The time-span of this phase varies greatly among individuals, from weeks to decades, with an average of 10 years[188]. These differences are probably due to several factors, including host genetics, such as the expression of viral coreceptors[189-191], certain cytokines and chemokines[192, 193] or specific alleles on MHC class I[194, 195], and viral factors, including attenuating mutations[196]. The overall state of the immune system also affects the duration in the chronic phase. Older infected individuals and vertically transmitted children have a shorter chronic phase. There are a few infected individuals who seem to stay in the chronic phase and can control their infection without medical treatment, the so called long-term nonprogressors.

Even without symptoms, there is a constant turnover of the T cells during the chronic phase, with a gradual decay of CD4+ T cells, and the regenerative capacity is lost. Simultaneously, the chronic immune activation is elevated and is not only

specific for HIV, but instead demonstrates a general increase in activated immune cells and production of inflammatory cytokines[183].

AIDS phase

The infected individual will develop AIDS when the CD4+ T cells have declined to a level where the cellular and the humoral immune responses can no longer be supported, approximately at 200 CD4+ T cells/µl. Infections caused by different opportunistic microbes will appear and eventually, if untreated, lead to death within approximately one year[188]. The cause of the CD4+ T cell depletion is not fully understood. The virus-mediated killing of target cells or cytotoxic immune response may not give the whole explanation. Additionally, it has been proposed that the chronic immune activation during the infection leads to an exhaustion of the naïve T cells, which cannot compensate for the death of the effector and memory T cells[197, 198]. The chronic immune activation may be caused by different factors, including plasmacytoid DC hyper-responsiveness and the rapid depletion of GALT CD4+ T cells, resulting in microbial translocation[199, 200].

HIV-1 infection in children

In vertically transmitted children, HIV-1 infection progresses as it does in adults, but the progression rate is generally much faster[201-203]. However, the disease progression also here differs in different individuals. The causes of this difference are not clear, but the timing of transmission, host factors or virus phenotype might influence it. Both the maternal and the infant immune responses have the ability to control the infection in the child. Maternal IgG antibodies specific for HIV-1 will be passively transferred to the child through the placenta. The neutralizing activity of such antibodies has been coupled to a lower risk of MTCT (reviewed in[172]). The infant's immune response against HIV can be detected in cord blood and includes both innate and cellular activity. However, the immune response in the infant is not fully developed, and the genetic similarity to the maternal response possibly makes it impossible to block virus variants already evading the maternal response.

Immune response to HIV-1

Throughout the HIV-1 infection, the host immune system is working intensively to control the infection. Early after infection, the innate immunity, including increased levels of inflammatory cytokines and chemokines produced by DCs, macrophages, natural killer cells and T cells, can be detected in plasma[204]. These factors will activate other players in the innate immune response but will also prime the adaptive response. However, the increased levels of cytokines and chemokines will also promote viral replication by recruiting susceptible target cells to the site of infection[204].

The adaptive immune response is detectable just before the peak viremia in the acute phase[204-206]. When the T cell response peaks 1-2 weeks later, viremia is declining to the viral set point, with the CD8+ T cells playing a central role, and the viral selection of escape mutants is already in progress[207, 208]. The T cell response continues to participate in the control of the infection during the chronic phase of the disease. The importance of the T cell response has been demonstrated in macaques depleted of CD8+ T cells and infected with SIV, resulting in a loss of viral control at the acute phase and increased viral load during the chronic phase[209, 210].

Much like the T cell response, the humoral response, with the production of antibodies, has been demonstrated to contribute to the control of the SIV virus load[211]. Such early induced antibodies are probably of the non-neutralizing type and use FC-receptors or complement to mediate their effect. Through the interaction between HIV-specific antibodies coating target cells and natural killer (NK) cells, the infected cells can be lysed via antibody-dependent cellular cytotoxicity (ADCC).

HIV-1 neutralizing antibodies

The first antibodies to neutralize autologous HIV-1 are not detected until ~12 weeks after transmission[204]. The term "neutralizing antibody" refers to an antibody capable of binding to virus surface proteins and thereby directly blocking or reducing the infection. Such antibodies are developed in most infected individuals, but contemporaneous autologous neutralizing antibodies are rarely found in HIV-1-infected individuals due to rapid virus escape[212-215]. Initially, the antibody response is specific for autologous virus variants, but with time, it can develop into a heterologous responses, i.e., broadly neutralizing antibodies, which are neutralizing viruses obtained at different time points and from other individuals[216]. The effect of the humoral antibody response on the virus infection is demonstrated by the rapid emergence of escape mutants. Alterations of the envelope glycoprotein's variable loops and an increasing glycan shield are primarily responsible for the escape mutants[33, 159, 214, 217].

Individuals that develop broadly neutralizing antibodies have recently gained considerable focus. Such antibodies can neutralize infection by HIV-1 variants from different subtypes[218], and they are directed against conserved epitopes of the envelope glycoproteins. Approximately 20% of infected individuals will develop broadly neutralizing antibodies to some degree[219]. However, harboring such antibodies is unfortunately not associated with a prolonged chronic asymptomatic phase of the infection[220, 221]. There are virus variants that can also escape neutralization by broadly neutralizing antibodies, and these variants have been demonstrated to have unaltered replication capacity[222, 223]. Still, in regard to the development of a prophylactic antibody-based HIV-1 vaccine, it is of great interest to identify the specific epitopes these broadly neutralizing antibodies are targeting.

A few of these antibodies have been isolated from infected individuals and characterized by their epitopes[224-231]. These broadly neutralizing antibodies have been demonstrated to protect from infection after passive transfer in several macaque models[232-235]. Such studies give hope that if a future vaccine could elicit similar antibodies, it would have a potential protective effect. Modern techniques have made it possible to isolate several new broadly neutralizing antibodies, and several have been investigated for epitope definition and their neutralizing potential (reviewed in[236]). When summarizing today's knowledge of targets for broadly neutralizing antibodies, four distinct Env regions merit special interest: the gp41 MPER, the CD4 binding site, the quaternary V2/V3 loop structure and the carbohydrates of gp120.

HIV-1 therapy and prevention

Antiretroviral medication offered to HIV-1 infected individuals today does not cure the infection, but efficiently helps in controlling the infection. The first treatment used in infected patients consisted of monotherapy. First such therapies were AZT and didanosine (ddI), introduced 1987 and 1991 respectively[237, 238], which both inhibit the reverse transcription. However, the effects of monotherapy was soon discovered to be brief and limited, due to rapid emergence of resistant viruses with point mutations in the reverse transcription gene[165]. Today's treatment consists of combination antiretroviral therapy (cART) and it has been very beneficial to many infected individuals since it was introduced in 1996. A recent study conducted in a Danish cohort revealed that mortality in patients with successful cART and without other risk factors, is almost identical to that of the non-infected population[239]. The treatment is a combination of at least three different drugs, targeting at least two different viral enzymes. Up to date the main viral targets have been to block or interfere with the reverse transcription and to inhibit viral protease. Since escape virus mutants will develop under the selection pressure of the treatment, it is important to have alternative options when the treatment fails. New antiviral drugs, including integrase inhibitors and entry inhibitors, have been licensed and others are under development[240].

Development of prophylactic measures is also ongoing. In 2010 one study demonstrated a 39% reduction in HIV-1 transmission rate by introducing a vaginal gel containing a RT inhibitor, tenofovir, that should be applied in close proximity to time of exposure[241]. In addition, use of pre-exposure prophylaxis (PrEP) has recently been proven successfully to prevent transmission in highly exposed uninfected individuals[242, 243].

