Within-host HIV-1 evolution in relation to viral coreceptor use and host environment
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General Discussion
The study of the interplay between the human immunodeficiency virus type-1 (HIV-1) and its host environment helps to increase our understanding of HIV-1 pathogenesis and the factors determining the rate of disease progression. In this concluding chapter, HIV-1 evolution throughout the disease course in relation to viral coreceptor use and the potential host selective forces driving this process are being put in perspective. Moreover, the consequences of the continuous adaption of HIV-1 to its host environment for the interaction with its coreceptors and its implications for treatment with HIV-1 coreceptor inhibitors will be discussed.

**HIV-1 transmission and coreceptor use early in HIV-1 infection**

Establishment of the majority of sexually transmitted HIV-1 infections results from infection with and/or expansion of a single virus as has been shown by detailed studies of primary HIV-1 infection with sampling prior and around seroconversion (Fiebig stage I-V)\(^1,2\). This underscores the existence of a severe population bottleneck associated with mucosal HIV-1 transmission and establishment of infection in a new host, and is consistent with the genetically homogeneous viral population observed early in infection, which becomes more heterogeneous during the chronic phase\(^3\) (Chapter 5), the latter probably being the result of adaptation to the continuously changing new host environment.

This transmission bottleneck could offer an explanation for the predominance of R5 variants in acute infection. Several mechanisms (reviewed in Margolis et al.) acting at the mucosal level such as preferential trapping and inactivation of CXCR4-using variants by mucin and innate antiviral proteins, preferential infection of macrophages, dendritic cells (DCs) and Langerhans cells (LCs) by R5 variants, preferential capture and transfer of R5 variants from DCs and LCs to CD4+ T cells, and large availability of CCR5 expressing CD4+ T cells residing in the mucosa, could explain the advantage of viral variants with a CCR5-using phenotype to be transmitted and/or preferentially expanded. These mechanisms, however, cannot explain why CXCR4-using variants would be counter selected also when the route of transmission is via blood-to-blood contact, for instance after sharing of contaminated needles\(^5,6\), suggesting a role for non-mucosal barriers to the expansion of CXCR4-using viruses such as a higher susceptibility of these variants to humoral immunity or the existence of a viral reservoir in macrophages that favors the persistence of R5, and not CXCR4-using, variants (reviewed in Margolis et al.). The fact that individuals with a CCR5 Δ32/Δ32 genotype who completely lack CCR5 expression are highly resistant to HIV-1 infection\(^7,7-10\), supports that CXCR4-using variants are not easily transmitted. However, although unlikely, the transmission of CXCR4-using variants is possible as shown by the few cases of HIV-1 infected individuals with a CCR5 Δ32/Δ32 genotype from whom CXCR4-using variants have been isolated\(^11-18\). Another factor contributing to the predominance of R5 variants early in infection could be the higher likelihood that HIV-1 is transmitted by acutely infected individuals, generally unaware of their seropositive status, with a high viral load and likely to harbor only R5 variants\(^19-24\).

It has been reported that envelopes from newly transmitted subtype A and C HIV-1 are shorter, less glycosylated, and more sensitive to neutralization by plasma from the chronically infected partner than viral envelopes from the donor prior to transmission\(^25,26\). These features are not always observed for subtype B HIV-1 variants\(^1,26-28\), however,
several studies have shown that in HIV-1 subtype B infections early stage viruses, as compared to viruses that are present during the chronic phase of infection, display lower efficiency of CCR5 use, lower replicative capacity and cytopathicity and higher sensitivity to antibody neutralization. This raises the question whether viral characteristics of early stage viruses are advantageous for transmission. In Chapter 7, we show however, that adaptations acquired by viruses at the chronic phase of infection, such as increased efficiency of CCR5 use and a more glycosylated envelope with higher V3 charge, can be preserved upon transmission. If the possession of these characteristics that support more efficient virus replication, more efficient use of the cellular entry complex, and evasion from the immune system is not disadvantageous for transmission of the virus, then the higher risk of transmission during the acute phase of HIV-1 infection is more likely the responsible factor for the predominance of R5 HIV-1 variants in early infection. Transmission of viruses with a more “late stage” phenotype may contribute to the evolution of HIV-1 at a population level. Studies of HIV-1 transmission chains and knowledge of the characteristics of the transmitted virus may help to better understand evolution of HIV-1 molecular and biological properties both within a host and throughout the epidemic.

