HIV-1 Tropism and Its Detection – Impact on Infection, Transmission and Treatment

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The HIV-1 Receptor and Co-receptors

The outcome of exposure to HIV-1 varies greatly between individuals. One of the factors determining this variability in outcome is the cellular tropism or viral phenotype, as damage caused by HIV-1 is critically influenced by the cell types the virus is capable of infecting (see Figure 1). HIV-1 requires two cellular receptors for entry: CD4 and one of a family of chemokine receptors (co-receptor). In vivo, the major co-receptors used by HIV-1 are CC chemokine receptor 5 (CCR5)3,5 and CXC chemokine receptor 4 (CXCR-4).6 Individual viruses are classified based on their ability to use the former (R5) or the latter (X4) or both (R5X4) of these co-receptors.7 Bulk virus isolates capable of using both co-receptors are designated dualtropic (D/M) as they may contain any mixture of these three types.8 Before the identification of the co-receptors, viruses were classified based on their ability to infect induce syncytia (multinucleated giant cells) in MT-2 cells,9,10 which do not express CCR-5.11 Thus, viruses that do not infect MT-2 cells are non-syncytium-inducing (NSI) and R5, while viruses that do infect MT-2 cells are syncytium-inducing (SI) and either X4 or R5X4.12

Co-receptor Expression and Pathogenesis

Of note, this ability to induce syncytia in MT-2 cells reflects the viral co-receptor use and not enhanced cytopathicity of X4 virus per se, as R5 virus can at least be equally cytopathic to its target cells.13–15 The differential pathogenicity of R5 and X4 viruses in vivo lies mainly in the co-receptor expression patterns of the host cells. CXCR-4 is expressed on many more CD4+ cells in the body (including haematopoeitic progenitor cells, thymocytes and naïve T cells) than CCR-5 (which is mostly found on memory T cells and macrophages).16,17 Thus, switching to CXCR-4 use potentially allows the virus to access a large and critical pool of target cells affecting T-cell ontogeny, a fact that may help explain the accelerated CD4+ T-cell decline associated with the emergence of CXCR-4-using virus.18 Recently, it was even suggested that X4 virus actually emerges from the thymus.19

Cell Tropism

Virus use of co-receptor(s) and virus target cell tropism (lymphocytes, macrophages and/or transformed T-cell lines) are highly related but distinct characteristics of the viral envelopes.20 There is a cell-type-specific component to co-receptor use so that not all R5 viruses can infect macrophages, despite the fact that these cells express CCR-5.21 X4 viruses are not able to infect all CD4+ T-cell lines, despite the expression of CXCR-4 on these cells.22 Moreover, R5X4 viruses differ in terms of which co-receptor they use to infect different target cells.23 At least some of these discrepancies can be explained by CD4 expression levels on the different target cells and the CD4 dependencies of viral envelopes.24,25 To capture the discrepancies, an additional classification scheme that addresses both cell tropism and co-receptor use has been proposed (e.g. L-R5 denotes the lymphocytotropic R5 variant, M-R5 denotes the macrophagetropic R5 variant, D-R5X4 denotes the dual-tropic R5X4 variant and T-X4 denotes the T-cell-line-tropic X4 variant).26

Transmission and Evolution of Co-receptor Use

Independently of the route of transmission, R5 virus seems to be more efficiently transmitted and to predominate in the early stages of infection.26,27 A combination of multiple mechanisms – including preferential trapping and inactivation of X4 virus by mucin and innate antiviral proteins, preferential transcytosis of R5 virus, preferential amplification/binding of R5 viruses by macrophages, dendritic cells and Langerhans cells and preferential neutralisation of X4 virus – most likely explains this asymmetric transmission.26 In 80–90% of asymptomatic treatment-naive patients, only R5 virus is found.29,30 In approximately half of patients, virus capable of using CXCR4 emerges prior to the diagnosis of AIDS.1,2 The introduction of highly active antiretroviral treatment (HAART) has greatly reduced progression to AIDS in treated individuals. Recent cross-sectional studies have found X4 virus in 40–55% of patients with previous antiretroviral exposure,31–33 probably reflecting the on average lower CD4 counts in treated individuals (see below).34 It is important to note here that R5 virus remains present after the emergence of X4 virus.35 Pure X4 virus populations are rare and are often associated with late-stage disease, instances of known X4 transmission or absence of CCR-5 expression in the host, as discussed in more detail below.35,36,37 Indeed, the heterogeneity of co-receptor expression among specific cellular targets allows for continued evolution of both R5 and X4 virus in an infected individual.38 This evolution is accompanied by improved virus–co-receptor affinity, which in turn is reflected in decreased sensitivity for their respective co-receptor antagonists.39–41 It is unclear what governs whether X4 virus emerges. It also remains largely unexplained whether the switch is responsible for disease progression or whether it occurs as a consequence of progressive immunodeficiency. However, recent evidence suggests that the newly emerging X4 virus is more sensitive to autologous neutralisation than the co-existing R5 virus,42 providing some support for the latter view.
It should also be mentioned that the shift from R5 to X4 use is not a prerequisite for disease progression. Approximately half of patients infected with subtype B never experience a shift prior to AIDS diagnosis,\(^7\) and R5 variants show considerable variation in their cytopathological properties and replicative fitness.\(^{40,44-47}\) However, X4 virus can emerge at any stage of disease, even after AIDS diagnosis, as well as in individuals who have long maintained relatively stable CD4 counts.\(^{25,46}\) Application of ultra-deep-sequencing (UDS) technology may provide more insight into the evolutionary kinetics of X4 emergence.\(^{49}\) UDS has already revealed the low-level presence of X4-like sequences in samples not scored X4 using either phenotypic or genotypic assays.\(^{51,51}\) Future UDS analysis of longitudinal samples from patients who either did or did not develop X4 virus prior to AIDS diagnosis may reveal the clinical relevance of this low-level presence and provide clues to the factors that influence their outgrowth.

