Within-host HIV-1 evolution in relation to viral coreceptor use and host environment
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SUMMARY

The high mutation rate and rapid replication dynamics of human immunodeficiency virus type 1 (HIV-1) contribute to its high genetic variability by causing continuous emergence of HIV-1 variants within an HIV-1 infected individual that are genetically highly related but distinct from each other (viral quasispecies). The continuously changing host environment acts on the viral quasispecies, resulting in the selection of viral biological properties, such as higher replication capacity and more efficient use of the entry complex, and facilitating escape from the host immune response. HIV-1 entry into the target cell is mediated by the viral envelope protein, which binds to CD4 and a chemokine receptor (CCR5 or CXCR4), which functions as a coreceptor. The differential expression of CCR5 and CXCR4 on T cell subsets, in combination with the ability of HIV-1 to use one or both of these coreceptors, strongly contributes to the cell tropism of the virus. Moreover HIV-1 coreceptor use has an impact on disease progression. In this thesis, the evolution of HIV-1 and the composition of the viral population within an HIV-1 infected individual were studied in relation to viral coreceptor use and host environment.

In vivo HIV-1 replicates predominantly in macrophages and CD4+ T cells that reside in tissues or peripheral blood. Viral DNA that is present in these cells may represent actively replicating or non-replicating virus, either transiently (latent virus) or permanently (defective archived HIV-1 that will remain present as long as the infected cell lives). In addition to a cell-associated state, HIV-1 virions can be present in a cell-free state in serum, their short half-life indicating recent production, and therefore the viral quasispecies in serum is assumed to be biologically the most relevant (in contrast to the latent/incompetent cell-associated quasispecies). If these virions are produced by infected target cells from peripheral blood, one would expect the viral quasispecies of actively replicating viruses in PBMC and serum to be highly similar. In Chapter 2, we investigated the genetic relationship between the total HIV-1 quasispecies in PBMC (PBMC-DNA), the replication competent HIV-1 variants isolated from PBMC, and the cell-free HIV-1 quasispecies in serum (serum-RNA) throughout the disease course of four therapy naïve HIV-1 infected individuals. HIV-1 variants from the three different sources formed a single virus population at most time points analyzed. Interestingly, however, viral variants from serum and PBMC occasionally did not reflect the same fraction of viruses present in peripheral blood, as shown by the underrepresentation of CXCR4-using variants in serum or the inability of certain viral variants in serum to persist over time. Based on these results we concluded that replication competent clonal HIV-1 variants isolated from PBMC may be equally representative of the viral variants present in blood as sequences obtained from serum-RNA or PBMC-DNA. However, certain selective forces may drive differential evolution of the cell-free and cell-associated virus pool, in which case, sequences from both sources would be required to obtain a more complete picture of the viral population in peripheral blood in vivo.

The independent evolution of HIV-1 variants from PBMC and serum in an HIV-1 infected patient with cross-reactive neutralizing activity in serum is described in Chapter 3. The majority of viral variants that were obtained from serum between 4 and 7 years after seroconversion were unable to persist in peripheral blood. Interestingly,
these viral variants were more sensitive to autologous serum neutralization, had shorter envelopes with fewer potential N-linked glycosylation sites (PNGS), and showed lower replication kinetics than the HIV-1 variants that did evolve successfully, suggestive for a role for neutralizing antibody pressure on the negative selection of those viral variants. These data clearly reflect the dynamic interaction between HIV-1 and host humoral immunity.

Target cell and coreceptor availability may as well be one of the host selective pressures driving HIV-1 evolution. Indeed, one could argue that low expression of CCR5 may select for HIV-1 variants with the highest efficiency to use this coreceptor. Interestingly, heterozygosity for a 32 base pair deletion in CCR5 (CCR5delta32), a genotype that is observed in about 18% of the Caucasian population, is associated with lower CCR5 expression and slower disease progression. In Chapter 4, we compared the evolution of HIV-1 variants in progressing and long-term non-progressing individuals with either a CCR5 wt/wt or CCR5 wt/Δ32 genotype who only harbored CCR5-using (R5) HIV-1 variants during their course of infection. An approach using hierarchical phylogenetic models (HPMs) that incorporate fixed-effects was developed to estimate viral evolutionary rates and selection pressure ($d_N/d_S$) within hosts and evaluate whether these parameters differed with respect to CCR5 host genetic background and disease progression. We investigated whether the CCR5 wt/Δ32 genotype had an impact on the evolutionary rate of the virus and the selection pressure directed against the viral envelope protein. Unexpectedly, the CCR5 genotype had no influence on viral evolutionary rate or $d_N/d_S$. Indeed, although CCR5 genotype has a measurable impact on disease progression, there is no absolute relationship as some CCR5 wt/Δ32 HIV-1 infected individuals show rapid or typical progression to disease. We did observe an association between viral evolutionary rate and rate of disease progression. Moreover, given the absence of clear $d_N/d_S$ differences between HIV-1 individuals with a slow or typical disease progression, delayed onset of AIDS symptoms was solely associated with lower viral replication rates rather than with differences in selection on amino acid fixation. Our study implies that although CCR5 availability may have an impact on HIV-1 evolution, other host factors are likely to determine viral replication rate and exert selection pressure on the virus as well.