Origin of CXCR4-using variants

Several studies have suggested that CXCR4-using variants evolve from R5 variants. Recent findings in different cohorts have shown that CXCR4-using variants are not necessarily rare during primary infection, with varying frequencies depending on whether a phenotypic (3-6.4%) or a genotypic (13-17%) assay was used to determine viral coreceptor use. Although this would argue for a more frequent transmission of CXCR4-using variants than traditionally believed, it cannot be excluded that the prevalence of CXCR4-using variants may have been overestimated due to the use of genotypic assays and coreceptor use prediction tools. The observation that failure under treatment with the CCR5 inhibitor Maraviroc can be caused by the outgrowth of CXCR4-using HIV-1 variants that existed already before the onset of therapy raises the concern that both R5 and CXCR4-using variants are initially transmitted and while R5 variants are initially preferentially expanded, CXCR4-using variants may be maintained in a reservoir from which active replication is only started when R5 variants are suppressed. In Chapter 5, we confirm de novo evolution of CXCR4-using variants in 10 HIV-1 infected individuals by showing that newly detected CXCR4-using variants are genetically more closely related to the pre-existing R5 variants. Indeed, this is further supported by the fact that in one of those HIV-1 infected individuals, emergence of CXCR4-using variants occurred despite the fact that the donor only harbored R5 variants during and after transmission (Chapter 7). Even with the use of ultra-deep sequencing, which allows for the detection of minor variants, detection of sequences with predicted CXCR4 use followed a period in which only R5 variants were detected (Chapter 8). Moreover, the ability of recently emerged CXCR4-using variants to use CCR5, even if only in highly expressing CCR5 cell lines, which can be lost later in infection (Chapter 5) or the detection of intermediates between CCR5 and CXCR4 (Chapter 8), supports a transition from CCR5 to CXCR4 use. Taken together, these observations point towards a selective transmission and/or initial outgrowth of R5 variants, which may acquire the ability to use CXCR4, through evolution in some
HIV-1 infected individuals, rather than the (re-)appearance of these viral variants from a reservoir that was established at the time of infection.

**Transition from a CCR5- to a CXCR4-using phenotype**

The likelihood that an R5 variant acquires CXCR4 use should theoretically be high considering the minimal required sequence changes for a switch from CCR5 to CXCR4 co-receptor use\(^ {53-59}\) and that these mutations must be occurring continuously *in vivo* due to the exceptionally high mutation rate of HIV-1\(^ {60}\). Based on this, emergence of CXCR4-using variants would be expected to occur frequently and relatively early in the course of infection. However, CXCR4-using viruses do not emerge in all infected individuals with HIV-1 subtype B\(^ {61,62}\) and when they do, they are not generally detected at an early disease stage\(^ {51}\). Indeed, CXCR4-using variants emerge relatively slow in the majority of HIV-1 subtype B infections. Moreover, although the transition from CCR5 to CXCR4 use may occur several times throughout the disease course (Chapter 5, 6 and 8), the emergence of CXCR4-using variants that subsequently persist as successfully replicating viruses occurs only once (Chapter 5). Altogether this suggests that the evolution towards a CXCR4-using phenotype is strongly disfavored *in vivo*. Indeed, the R5X4 variants isolated close to the estimated emergence of CXCR4-using variants replicated slower in a CCR5 expressing cell line as compared to their co-existing R5 variants (Chapter 5). The transition from CCR5 to CXCR4 use may involve a stage of reduced viral fitness, as reflected by the lower efficiency of coreceptor-mediated entry and the higher susceptibility to neutralizing antibodies of intermediate variants\(^ {63,64}\). Once a transition from CCR5 to CXCR4-use results in successfully replicating CXCR4-using variants, newly emerging intermediates are probably outcompeted by the already existing replication competent CXCR4-using variants.