**CCR5 Δ32**

The clinical importance of co-receptor use in HIV pathogenesis is further illustrated by several cohorts of long-term non-progressors harbouiring only R5 virus and by exposed but uninfected individuals who lack the CCR-5 co-receptor.\(^{50-54}\) The genetic variant responsible for this latter phenotype – a 32-base pair deletion (designated CCR-5 Δ32) resulting in a frameshift and premature stop codon – is not associated with any obvious clinical disease, although impaired immune responses to certain virus infections are now being reported.\(^{50}\) In contrast, loss of CXCR-4 function in mice results in defects in vascular and cerebellar development, haematopoiesis and cardiogenesis.\(^{55-56}\) The protective effect of the CCR-5 Δ32 genotype in HIV-1 infection is not limited to individuals with two mutant alleles. HIV-1-infected individuals heterozygous for the CCR-5 deletion have significantly delayed disease progression,\(^{56}\) confirmed in a meta-analysis of 19 studies of HIV-1-infected adults from the US, Europe and Australia.\(^{57}\) This was especially true for those heterozygous individuals harbouring only R5 virus.\(^{55,60}\) Interestingly, the protective effect of the CCR5 Δ32 genotype in children was observed only in the first years of life, indicating that disease progression may be accelerated disproportionately once X4 virus has developed in HIV-1-infected children with CCR-5 Δ32.\(^{52}\)

**Molecular Determinants of Co-receptor Use**

Early studies mapped the determinants that govern SI phenotype and cell tropism mainly to the envelope gene, especially to the variable regions, and in particular the third variable (V3) region, the V3 loop. Both overall V3 loop charge\(^61\) and the specific presence of positively charged amino acids at position 11 and/or 25 in the V3 loop were shown to be strongly associated with SI phenotype.\(^{64,65}\) Additional determinants for co-receptor usage (CU), especially V2 length and the presence of glycosylation sites, have been reported in other envelope regions, but no single amino acid changes have been pinpointed that determine phenotype in all genetic backgrounds.\(^{53,66-68}\) As expected, CXCR-4 use was also conferred by changes in the envelope variable regions.\(^{69-71}\) Evidence suggests that the evolutionary changes in the V3 loop involved in the switch are accentric and gradual, and that dual CU with equal efficiency is a phase through which virus evolution passes but does not linger. As mentioned above, there is continued evolution of co-receptor use, resulting in very different co-receptor affinities within the quasispecies.\(^{46}\)

**Non-B Subtypes**

HIV-1 subtype B is by far the most widely studied subtype with respect to CU. However, the molecular and epidemiological characteristics of tropism can differ strikingly between subtype B and non-B subtypes. For example, X4 virus prevalence among subtype C infections is 5–10% even in AIDS patients – much lower than that for subtype B.\(^{73,81}\) Subtype C infections account for over half of worldwide HIV-1 prevalence,\(^{52}\) and are as deadly as subtype B. In contrast, in subtype D infections there is a higher prevalence of X4 virus,\(^72,83,84\) which is correlated with faster disease progression in this subtype compared with other subtypes.\(^{85,87}\) It is clear that the V3 loop is also the principal genetic determinant of phenotype in the other HIV-1 subtypes,\(^{85}\) although subtype C X4 virus does not show the same dependence on positive charge.\(^{83,84}\) While the lower prevalence of X4 viruses in subtype C infections may bode well for the potential effectiveness and useful life of CCR-5 inhibitors (see below) in the developing world, recent evidence suggests that the prevalence of X4 in subtype C infections is increasing.\(^{88}\) Moreover, the genetic differences between subtypes in naturally arising viruses also suggest that resistance to these agents may arise through mutational pathways that are unlike those that have been worked out for subtype B.\(^{53}\)

**Highly Active Antiretroviral Therapy and Co-receptor Use**

Several studies have suggested that HAART influences viral co-receptor use with both increased and decreased frequencies of X4 virus observed post-treatment.\(^{89,90}\) However, a more recent study comparing treatment-naïve and -experienced patients showed a similar frequency of X4 virus in both groups. Instead, emergence of X4 virus was significantly associated with lower CD4 counts regardless of antiretroviral treatment exposure.\(^{34}\)