Development of an HIV-1 vaccine has been prioritised since the discovery of the virus[244]. One of the major obstacles to overcome is the extreme antigenic

variability of HIV-1. However, the observation that HIV-1 infection is typically established by a single virus particle offers hope that a vaccine only has to block a limited number of viral strains. Unfortunately, so far no unique character of transmitting viruses has been identified. Instead an efficient vaccine has to elicit broad neutralizing antibodies that can protect infection of many different virus variants. In addition, since the virus is quickly absorbed upon transmission and transported to various lymphoid tissues and sub-sequentially integrated into host cells, the protecting antibodies have to be in place at high concentrations at the mucosal site to hinder the first interactions. Today it is generally assumed that an efficient vaccine should elicit both humoral and cellular immune response. So far only three vaccine approaches have been tested in clinical trials, in phase 2b and 3, and only one of these trials (RV144) demonstrated a modest reduction, around 30%, in HIV-1 aquisition[245]. With experience and knowledge learned from previous trials, future vaccine development need to focus on understanding the immune factors influencing an effective response, how to induce a broad response, how to use new adjuvants and vectors, and how to learn more from animal models[246].

Materials and methods

Viruses

HIV-1 isolates used in this thesis were obtained from infected patients attending clinics in Stockholm, Sweden, from 1987 until 1995. Since this was before the introduction of cART, none of the patients were under effective treatment, but some received antiretroviral monotherapy.

Paper I, II and **III** are based on virus isolates from 15 homo- and bisexual men, selected from a larger cohort of patients, who attended the South Hospital, Stockholm, Sweden[111]. These patients were followed continuously for several years with virus isolation and CD4+ T cell counts. In **paper I** and **II**, patients, seven and six respectively, harbouring viruses with exclusive use of CCR5 as coreceptor throughout the disease course were studied, so called "non-switch virus patients". The R5 phenotype was determined by virus infection of coreceptor indicator cell lines GHOST and U87[116]. Studied patients were selected on the basis of their virus phenotype, their disease progression to AIDS and severe depletion of CD4+ T cells[116]. Isolates from two sample time points from each patient were studied, one from the chronic and asymptomatic phase of the disease, and the second from the end-stage of the disease when the patients were severely immunosuppressed, with very low CD4+ T cell counts (≤50 cells/μl). In **paper III**, HIV-1 from eight patients that had developed CXCR4-using virus variants during disease progression, "switch virus patients", were studied. From each patient two virus isolates, previously determined to be CXCR4-using[247], were selected; the earlier isolate was the first CXCR4-using isolate detected, and the later isolate was obtained when the CD4+ T cell count was very low. The timing of CXCR4-using virus isolations was also matched according to the immune status, i.e. CD4+ T cell count, at the time of R5 virus isolation from "non-switch virus patients" studied in **paper I** and **II**. Primary HIV-1 isolates for **paper I-III** were obtained from PBMC of infected individuals[111].

In **paper IV** HIV-1 primary isolates from vertically infected children and their infected mothers were analyzed. Viruses from these mothers and children have been analyzed in previous studies in relation to clinical, immunological and virus parameters[248-251]. All virus isolates were isolated from patient PBMC or plasma and determined to be of the R5 phenotype[249]. From each mother one virus isolate was studied, sampled at or close to delivery, and from each child two virus isolates were obtained, sampled close to birth and in the subsequent phase of immunodeficiency.

Virus biological cloning system

Primary virus isolates used in **paper III** where all previously determined to be CXCR4-using[247] and could potentially included both monotropic R5 and X4 viruses as well as dualtropic R5X4 virus variants. Thus, in order to evaluate the evolution of CXCR4-using viruses we preformed virus biological cloning. By the use of a modified protocol based on limiting dilution and PBMC cultures[252], we could generate CXCR4-using virus biological clones from our primary virus isolates. These were subsequently tested for coreceptor phenotype (described below) and only the CXCR4-using virus variants were included in the study.

Characterization of viral phenotypic properties

Determination of coreceptor tropism

The coreceptor tropism of HIV-1 isolates used in **paper I, II** and **IV** had previously been determined[116, 249]. However, in order to exclude the R5 monotropic virus variants in **paper III**, the U87.CD4 cell line expressing CCR5 or CXCR4 was used for virus coreceptor determination. Detection of infection was based on both visible syncytium formation and a significant increase in p24 antigen production. In addition, coreceptor usage was also predicted by gp120 V3 loop sequences using the bioinformatic algorithm position-specific scoring matrix $PSSM_{NSI/SI}$[105].

Virus infection assays

Direct infection of target cells was used in all papers. In **paper I** the target cells were PBMC and the C6 T-cell line, in **paper II** the target cell was the U87.CD4-CCR5 cell line, in **paper III** target cells included PBMC and the MT-2 cell line and in **paper IV** the target cells were PBMC. The reason for use of different target cells was practical. First choice of cells was PBMC to resemble the cell mosaic in the *in vivo* situation as much as possible. In addition to PBMC, cell lines were used in parallel to compare and verify the results obtained with PBMC targets. Initial experiments performed in the study of **paper II** also included PBMC, however, due to wide variation in parallel PBMC infections during neutralization assays the U87.CD4-CCR5 cell line was instead chosen.

The same principle of infection protocol was used for all target cells: 1) Prior to infection, the virus was normalized according to functional viral RT. 2) Next virus was added to target cells and allowed to incubate over night. 3) Following morning, the infected cultures were washed to remove residual inoculum virus. Virus replication was analysed by detection of the viral p24 antigen in cell supernatant at day 6-7 for suspension cells (PBMC, C6 and MT-2), or

determination of visible plaque forming units, syncytia, after 4 days for adherent cells (U87.CD4-CCR5 or -CXCR4). In **paper III** viral infectivity was determined by 50% tissue culture infective dose[253] (TCID50) and replication capacity was detected by p24 antigen release.

When normalizing inoculum virus dose in the infection assays, functional viral RT was chosen. RT activity has been demonstrated to correlate well with viral RNA levels, and would thus be an appropriate measurement of infectious viral particles[254, 255]. Viral p24 content would also be possible to use but p24 levels show lower correlation with RNA levels, since p24-capturing assays measure all p24 included in infectious, non-infectious and decaying viruses.

Virus *trans*-infection assays

In **paper I**, **III** and **IV**, direct infection of target cells were run in parallel with DC-SIGN mediated *trans*-infection of target cells, enabling comparison of relative use of DC-SIGN in *trans*-infections. PBMC and C6 cells were used as target cells in **paper I**, MT-2 cells were used as target cells in **paper III** and PBMC was used as target cells in **paper IV**. Ramos cells, a B-cell line, engineered to express DC-SIGN[256] was used to mediate the *trans*-infection. In the *trans*-infection setup RT normalized virus was added to irradiated Ramos/DC-SIGN cells for adherence during three hours. Next, the virus-pulsed Ramos/DC-SIGN cells were washed thoroughly to remove unbound virus, before target cells were added to the culture and the *trans*-infection was analysed after 6-7 days of culture. Relative efficiency of DC-SIGN mediated *trans*-infection was evaluated as the ratio between *trans*-infection and direct infection.

Virus binding assay

Determination of virus binding ability to the DC-SIGN receptor was evaluated in **paper I**. Both Ramos/DC-SIGN and wild type Ramos cells, without DC-SIGN, were used to evaluate the specific binding of virus to DC-SIGN. RT-normalized virus was incubated with the Ramos cells for three hours. Then the cells were extensively washed to remove unbound virus, before they were lysed and the lysate, as well as the inoculum virus, was analysed for viral p24 content.

Head-to-head competition assay

To compare the fitness of viruses in direct infections and in DC-SIGN mediated *trans*-infections in more detail, we allowed viruses to compete side-by-side in direct infections and *trans*-infections in **paper I** and **IV** (Figure 13). Virus isolates were mixed at a 1:1 ratio according to RT content. Next the mixture was diluted in several steps to limiting inoculum virus dose. The serial diluted mixtures were added directly to target cells, PBMC, or to Ramos/DC-SIGN cells for three hours pulsing and subsequent *trans*-infection. The infections followed the above described assays, direct and *trans*-infection respectively. To identify which virus

that dominated in the competition after seven days, sequencing of integrated provirus was done. Prior to the competition assay, gp120 V2 region of tested primary isolates had been sequenced for identification of unique virus signature sequences. Thus, replicating virus in the competitions was identified according to these known gp120 V2 signature sequences.