In Chapters 5, 6 and 8 we show that multiple mutational pathways can lead to the acquisition of CXCR4 use by R5 variants, but that there are certain requirements for the evolution towards a successfully persisting CXCR4-using phenotype *in vivo* (Chapter 5 and 6) which involve the gain of at least one positively charged amino acid in the V3 region resulting in a higher V3 charge, which may facilitate the interaction with acidic residues in CXCR4\(^ {65}\). Interestingly, the requirement of positively charged amino acids at V3 positions 11 or 25 for CXCR4 use, was not absolute (Chapter 5). Consistent with other studies\(^ {57,63,66-68,75}\), amino acid residues in gp120 envelope regions outside V3 also appeared to be implicated in a successfully replicating CXCR4-using phenotype in half of the subjects studied. These may be additional mutations that compensate for the impact that the essential V3 region mutations required for the interaction with CXCR4 have on the envelope conformation. Therefore, they likely contribute to maintain its function as mediator of viral entry. Specific characteristics of the humoral immune response of each HIV-1 infected individual may explain the range of possibilities by which an R5 variant can acquire CXCR4 use, given that changes in the envelope conformation may also compromise the exposure of epitopes targeted by neutralizing antibodies.
Optimization of coreceptor use throughout HIV-1 infection and implications for the use of coreceptor inhibitors

In HIV-1 infected individuals in whom CXCR4-using variants are absent throughout the entire disease course, R5 variants evolve towards an increased efficiency of CCR5 use as reflected by an overtime increasing resistance to CCR5 inhibitors and natural ligands (RANTES, MIP-1α and MIP-1β) or decreasing dependence on specific binding regions in CCR5. Similarly, evolution towards a more efficient CXCR4 use has also been observed for CXCR4-using variants and for the respective CCR5 and CXCR4 use of co-existing R5 and CXCR4-using variants within the same HIV-1 infected individual (Chapter 5). Optimization of CCR5 use throughout HIV-1 infection occurs independently of the emergence of a viral population that can use CXCR4 (Chapter 5) and involves a decreased dependence on N-terminus, ECL1 and ECL2 of CCR5, while independence of ECL1 and ECL2 of CXCR4 leads to optimization of viral CXCR4 use. Alternatively or in addition to an increased binding affinity for the chemokine receptor, the ability to change the binding domains used for the interaction with the coreceptor may explain the increased resistance of late stage R5 variants to CCR5 natural ligands that bind the N-terminus and ECL2 or of late stage CXCR4-using variants to AMD3100 that binds to ECL2 and ECL3. Moreover, R5 variants can become resistant to small molecule CCR5 inhibitors, including Maraviroc that is currently used as antiretroviral, by recognizing the complex of CCR5 and inhibitor while maintaining their ability to use CCR5. This mechanism is consistent with the mode of action of these inhibitors, which bind to the extracellular domains of CCR5, ECL2 in the case of Maraviroc, and alter their conformation. The increasing flexibility of HIV-1 to use CCR5 throughout the natural disease course suggests limitations to the use of Maraviroc in patients who harbor such late-stage variants. Characterization of CCR5 use and Maraviroc sensitivity in therapy naïve HIV-1 infected individuals could therefore elucidate whether resistance to Maraviroc can arise due to optimization of CCR5 use during the natural course of infection. CCR5 inhibitors are also being considered as candidates for the development of microbiocides, agents that can be applied locally and that can prevent sexual HIV-1 transmission. Efficiency of CCR5 use of the viral strains from the transmitter, which as discussed earlier is a viral characteristic that can be transmitted, may have an impact on the effectiveness of these CCR5 inhibitors.