In the natural course of HIV-1 infection, the early phase is dominated by R5 viruses but in about 50% of subtype B infected subjects, CXCR4-using variants emerge at later stages, preceding an accelerated loss of CD4+ T cells and more rapid disease progression. The evolution of HIV-1 from an R5 to a CXCR4-using phenotype is not yet fully understood. For the study in Chapter 5, clonal HIV-1 variants were isolated from ten individuals at multiple time points throughout their entire natural disease course, from before and after the first appearance of CXCR4-using variants. Phylogenetic analysis showed that CXCR4-using variants evolved from R5 variants. Although this occurred more than once in some subjects, the emergence of CXCR4-using variants that successfully persisted over time was the result of a single evolutionary process, which commonly involved the gain of at least one positively charged amino acid in the V3 region and additional subject-specific changes outside the V3 region, suggestive for a role of host-specific selection pressures on this process. After the emergence of CXCR4-using variants, R5 variants remained present and co-existing R5 and CXCR4-using variants evolved independently, also with respect to their mode of CCR5 and
CXCR4 use as reflected in a decreasing dependence on particular binding regions in the coreceptors. This changing interaction with the HIV-1 coreceptors over time within an infected individual suggests limitations to the use of inhibitors targeting the coreceptors in patients harboring such late-stage variants.

Acquisition of the ability to use CXCR4 is accompanied by only minimal amino acid changes in the viral envelope which suggests that this change could easily be established in vitro by target cell selection. In Chapter 6 it is shown, however, that the R5 variants from only one out of four HIV-1 infected individuals, who developed CXCR4-using variants in vivo, could evolve to a CXCR4-using phenotype in vitro. The specific R5 variants had an envelope with higher V3 charge and higher number of potential N-linked glycosylation sites than the R5 variants that failed to acquire CXCR4 use in vitro. The acquisition of CXCR4 use in vitro and in vivo was associated with multiple mutational patterns that not necessarily involved the V3 region. However, changes in V3 at positions 11 and 24 were a prerequisite for successful persistence of CXCR4-using variants in vivo. This suggests that acquisition of CXCR4 use is more complicated than the minimal amino acid requirements would suggest and that positive selection pressures in vivo, mainly targeting the V3 loop, are a prerequisite for the emergence of CXCR4-using variants during the natural course of infection.

CXCR4-using viruses do not emerge in all infected individuals with HIV-1 subtype B and when they do, they are not generally detected at an early disease stage. Considering the minimal required sequence changes for a switch from CCR5 to CXCR4 co-receptor usage and that these mutations must be occurring continuously in vivo due to the exceptionally high mutation rate of HIV-1, the relatively slow emergence of CXCR4-using variants suggests that evolution towards a CXCR4-using phenotype is strongly disfavored in vivo and it is very likely that both host and viral factors influence the outcome of this process. In Chapter 7, HIV-1 evolution was studied in an HIV-1 transmission couple of which the donor exclusively had R5 variants during his entire disease course while the recipient developed CXCR4-using variants. Over time, R5 variants in the donor optimized co-receptor use and increased the number of PNGS and V3 charge of their envelopes. The earliest R5 variants in the recipient showed similar CCR5 efficiency and envelope molecular properties as the late R5 variants in the donor, characteristics that apparently can be conserved upon transmission. Emergence of CXCR4-using variants in the recipient was preceded by a selective sweep and an increase in the envelope number of PNGS and V3 charge of the R5 viral population. The high efficiency in CCR5 use and the more positively charged V3 envelope region of the recently transmitted R5 variants may have been instrumental to the emergence of CXCR4-using viruses under the specific recipient's immune environment. However, larger studies are required to value the contribution of viral and host factors in this evolutionary process.

Although phylogenetic analyses clearly support that CXCR4-using HIV-1 variants evolve from R5 HIV-1 variants, the intermediate HIV-1 variants are only seldom observed which may reflect their relatively low fitness. As a consequence, the kinetics and mutational pathways involved in the evolutionary process of CCR5- to CXCR4-using transition have been difficult to study and are therefore poorly understood. In Chapter 8 ultra-deep sequencing of the V3 loop of the viral envelope in combination with V3-based coreceptor prediction tools was used to detect HIV-1 variants during
the transition from CCR5- to CXCR4-usage. PBMC and serum samples that were obtained from eight HIV-1-infected individuals at three-monthly intervals around the moment of first detection of CXCR4-using variants were analyzed. Analysis of the genetic relationships of the V3 sequences using minimum spanning trees revealed that the transition in coreceptor usage followed a stepwise mutational pathway involving sequential intermediate variants, which were generally present at relatively low frequencies compared to the major predicted CCR5- and CXCR4-using variants. In addition, individual patients differed with respect to the number of predicted CXCR4-using variants, the diversity among major predicted CCR5-using variants, and the presence or absence of intermediate variants with discordant phenotype predictions. These results provide the first detailed description of the mutational pathways in V3 during the transition from CCR5- to CXCR4-use in natural HIV-1 infection. They also confirm the low frequency of the transitional variants most likely reflecting decreased *in vivo* fitness and possibly explaining why CXCR4-using variants, unlike CCR5-using variants, are not detected in all patients at every stage of disease.

The implications of all findings in this thesis are put in perspective in the *General Discussion.*