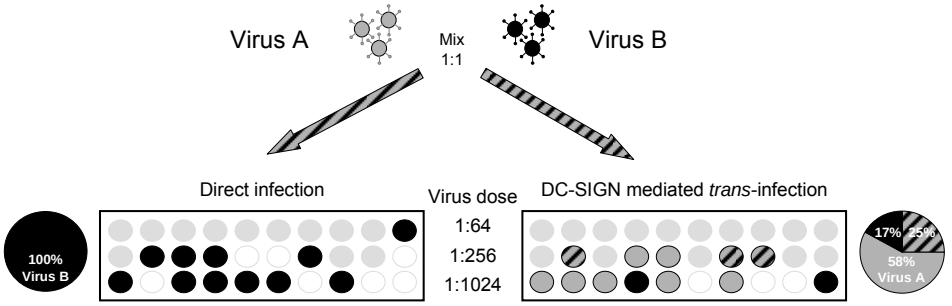

Figure 13. Head-to-head competition assay. Two different virus isolates, A and B, were mixed at a 1:1 ratio. The mixture was diluted and used in PBMC infection, either direct infections or DC-SIGN mediated *trans*-infections. Replicating virus in the competition was determined by sequencing, illustrated as black "cultures" if virus B was identified, dark gray if virus A was identified and black/dark gray if both viruses were identified. Light grey and white illustrate cultures infected but not sequenced or not infected, respectively.

Virus neutralization assay

Neutralization sensitivity was evaluated for R5 viruses in **paper II**, by use of the previously published U87.CD4 plaque reduction assay[257]. For the analysis of virus sensitivity to broadly neutralizing monoclonal antibodies (mAbs) we used TriMAb. TriMAb is an equal molar ratio mixture of three broadly neutralizing monoclonal antibodies, IgG1b12[224], 2G12[230] and 2F5[258]. The TriMAb mix or the mAbs alone were incubated with virus for one hour, to allow neutralization, before addition to target cells, U87.CD4-CCR5 cells. Then the neutralization assay followed the protocol described by Shi *et al.*[257]. Neutralization of virus was measured as plaque reduction in the presence of antibodies as compared with control infections without antibodies, and presented as the concentration needed to inhibit 50% of virus replication (IC50).

Characterization of Env molecular properties

Generation of *env* clones

In order to study the evolution of Env properties of HIV-1 in **paper I-III**, we preformed cloning of *env* of our primary isolates. For the CXCR4-using viruses

studied in **paper III**, this was accomplished along with the biological cloning. For the R5 viruses used in **paper I** and **II**, the *env* gene was molecular cloned. Viral DNA was isolated from PBMC infected with the R5 viruses. The *env* gene was amplified using a nested PCR approach. The amplified product was cloned into the pSVIIIenv expression plasmid and four clones from each isolate were selected and sequenced for subsequent analysis.

Sequence analysis of clones

Sequence studies of R5 viruses in **paper I** and **II**, was performed on clones containing the full-length *env* gene including both gp120 and gp41. Sequence analysis of the CXCR4-using viruses in **paper III** was limited to the *env* fragment encoding gp120. Prior to sequence analysis of the separate R5 and CXCR4-using viruses, a maximum likelihood tree, containing all clones, was constructed which ruled out any contamination between specimens. Sequence analysis in **paper I** included finding potential correlates between Env PNGS and efficient virus DC-SIGN use. In **paper II** the total numbers of Env PNGS for each isolate was analyzed, by using the N-glycosite tool in the HIV-1 sequence database [http://www.HIV-1.lanl.gov]. In **paper III** numbers of gp120 PNGS, amino acid length and net positive charge of the different isolates were analyzed. The gp120 net positive charge was calculated according to the amino acids lysine (K) and arginine (R), contributing with +1 each, and the amino acids aspartic acid (D) and glutamic acid (E), contributing with -1 each.

Molecular modeling of gp120

Molecular modelling of trimeric gp120 performed in **paper II** was based on the crystal structure of the CD4 bound gp120 trimer which excludes the V1V2 region[23]. Glycan alterations were modelled on the structure and the essential glycans for the epitope of the 2G12 mAb were illustrated[227, 228].

Results and discussion

Viral evolution during late stage disease

Main findings

- Viral infectivity and replication capacity increase at end-stage disease, both in R5 and CXCR4-using HIV-1 populations (**paper I, III**)
- Increase in viral infectivity correlates with Env modifications; decreased numbers of PNGS in gp120 of R5 HIV-1, and increased net positive charge in gp120 of both R5 and CXCR4-using HIV-1 (**paper II, III**)
- Sensitivity to broadly neutralizing antibodies increase at end-stage disease, in R5 HIV-1 population (**paper II**)
- Increase in R5 HIV-1 sensitivity to broadly neutralizing antibodies, including 2G12, correlates with decreased numbers of PNGS and increased net positive charge of gp120 (**paper II**)

With the aim of understanding how HIV-1 evolves both phenotypically and molecularly during late-stage disease, virus isolates from 15 patients were studied. Seven of these patients harbored virus isolates exclusively using CCR5 as a coreceptor throughout the entire disease (**paper I** and **II**), and the remaining eight had viruses that switched their coreceptor use to include CXCR4 (**paper III**).

Viral infectivity

To study whether viral infectivity evolves during late-stage disease, we allowed primary R5 viruses from different stages of the disease, the chronic and the end-stage phases, to compete with each other in the same infection, called head-to-head competition (**paper I**). The end-stage viruses displayed enhanced fitness over the chronic viruses, by dominating in direct PBMC infections (Figure 14). This result was observed both in intra-patient competition, where chronic and end-stage viruses were isolated from the same patient, and inter-patient competition, where chronic and end-stage viruses were isolated from different patients. The enhanced fitness of end-stage R5 HIV-1 for direct infection is in agreement with previous R5 HIV-1 studies of us and others[115, 120, 121].

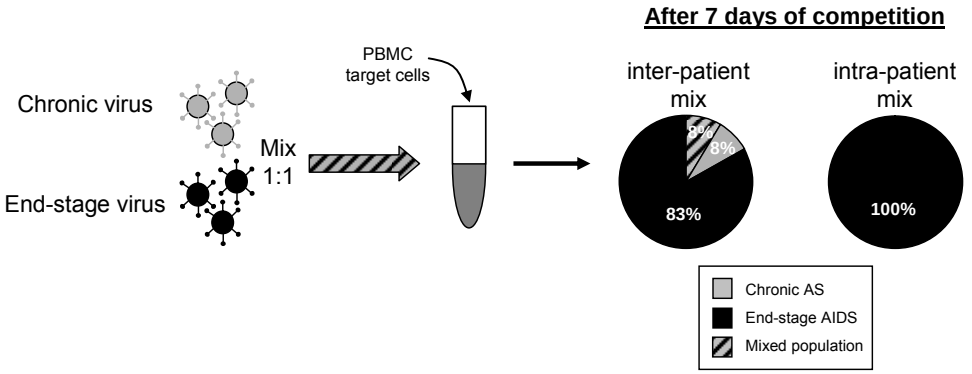

Figure 14. End-stage R5 viruses dominate in direct PBMC head-to-head competition infections. Chronic and end-stage R5 viruses, isolated from different patients (inter-patient) or from the same patient (intra-patient), were mixed at a 1:1 ratio and competed head-to-head in direct PBMC infections. Replicating virus was determined after seven days and presented percentages were calculated from parallel infections.

Studies of changes in infectivity of CXCR4-using viruses emerging after the coreceptor switch is limited[259]. In **paper III,** we aimed to investigate if a similar increase in viral infectivity, as seen in the end-stage R5 viruses, was observed in the CXCR4-using virus population. Prior to the infectivity studies, CXCR4-using virus biological clones were generated from isolates obtained during the chronic and end-stage disease. Considering the average infectivity and replicative capacity of CXCR4-using virus clones from individual isolates, we observed an increase in the T cell infectivity and replication capacity of the end-stage viruses compared with corresponding chronic isolates (Figure 15). Increased infectivity and replication also correlated inversely with decreasing CD4+ T cell count, suggesting that the observed phenotypic alteration is a consequence of a declining immune response in the host.