HIV-1 CXCR4 use and antiretroviral therapy

A role for HIV-1 coreceptor use in response to antiretroviral drugs that do not target HIV-1 coreceptors has been reported. Monotherapy with reverse transcriptase inhibitor zidovudine was shown to be more effective against R5 variants, while didanosine was more effective against CXCR4-using viruses. This could be explained by the differential cell tropism of R5 and CXCR4-using variants, as these compounds are differently active in activated CCR5- and resting CXCR4- expressing HIV-1 target cells. In general, however, drugs that are activated in both types of target cells or that do not require activation, such as lamivudin or the protease inhibitor ritonavir, respectively, have a similar effect in both R5 and CXCR4-using variants. Current antiretroviral therapy combines at least three different compounds (cART), each targeting a different step on the virus life cycle. Evaluation of the impact of cART on viral coreceptor use
and vice versa is difficult given that initiation of therapy depends on the CD4 count\textsuperscript{85}, and therefore at least in HIV-1 subtype B infection, there is a higher likelihood to have or develop CXCR4-using variants at the time of initiation of cART. Up to now, it has not been clearly demonstrated that the effectiveness of drug regimens is influenced by HIV-1 coreceptor use, neither whether cART can influence a change in viral population coreceptor use\textsuperscript{86-88}. Thus, although emergence of CXCR4-using variants clearly has negative implications for the natural course of HIV-1 infection, the study of HIV-1 coreceptor use may not seem relevant for patients who can be treated with cART. However, the recent availability of CCR5 antagonists as antiretroviral therapy emphasizes the need to accurately identify CXCR4-using variants in patient samples when considering the use of this new drug class, given that this treatment is likely ineffective in patients harboring CXCR4-using variants\textsuperscript{89,90} and that intermediates between CCR5- and CXCR4-using variants, even if present at very low levels, unable to persist in co-existence with R5 variants (Chapter 8), may be selected upon suppression of the R5 variants population. Indeed CXCR4-using variants present at less than 1% of the total pre-treatment viral population may be selected upon CCR5 antagonist treatment\textsuperscript{89,91-93}. Chapter 8 shows that prediction of viral coreceptor phenotype using V3 sequences generated by ultra-deep sequencing allows a more sensitive detection of CXCR4-using variants present at levels below approximately 2.5% of the total virus population during natural infection as compared to the phenotypic MT-2 assay and enhanced-sensitivity Trofile assays (ESTA). Although the use of genotypic methods for the detection of CXCR4-using variants seems to be a promising tool, it has certain limitations due to the inaccuracy of the various bioinformatic tools for predicting the correct viral coreceptor phenotype. Currently available coreceptor prediction tools therefore need to be improved before this technique can completely replace the phenotypic methods that are presently used in the clinic.

**Host selective pressures driving HIV-1 (envelope) evolution**

The fact that HIV-1 envelope protein is involved in viral entry and that it is the target for neutralizing antibodies and, to a lesser extend, CTL responses, implies that the changes in the viral envelope required for HIV-1 escape or adaptation to immune responses will be conditioned by the maintenance of the viral envelope function as mediator of viral entry and vice versa. The selective forces driving HIV-1 evolution, and that are acting on the viral envelope, can differ within an HIV-1 infected individual depending on the body compartment. Although we observed similar evolution of HIV-1 variants obtained from serum and PBMC (Chapter 2 and 3), evidence for differential selective forces acting on the viruses from those two sources was found occasionally. The differential effect of the humoral immune response on cell-free (serum) and cell-associated (PBMC) viruses is shown in Chapter 3, where the majority of HIV-1 variants present in serum between 4 and 7 years after seroconversion in an HIV-1 infected individual were unable to persist in peripheral blood. These unsuccessful viruses were more sensitive to autologous serum neutralization, had shorter envelopes with fewer potential N-linked glycosylation sites, and showed lower replication kinetics than the HIV-1 variants that did evolve successfully, suggesting that humoral immunity played a role in the negative selection of those viral variants. In addition, underrepresentation of CXCR4-using variants in serum\textsuperscript{94} (and Chapters 2 and 8), suggests that CXCR4-using
variants may preferentially be present in a cell-associated state and that they are more restricted to cell-to-cell spread. The fact that this is observed in the HIV-1 infected individual with broad neutralizing activity in serum studied in Chapter 3 together with the higher susceptibility of newly emerged CXCR4-using variants to antibody neutralization as compared to co-existing R5 variants\(^{64}\) makes it likely that humoral immunity contributes to the suppression of CXCR4-using variants as soon as they bud from infected cells into the serum as free virions.

HIV-1 has to compete with the natural ligands of CCR5 and CXCR4 for binding to the coreceptors. Moreover, CCR5 and CXCR4 expression levels as well as availability of CCR5 and CXCR4 expressing target cells may be limited depending on the disease stage. Altogether, coreceptor availability could be one of the underlying selective pressures driving optimization of viral coreceptor use throughout HIV-1 infection. Given the reported lower percentages of CCR5 expressing target cells and higher levels of RANTES production in HIV-1 infected individuals with a CCR5 wt/Δ32 genotype\(^{95,96}\), the comparison of HIV-1 evolution in infected individuals with either a CCR5 wt/wt or with a CCR5 wt/Δ32 genotype (Chapter 4) offered the opportunity to investigate the contribution of CCR5 and target cell availability on the selection pressure directed against the viral envelope and therefore its impact on HIV-1 intra-host evolution. Delayed onset of AIDS symptoms is correlated with a slower rate of synonymous substitution, indicative of a slower replication rate\(^{97}\). The association between HIV-1 infection in individuals with a CCR5 wt/Δ32 genotype and a lower viral load set point and a slower HIV-1 disease course\(^{98,99}\), could suggest that target cell and CCR5 availability can influence progression to AIDS by limiting the viral replication rate. However, CCR5 genotype appeared to have no influence on viral evolutionary rate, which is the resultant of the viral mutation rate, viral generation time and the selective pressure acting on the virus, or on the selection pressure on the viral envelope (Chapter 4). This implies that although CCR5 availability may have an impact on HIV-1 evolution, it is the combination with other factors, such as cellular and humoral immune pressures and immune activation, that likely determines viral replication rate and progression of the disease, and the overall selection pressure on the virus. Therefore, the selection of viral variants with an enhanced coreceptor use cannot be solely explained by the depletion of target cells, a phenomenon that characterizes the clinical course of HIV-1 infection.

**Emergence of CXCR4-using variants: cause or consequence of immune impairment?**

The emergence of CXCR4-using variants at any stage of HIV-1 infection precedes a decline in CD4\(^+\) T cells and accelerated disease progression\(^{61}\). The factors determining the time required for the emergence of CXCR4-using variants are still unclear. The higher sensitivity to antibody neutralization of newly emerged CXCR4-using variants as compared to their co-existing R5 variants\(^{64}\) and their underrepresentation in serum\(^{64}\) (Chapter 2 and 8) may imply a role for the humoral immune response on suppressing the appearance of CXCR4-using variants. However, emergence of CXCR4-using HIV in the face of potent humoral immunity has also been observed\(^{64}\) (Chapter 3). The decrease in R5 HIV-1 population diversity (Chapter 5) and CD4\(^+\) T cell counts observed around the emergence of CXCR4-using variants could be a sign of the immune system’s
deterioration. This is also illustrated in an HIV-1 infected individual in Chapter 7, by the decline in number of PNGS in the viral envelopes of R5 and CXCR4-using variants after a phase of immune escape preceding the emergence of CXCR4-using variants; with a decline of the humoral immune response the occlusion of antibody epitopes by sugars on the viral envelope may not be as essential and the loss of those sugars may result in gain of a more efficient use of the entry complex. The low-stage fitness of CCR5- and CXCR4-using variants intermediates together with the relative slow emergence of CXCR4-using variants in HIV-1 infection, despite the potential advantage that CXCR4 use confers to the virus by broadening its target CD4+ T cell range, suggest that when the host selective pressures that earlier in infection favored the outgrowth of R5 variants or selected against the emergence of CXCR4-using variants begin to fade as a consequence of disease progression, the highly disfavored transition from CCR5 to CXCR4 usage will occur. The infection of a larger target cell range within the CD4+ T lymphocytes subsets and the interference of CXCR4-using variants with CD4+ T cell renewal by infection of naive CD4+ CXCR4+ T cells may just then contribute to the immune system collapse. The advantage of a CXCR4-using variant in such environmental conditions may explain why there is no reversion from CXCR4 use to CCR5 use. The observation that ultimately the prevalence of CXCR4-using variants in individuals with a CCR5 wt/Δ32 genotype, whom have lower expression CCR5 levels and slower disease progression, is comparable to individuals with a CCR5 wt/wt genotype, although the emergence of CXCR4-using variants is delayed in individuals with a CCR5 wt/Δ32 genotype, supports the hypothesis that a functional immune system prevents the emergence of these variants. Although host immune environment may probably have a large impact on the emergence of CXCR4-using variants, in Chapter 7 we emphasize the relevance of the viral background in this process.

Concluding remarks

The identification of CCR5 and CXCR4 as coreceptors for HIV-1 and their differential expression patterns in CD4+ T cell subsets, and the discovery of the ability of HIV-1 to use one or both of these coreceptors, which in combination are strong determinants of the cell tropism of the virus, has increased our understanding of HIV-1 pathogenesis and the factors determining the rate of disease progression. Further insight on HIV-1 coreceptor use evolution has recently become relevant as the presence of CXCR4-using variants has negative implications for the treatment with a new class of antiretroviral drugs targeting the CCR5 coreceptor. Despite enormous progress, the underlying mechanism on the evolution towards CXCR4 use remains largely unresolved. The study of the interplay between HIV-1 and its host environment in the context of HIV-1 coreceptor use in the absence of antiretroviral therapy may help to resolve parts of this puzzle.
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