CXCR4-using viruses have been demonstrated to be more replicative then R5 virus *in vitro*[260], however we could not verify this when comparing results previously published[121] and obtained in **paper III**. Instead, we noted a continuous evolution of the infectivity and replicative capacity of CXCR4-using viruses after the coreceptor switch and during the progression to end-stage AIDS. The first emerging CXCR4-using viruses have been suggested to develop through less fit intermediate stages, which then would evolve toward better replicative competence[261]. Thus, our results are in agreement with Shakirzyanova *et al.*[261], as our chronic virus variants with lower infectivity and replication capacity were isolated when CXCR4 use was first detected. The concordant pattern toward increased infectivity and replicative capacity in both R5- and CXCR4-using virus populations indicates a similar immune pressure exerted on these different types of virus by the host. One possible explanation for the development of increased

infectivity seen at the end-stage of the disease could be the presence of extremely low numbers of target cells. HIV-1 variants that infect target cells more efficiently may be selectively favored when target cells are declining, resulting in increased viral fitness. Indeed, we were able to correlate the infectivity and replicative capacity with the CD4+ T cell count at the time of virus isolation for the CXCR4-using viruses, and the same correlation has previously been published by our group in regard to evolution of R5 viruses[121]. In agreement with these findings, a recent publication by Bunnik *et al.* also confirms that virus isolated when the CD4+ T cell count was below 100 cells/µl showed higher replication than virus isolated above 100 cells/µl[262]. Another explanation for the emergence of more infectious viruses might be the waning immune pressure. A functional immune system selects for virus variants good at hiding, i.e., virus immune escape variants. Features of HIV-1 escape variants might influence and reduce the infectivity. More infectious virus variants might be inhibited and held under control when the immune system is relatively intact during the chronic phase. When severe immunodeficiency develops, these virus variants may instead be allowed to replicate and dominate in the host. Indeed, in the absence of immune pressure, such as during transmission to a new host, the reversion of escape mutations has been demonstrated and associated with viral fitness[263, 264].

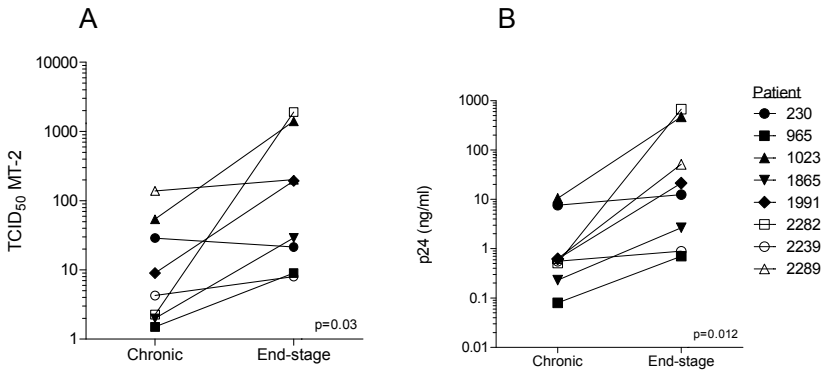

Figure 15. Increase in T cell infectivity and replicative capacity of CXCR4-using viruses at end-stage disease. CXCR4-using virus clones derived from isolates obtained at chronic and end-stage phase of disease were used in infection of MT-2 T cell line and compared in A) viral infectivity, measured by TCID50, and B) replicative capacity, detected by p24 antigen release.

Viral sensitivity to broadly neutralizing antibodies

To evaluate whether virus variants emerging at end-stage disease may display altered sensitivity to neutralizing antibodies, i.e., display reversion of immune escape, we analyzed whether chronic and end-stage R5 isolates were differently sensitive to broadly neutralizing antibodies (**paper II**). Our R5 virus panel was

tested for sensitivity to the well-known broadly neutralizing monoclonal antibodies 2G12, IgG1b12 and 2F5, separately or mixed as TriMAb. The end-stage R5 viruses were demonstrated to be of higher sensitivity to neutralization by the TriMAb mix than the corresponding chronic viruses (Figure 16a). When virus sensitivity to the separate monoclonal antibodies was tested, differences in sensitivity between chronic and end-stage viruses did not reach statistical significance, as several viruses were not neutralized to 50%. However, the 2G12 antibody tended to inhibit the end-stage viruses more than the chronic stage viruses (Figure 16b).

The increased sensitivity of end-stage R5 viruses to neutralization by the TriMAb mix of broadly neutralizing mAbs supports the theory that viruses with diminished ability to protect themselves from binding antibodies have increased infectivity. Indeed, it has been shown that in a newly infected individual with no pre-existing immune response, neutralization-sensitive viruses are responsible for establishing the infection[160]. This result suggests that neutralization-sensitive viruses have a selective advantage, maybe due to higher infectivity, over neutralization-resistant viruses. Our end-stage viruses were isolated at a time point when the virus-specific immune system was not efficient and likely did not exert a strong immune pressure, which shares some similarities with the environment of the newly infected host. In addition, it has been demonstrated that primary virus passaged repeatedly in T cell lines, in the absence of immune pressure, adapts to a more neutralization-sensitive phenotype[265-267]. In contrast, data have been published showing that escape from neutralization is not correlated with a decrease in replication capacity[222, 262]. However, different outcomes from these studies and our study might be due to the isolation time point of viruses, as our end-stage viruses were isolated at a very low CD4+ T cell count.

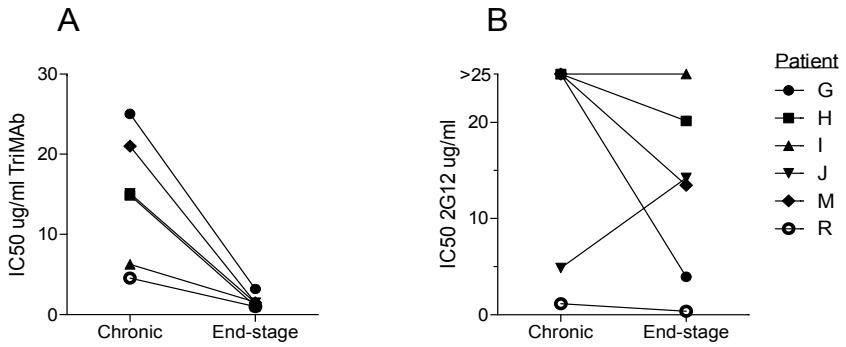

Figure 16. End-stage R5 viruses are more sensitive to neutralization by TriMAb. Chronic and end-stage R5 viruses were compared for sensitivity to neutralization by A) TriMAb mixture and B) monoclonal antibody 2G12, determined by calculation of IC50.

Of the monoclonal antibodies included in TriMAb, 2G12 demonstrated the largest differences between chronic and end-stage viruses. Thus, it seems that the different effects of TriMAb on our chronic and end-stage viruses were mainly due to the action of 2G12, even though the antibody alone appeared less potent. Indeed, it has been shown that when the three antibodies are mixed, they work synergistically[268-271], although these data are contradicted by other studies[218, 272]. The 2G12 antibody itself is a very special antibody, as it actually does not bind to the Env peptide backbone, but instead binds to the carbohydrates attached to gp120[227, 228]. The Env-attached carbohydrates are produced by the host cell machinery and are generally consider to be of low immunogenicity. However, because several of the gp120 carbohydrates are clusters of high-mannose type, which do not occur on mammalian glycoproteins[49], they provide a potential target for antibodies. In addition, 2G12 displays cross-reactivity to other pathogens, including opportunistic ones, such as *Candida albicans*, that are associated with HIV-1 infection[273]. Possibly an opportunistic infection parallel to the HIV-1 infection initially induced the 2G12 antibody production in the patient from whom the antibody was isolated. Although 2G12 neutralizes a wide range of virus isolates, it is not equally potent against all types of viruses due to variations in glycosylation[218, 274]. For instance, subtype C HIV-1 often lacks one or two of the essential glycans for 2G12 and is thus less sensitive to neutralization by this antibody.

Env glycosylation and charge

The most likely explanation for the observed phenotypical differences of the studied chronic and end-stage R5 and CXCR4-using viruses would be the alteration of Env. To explore the correlations between Env modifications and the evolution of the different phenotypes, we sequenced the *env* gene from molecular and biological clones of R5 and CXCR4-using isolates, respectively (**paper I-III**).

Within "non-switch virus patients" (**paper I** and **II**), we noted that the R5 virus population evolved toward decreasing numbers of gp120 PNGS with disease progression (Figure 17a). Previously, it has been demonstrated that HIV-1 Env will increase in numbers of PNGS with disease progression as a result of escape from the immune response[33, 159, 160]. However, our result of a decrease in Env PNGS has been confirmed by others to occur during severe immunodeficiency[159]. Thus, our observation adds to the model in which the R5 HIV-1 selected in the absence of immune pressure, in the acute phase and during severe immunodeficiency, has lost glycans in favor of replication efficiency. The PNGS numbers on our R5 viruses also correlated inversely with viral infectivity and neutralization sensitivity, i.e., viruses with fewer glycans were more infectious and easier to neutralize with the TriMAb mixture of broadly neutralizing antibodies (Figure 17b and c).

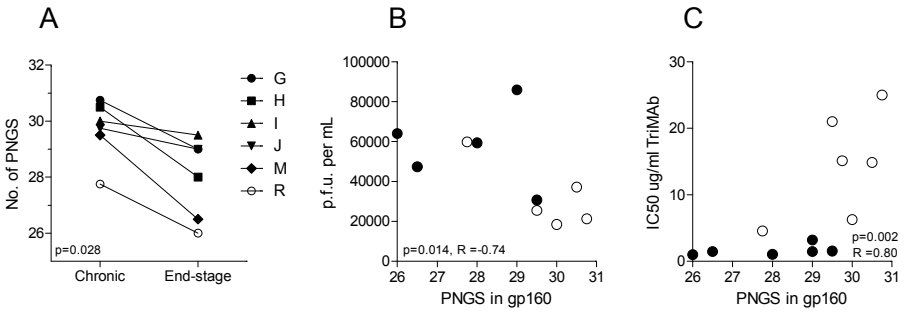

Figure 17. Evolution of PNGS numbers in R5 HIV-1 correlates with phenotypic modifications. A) Numbers of PNGS in gp160 decreases for end-stage R5 HIV-1. Numbers of PNGS correlates with B) viral infectivity and C) neutralization sensitivity.

In the "switch virus patients" with CXCR4-using viruses that evolved after the coreceptor switch (**paper III**), we did not find a clear general pattern of Env PNGS alterations with disease progression. However, six out of eight patients had a decrease in PNGS number over time. Instead, we noted that the observed increase in infectivity of individual CXCR4-using virus clones correlated with the net charge of gp120. The Env net positive charge of R5 HIV-1 has previously, by our group and others, been described to increase during the end-stage of the disease[122, 275]. A general pattern of gp120 net charge evolution was not observed when comparing chronic and end-stage CXCR4-using viruses, but six out of eight patients showed an increase in the positive net charge of gp120 over time. The connection between charge and infectivity could be explained by reduced electrostatic repulsion between the overall negatively charged target cells and virus particles, as previously suggested[276, 277]. An increase in the net charge of the gp120 peptide backbone, in addition to the loss of negatively charged glycans, would result in reduced repulsion and subsequently increase infectivity.

In summary, probably both numbers of PNGS and net charge of gp120 will affect viral infectivity and neutralization sensitivity. Perhaps the net charge is a larger modulator of infectivity, as it was correlated with both R5- and CXCR4-using viruses. The numbers of PNGS might be more important in the escape from neutralization. However, in the CXCR4-using virus population, the numbers of PNGS did not correlate with either infectivity or neutralization sensitivity for the clones tested (data not shown).

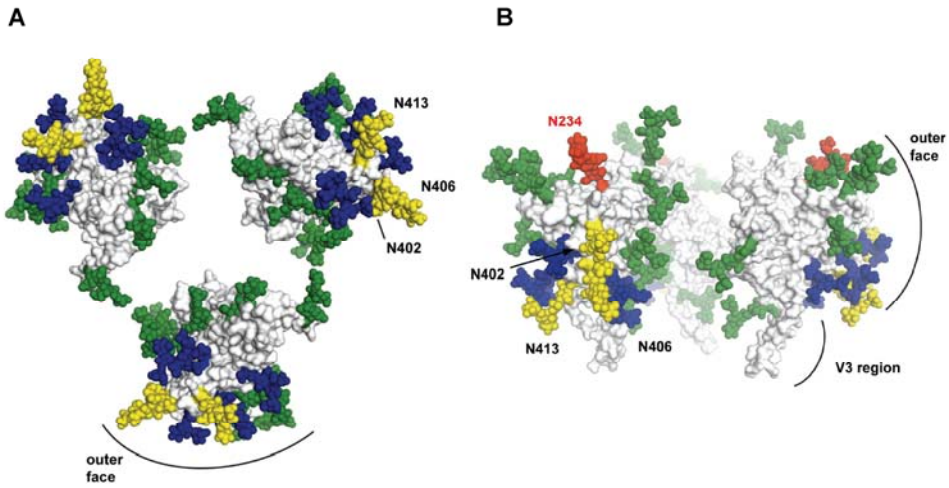

Figure 18. Lost PNGS of gp120 in end-stage R5 virus are located in the vicinity of the 2G12 epitope. Trimeric gp120 model with changes in glycans represented as spheres, derived from Env from patient M. Glycan changes is coloured as follow; yellow – loss of PNGS, red – gain of PNGS, green – conserved PNGS and blue – the 2G12 epitope. The trimer is seen from the target cell in A) and from the side in B) with the target side pointing downward.

Surprisingly, increased sensitivity to neutralization by the glycan targeting mAb 2G12 of end-stage R5 HIV-1 correlated with reduced numbers of PNGS and an increased net positive charge of Env (**paper II**). Because the end-stage R5 viruses displayed fewer glycans, it would be logical to assume that the glycan-binding antibody would bind less well to these viruses. Instead, the opposite was observed, and viruses harboring fewer glycans were more sensitive to 2G12 neutralization. This result might be explained by the epitope for 2G12 consisting of a few glycan sites on gp120 that are especially important for binding. These sites were mostly conserved on the end-stage R5 viruses, and instead, glycan sites surrounding the 2G12 epitope were lost (Figure 18). We speculate that the 2G12 epitope is more exposed if surrounding glycans are lost, thus making it easier for the antibody to bind. In line with our results and speculations, a recent publication demonstrated an increased resistance to 2G12 neutralization by viruses with elevated numbers of PNGS in the gp120 V1V2 and V3 regions[278]. Indeed, our analysis of PNGS lost in end-stage R5 viruses demonstrated that most modifications occurred in the variable regions, excluding the V3 region. Presumably, heavily glycosylated variable loops, such as V1V2, are likely to hide the 2G12-specific glycans and interrupt antibody binding. Similarly, it has also been reported that other determinants in gp120 affect 2G12 binding, as viruses resistant to 2G12 bind have been reported to harbor all the critical glycan sites[279-281]. According to molecular modeling, we noted that the elevated positive surface charge of gp120 of end-stage R5 HIV-1 clustered around the 2G12 epitope; however, as the V1V2 region is not

included in our model, we could not predict how charge alterations in this region would interfere with the 2G12 epitope. Nevertheless, the Env peptide backbone displaying a higher net positive charge significantly correlated with higher sensitivity to 2G12 neutralization. The connection between antibody binding and antigen surface charge is not well documented, but it has been shown that the positive charge of V3 region has a positive effect on antibody binding, and gp41-directed antibodies preferably bind to positively charged surfaces[282, 283]. Taken together, both loss of glycans and increase in surface charge seem to increase the sensitivity of R5 HIV-1 to 2G12 neutralization.

DC-SIGN use during transmission and disease progression

Main findings

- DC-SIGN binding and use for *trans*-infections is decreased in R5 HIV-1 emerging at end-stage disease of adults (**paper I**)
- Efficient binding and use of DC-SIGN correlates with the presence of a specific glycan site in the V2 region of gp120, in both R5 and CXCR4-using HIV-1 (**paper I, III**)
- Efficient DC-SIGN use appear not to correlate with vertical transmission of R5 HIV-1 (**paper IV**)
- Ability of R5 HIV-1 to use DC-SIGN increases within vertically transmitted children, from early after birth to established immunodeficiency (**paper IV**)

With the aim to understand how HIV-1 DC-SIGN use evolves during late-stage disease, 15 infected adult individuals were included, seven "non-switch virus patients" (**paper I**) and eight "switch virus patients" (**paper III**). Furthermore, R5 HIV-1 DC-SIGN use was studied during vertical transmission and the following pediatric disease progression in six mother-child pairs (**paper IV**).

Evolution of R5 HIV-1 DC-SIGN use during late stage disease

In **papers I** and **III**, we studied whether HIV-1 phenotypic evolution during late-stage disease also includes alterations in the use of DC-SIGN for *trans*-infection. DC-SIGN has been suggested to play a potential role in transporting HIV-1 to target cells and increasing the efficiency of T cell infections via the virological synapse[134]. Because the receptor is expressed in various possible transmission tissues *in vivo*, such as the rectum, female genital tract, colon, small intestine,

placenta and blood, DC-SIGN use has been suggested to play a role in the initiation of infection[148-152, 284-289]. However, how the viral ability to use DC-SIGN for *trans*-infection evolves with disease progression has not been extensively elucidated. In **paper I,** we demonstrated that R5 HIV-1 from end-stage disease had a diminished ability to bind to DC-SIGN compared to the corresponding chronic stage isolate. In addition, the ability of end-stage R5 isolates to use DC-SIGN for *trans*-infection was decreased (Figure 19a). The elevated ability of chronic stage R5 viruses to utilize DC-SIGN-mediated *trans*-infection was confirmed in head-to-head competition assays. In parallel to the direct infection competitions described above, *trans*-infection competitions were set up. In both intra-patient and inter-patient competitions, virus isolated during the chronic phase dominated over end-stage viruses (Figure 20). These findings indicate that chronic viruses have an advantage in *in vitro* infections mediated by DC-SIGN and would presumably benefit from using DC-SIGN *in vivo*. It has been suggested that DC-SIGN use serves as an escape from neutralizing antibodies, either by immune evasion from the neutralizing effects[147] or by blocking the neutralizing epitopes[146]. In addition, myeloid DCs expressing DC-SIGN have been shown to transmit virus to target cells in presence of neutralizing antibodies[290]. Thus, it seems that during the chronic phase of the disease, the relatively intact immune pressure may select for viruses with the ability to use alternative routes of infection, such as DC-SIGN mediated *trans*-infection. However, when the immune pressure wanes at the point of severe immunodeficiency, the benefits of using DC-SIGN are no longer needed, and other features, such as enhanced direct infectivity, are selected for.

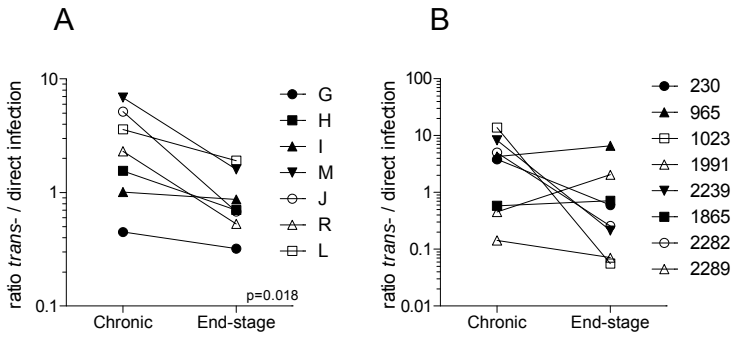

Figure 19. Different evolution of DC-SIGN use for R5 and CXCR4-using HIV-1. Relative use of DC-SIGN mediated *trans*-infection was compared for A) sequential R5 HIV-1 isolates and B) sequential CXCR4-using HIV-1 variants.

The observed evolution of the DC-SIGN use of R5 HIV-1 in "non-switch virus patients" was not confirmed in CXCR4-using viruses (**paper III**). Here, viruses from five out of eight patients showed a decrease in the use of DC-SIGN over

time, whereas the opposite was observed in viruses from three patients (Figure 19b). Possibly, CXCR4-using viruses are under higher immune pressure late in the disease compared to R5 viruses, and accordingly, viruses that maintain a strong ability to use DC-SIGN would be retained. Indeed, it has been shown that blocking *trans*-infection with CXCR4-using viruses requires more neutralizing mAb than blocking *trans*-infection with R5 HIV-1[291]. The same study suggested that CXCR4-using viruses used DC-SIGN-mediated *trans*-infection more efficiently than the R5 viruses. However, our results, obtained from *trans*-infections of R5- and CXCR4-using virus types, do not reveal such differences, as the use of DC-SIGN was within the same range for both viral phenotypes. Instead, we observed that the use of DC-SIGN varied widely within patients, depending on time point of virus isolation. Thus, we suggest that DC-SIGN use may, independent of coreceptor use, instead be influenced by other Env features.

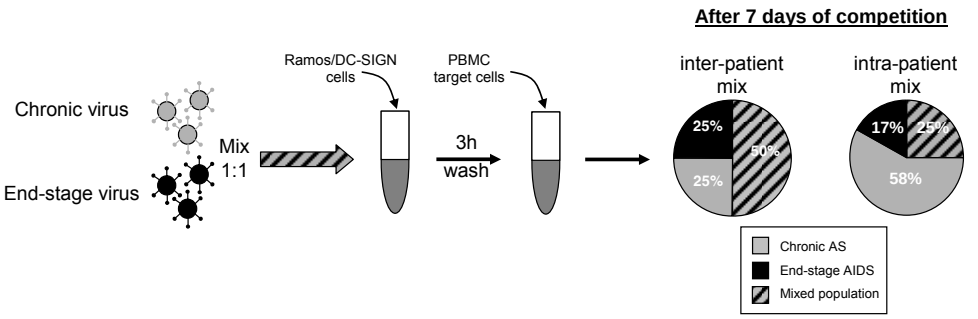

Figure 20. DC-SIGN mediated *in vitro* selection of chronic R5 viruses in head-to-head competitions. Chronic and end-stage R5 viruses, isolated from the different patients (inter-patient) or from the same patient (intra-patient), were mixed at a 1:1 ratio and competed head-to-head in DC-SIGN mediated *trans*-infections of PBMC. Replicating virus was determined after seven days and presented percentages were calculated from parallel infections.

DC-SIGN use of vertically transmitted R5 HIV-1

A role for DC-SIGN in HIV-1 transmission into a new host has been suggested, but little evidence except for the expression of DC-SIGN at sites of transmission has been presented[148-152, 284-289]. A possible role would be the "gatekeeper", resulting in selective transmission and a homogenous virus population initiating the infection in the new host. To study DC-SIGN use soon after transmission, we had the opportunity to study R5 HIV-1 isolates from vertically infected children and their transmitting mothers (**paper IV**). By the analysis of sequential isolates from the children, we could also follow the evolution of R5 HIV-1 DC-SIGN use from early after transmission until immunodeficiency had developed. All six children included were infected during pregnancy, either *in utero*, or during delivery, *intrapartum*, as they were not breastfed. Both types of infection involve

transmission across the placenta, as placental microtransfusions occur during delivery[175], and could possibly include DC-SIGN participation, as DC-SIGN is expressed by placental cells, both on the maternal side and on the fetal side[152]. During delivery, HIV-1 could potentially also transmit through the ingestion of maternal blood and genital secretions, and DC-SIGN is expressed in GALT[288]. The relative efficiency of DC-SIGN use was determined in parallel for maternal viruses, isolated at or close to delivery, and for viruses in the children, isolated close to birth and after immunodeficiency had developed. Surprisingly, the child's first virus, isolated early after birth, was found to use DC-SIGN for *trans*-infection less efficiently compared to the corresponding maternal virus in five out of six cases (Figure 21a). Moreover, when comparing the sequential isolates from the children, the follow-up virus obtained late in the disease displayed increased use of DC-SIGN for *trans*-infection in five out of five cases (Figure 21b). This finding was also confirmed in head-to-head competitions, where the child's first isolate was mixed with the maternal isolate or the child's follow-up isolate from late in the disease. Both the maternal isolate and the child isolate from the immunodeficiency period dominated in the competition over the first child virus isolate. Taken together, the use of DC-SIGN appears not to be an advantage during vertical R5 HIV-1 transmission, and the viral ability to utilize DC-SIGN for *trans*-infection seems instead to increase throughout the disease progression.

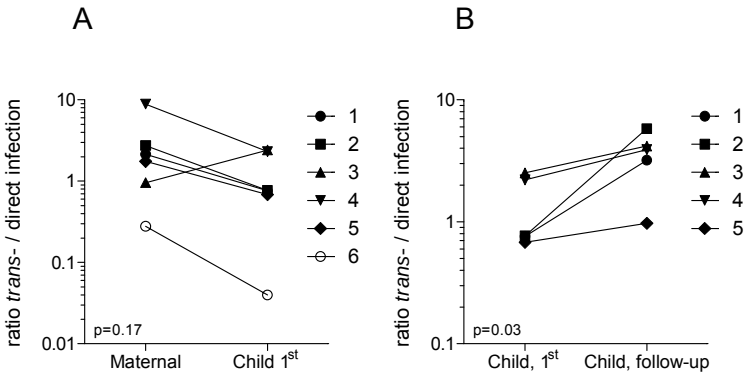

Figure 21. DC-SIGN use is diminished for virus obtained close to vertical transmission. R5 virus isolates from vertically infected children obtained at birth were tested in a DC-SIGN mediated *trans*-infection assay, and compared with A) maternal delivery isolate and B) the follow-up isolate from the same child. The mother-child pairs were numbered from 1-6.

In agreement with our observation on the diminished use of DC-SIGN for acute viruses, a recent publication by Boily-Larouche *et al.* demonstrated that a polymorphism influencing the expression of a DC-SIGN homologue, DC-SIGNR or L-SIGN[292], correlated with the risk of vertical transmission[293]. DC-SIGNR is

not expressed by DCs, but is expressed on endothelial cells in the placenta, in the lymph nodes and in the liver[294, 295]. DC-SIGNR binds the same ligands as DC-SIGN and can act as a *trans*-infection receptor for HIV-1[295] or an antigen capture receptor[296]. The study by Boily-Larouche *et al.* showed that the reduced expression of placental DC-SIGNR increased the risk of vertical transmission. This result suggests that DC-SIGNR may play a protective role during transmission across the placenta by preferentially degrading HIV-1 instead of transferring the virus to fetal target cells. Perhaps DC-SIGN plays a similar role during vertical transmission. The two receptors are not expressed by the same cells, but are in close proximity to each other and to the fetal target cells[297]. The factor that determines whether DC-SIGN-bound HIV-1 is degraded or transferred to target cells is unknown, but a recent publication showed that the composition of gp120 carbohydrates, either high-mannose or complex types, is a possible determinant for the outcome[298]. Higher expression of high-mannose types enhances DC capture but decreases the transmission to target cells, as the virus is degraded instead. Thus, we speculate that efficient DC-SIGN use may be a disadvantage in vertical transmission across the placenta, and virus variants with a diminished ability to bind DC-SIGN are selected for during vertical transmission.

The observed enhanced use of DC-SIGN for *trans*-infection for virus isolated during immunodeficiency in children indicates that DC-SIGN use also may evolve with disease progression in pediatric infections. This result is in agreement with the model in which efficient use of DC-SIGN protects viruses from an evolving immune response. However, the virus isolated during immunodeficiency in adults (**paper I**) demonstrated diminished use of DC-SIGN compared to chronic isolates. Unfortunately, viruses from the chronic phase of disease in the children were not possible to analyze, and it is plausible that viruses with even better use of DC-SIGN could have been demonstrated during the chronic phase. Alternatively, R5 HIV-1 evolution in children might not be the same as in adults, as children display different levels and functions of immune cells, and the disease progression can be accelerated[201-203].

Efficiency of DC-SIGN use related to the gp120 sequence

For the purpose of identifying determinants of efficient HIV-1 DC-SIGN use, in **paper I** and **III** we have correlated the gp120 sequence alteration in the tested R5- and CXCR4-using viruses with level of DC-SIGN use. Previously, DC-SIGN use has been coupled to alterations of specific regions of gp120, with respect to amino acid length, charge and numbers of PNGS[299]. However, neither amino acid length, net charge nor the total number of PNGS in gp120 of the tested R5- and CXCR4-using viruses correlated with DC-SIGN use. Instead, we found that the presence of a specific PNGS, amino acid 160 (aa160; numbered according to HxB2 sequence, Genbank accession number KO3455), in the gp120 V2 region, correlated with efficient use of DC-SIGN (**paper I** and **III**) (Figure 22). This result was observed

in both R5- and CXCR4-using viruses. Furthermore, this is in agreement with a previous study where the presence of a glycan site in aa160 of a SHIV-1 variant led to increased DC-SIGN binding and enhanced mucosal transmission[300]. The same study also demonstrated that glycan modifications that increased the interaction with DC-SIGN also conferred escape from neutralization, which also is in line with our results. The chronic R5 viruses studied, which often harbored PNGS in aa160, were better at using DC-SIGN and were also less sensitive to broadly neutralizing antibodies (**paper I** and **II**). Indeed, it has been reported of cases where PNGS in aa160 were not present in viruses found soon after infection during the acute phase. Instead the glycan site in aa160 seemed to be acquired under immune selection and evolved after a few months and then became fixed[301]. In fact, a majority of the sequences reported in the Los Alamos HIV-1 Sequence Database harbor this PNGS. Interestingly, a recent study by McLellan *et al.* showed that the PNGS in aa160 is critical for recognition by the recently discovered broadly neutralizing antibody, PG9[38]. Thus, it seems that one glycan can both be selected for during immune evasion, to escape the immune response, and at the same time be recognized by very potent antibodies. Other glycan sites important for DC-SIGN binding have been shown to overlap with the specific epitope for the 2G12 antibody[302, 303]. However, in the studied R5 viruses, the 2G12 epitope glycans were highly conserved (**paper II**), which was also true for the CXCR4-using viruses, and thus could not be related to variation in DC-SIGN use. All the maternal and child isolates analyzed in **paper IV** harbored the aa160 PNGS and thus could not explain the difference in DC-SIGN use observed in this case (data not shown). Therefore, results from the vertically transmitted children suggest that the PNGS in aa160 cannot be the only determinant for efficient DC-SIGN use. Probably several components, as suggested by us and by others[291, 299, 302, 303], contribute to the efficient use of DC-SIGN for mediating HIV-1 *trans*-infection.

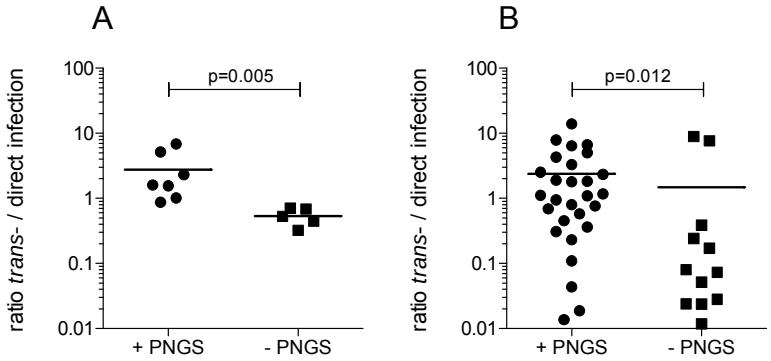

Figure 22. DC-SIGN mediated *trans*-infections correlate with presence of PNGS in aa160 of V2 gp120. Viruses were classified according to presence, +PNGS, or absence, -PNGS, of glycan site in aa160 in V2 region of gp120. A) DC-SIGN use of R5 virus isolates. B) DC-SIGN use of CXCR4-using virus variants.

Concluding remarks

Thirty years after the first cases of AIDS, we still do not have a cure or a vaccine against the HIV infection. More basic research and understanding of the biology of this complex virus seems to be the only promising approach for designing future successful therapies and HIV prevention strategies. In this thesis, I hope to have added to the understanding of how HIV-1 functions and evolves within infected individuals.

In parts of this work, we have focused on the viral evolution during late-stage disease, in "non-switch virus patients" and in "switch virus patients". Both R5 and CXCR4-using virus populations emerging during severe immunodeficiency demonstrated an increase in viral infectivity and replicative capacity. At the same time, R5 HIV-1 was found to be more sensitivity to broadly neutralizing antibodies during end-stage disease. These phenotypic alterations correlated with Env modifications. End-stage viruses harbored gp120 with fewer glycans and had an increase in positive net charge. The increase in Env positive charge and the reduced numbers of negatively charged glycans probably help the virus to infect the negatively charged target cells, due to diminished electrostatic repellent forces, and in turn more of epitopes sensitive to neutralizing antibodies are revealed (as modeled in the cartoon in Figure 23). The emergence of such viruses during immunodeficiency most likely reflects the waning of the immune response. Thus, the virus no longer needs the dense protective glycan shield of Env, and virus variants more efficient in infecting limited numbers of target cells are selected for. Because the lack of efficient antiviral immunity in the severely immunodeficient patients could resemble the status of the newly infected individual, it is possible that viruses initiating the infection in certain ways may resemble viruses emerging during end-stage disease. Therefore, knowledge from our studies could potentially help in designing new Env immunogens, thereby revealing more epitopes recognized by broadly neutralizing antibodies. It would also be of interest to investigate whether virus evolution during disease progression is similar in cART treated patients. There are still some patients where the current combination treatment will fail, and in these cases, new and individualized treatment strategies, possibly based on the inhibition of virus target cell entry, would be beneficial.

Studies on R5 viruses emerging during disease progression also demonstrated an evolution in the use of DC-SIGN, a C-type lectin expressed by dendritic cells, for *trans*-infection of target cells. In the adult individuals, we noted that end-stage R5 HIV-1 was less able to bind and use DC-SIGN for *trans*-infections than viruses from the chronic phase of the infection. This difference probably also reflects the immune status of the patient, as virus DC-SIGN-mediated *trans*-infection during

the immunocompetent phase could be a way for the virus to hide from the immune response (as modeled in Figure 23). In vertically R5 HIV-1-infected children, viruses isolated during immunodeficiency were more efficient in using DC-SIGN than viruses from early after birth, close to virus transmission. However, efficient use of DC-SIGN seemed not to be an advantage for viruses replicating early after birth, as corresponding maternal viruses in many cases displayed better use of DC-SIGN then virus transmitted to their children. This study is clearly restricted by the limited numbers of mother-child pairs and it would be of interest to follow this up with studies including viruses from more mother-child pairs. Another option is to include more and closer follow-up sequential isolates from these infected children to obtain a better picture of the evolution of DC-SIGN use during the complete disease course. Nevertheless, our study may still add to the understanding of, and also emphasize, the complex role of R5 HIV-1 DC-SIGN use during vertical transmission and the following pediatric disease progression.

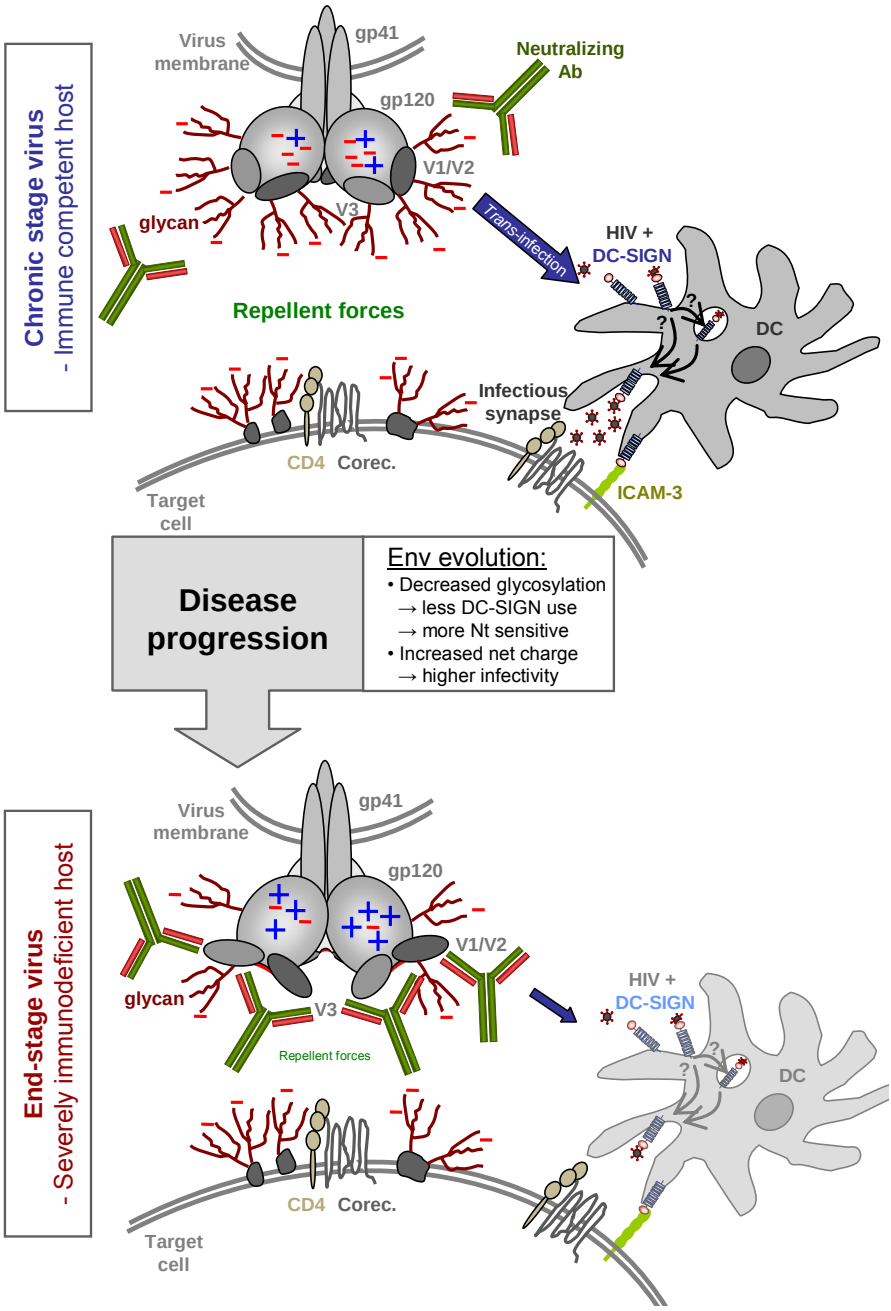

Figure 23. Cartoon on the evolution of HIV-1 Env and virus phenotypic properties during late-stage disease progression in relation to host interactions. Env evolution during late-stage disease progression results in gp120 with less glycans and increased net positive charge which translates into increased infectivity, less use of alternative ways of infection, such as DC-SIGN mediated *trans*-infection, and easier access for neutralizing antibodies to bind to Env epitopes.

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Appendices: Paper I-IV