**CCR-5 Inhibitors**

The findings for the CCR-5 Δ32 genotype described above have made CCR-5 a prime target for drug development. Several small-molecule CCR-5 inhibitors have been developed: maraviroc (MVC) has now been approved for clinical use, vicriviroc is in phase III trials and several others are at earlier stages in the drug-development process.\(^{10}\) Prior to initiating CCR-5 inhibitor treatment, patients are screened for the presence of virus capable of using CXCR-4. Both phenotypic and genotypic assays have been developed for screening, as outlined below, but the Monogram Trofile assay is the only clinically validated test and has been used to screen by far the largest number of patients.\(^{29-31}\) Resistance to MVC can emerge in one of two ways:

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**Table 1: Overview of Co-receptor Use and Cell Tropism of Different HIV-1 Variants**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Trofile</th>
<th>Clone</th>
<th>MT-2</th>
<th>TSM</th>
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</tbody>
</table>

**Legend**

- **CD4+ T cell**
- **Memory**
- **Naive and memory**
- **Naive and memory**

**Tropism**

- **CD4+ T cell**
- **Memory**
- **Naive and memory**
- **Naive and memory**

**Thymocytes**

- **-**
- **+++**
- **+++**

**Precursors**

- **-**
- **++**
- **+++**

**Macrophages**

- **+++**
- **+**
- **+/-**

**Dendritic cells**

- **+++**
- **+**
- **+/-**

**T cell lines**

- **-**
- **++**
- **+++**
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Table 1: Online Genotypic Co-receptor Predictors

<table>
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<th>URL</th>
<th>Method</th>
<th>Ref.</th>
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<td>PSSM</td>
<td>86, 114</td>
</tr>
<tr>
<td>genop2pheno</td>
<td>coreceptor.bioinf.mpi-inf.mpg.de</td>
<td>SVM</td>
<td>103</td>
</tr>
<tr>
<td>WetCat</td>
<td>genomica2.ucsd.edu:8080/wetcatv3.html</td>
<td>DT</td>
<td>115</td>
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<tr>
<td>RF</td>
<td>bioinfo.gnomeweb.net/HIV-1/PhenoPred.php</td>
<td>RF</td>
<td>106</td>
</tr>
</tbody>
</table>

CU = co-receptor usage.

Table 1: Online Genotypic Co-receptor Predictors

reduced susceptibility of the R5 virus associated with changes in the V3 loop that allow the virus to use the MVC-bound form of CCR-5, or the emergence of viruses that use CXCR-4. In this latter scenario, the CXCR-4-using virus in MVC-treated patients seemed to originate from a pre-treatment reservoir, indicating that screening assay sensitivity remains to be improved. Interestingly, in these patients circulating virus reverted to predominantly R5 following cessation of MVC, indicating that the selective pressure acting against CXCR-4-using virus was preserved. In vitro studies have shown that small-molecule CCR-5-inhibitor-resistant R5 viruses are cross-resistant to other small-molecule CCR-5 inhibitors, but sensitive to protein ligands and other antiretroviral drug classes and hypersensitive to neutralising monoclonal antibodies, suggesting the existence of further in vivo constraints on escape from CCR-5 inhibitors.

Phenotypic Assays

Traditionally, the MT-2 assay has served as a tool to determine tropism based on the expression of CXCR-4 on the cell surface. Two widely used versions of the MT-2 assay exist: one in which the patient cells are directly co-cultured with the MT-2 cells, and another in which virus stocks are first generated from the patient cells by co-culture with seronegative stimulated peripheral blood mononuclear cells (PBMCs). This latter version probably limits the sensitivity of the assay, as MT-2 cells are more sensitive to infection by CXCR-4-using virus than are PBMCs. Moreover, PBMC passaging of patient samples prior to MT-2 inoculation has the potential to distort the original in vivo HIV population. Using direct co-culture, the results of the MT-2 assay are highly concordant with those of the enhanced version of the Trofile assay described below. Although the MT-2 assay is not technically challenging, the need for viable patient cells (either fresh or cryopreserved) and biosafety level 3 laboratories has limited widespread implementation.

Besides the MT-2 assay, there are at least four recombinant phenotypic assays available to predict CU: Trofile (Monogram Biosciences), Phenoscript (VIRalliance), XtrackC/PhenX-R (inPheno) and VircoType (Virco). Patient plasma is used to generate pseudoviruses or infectious recombinant viruses that include full-length or partial viral envelopes derived from the patient’s virus population; these are subsequently tested on indicator cell lines expressing CD4 and either CCR-5 or CXCR-4. Unlike the MT-2 assay, recombinant phenotypic assays are able to distinguish between pure X4 and D/M populations (see Figure 1), although the clinical relevance of this distinction is yet to be determined. The methods and characteristics of these four assays have recently been reviewed in detail. There are two major advantages of recombinant phenotypic assays over genotypic prediction. First, the in vitro culture step on specific CCR-5- or CXCR-4-expressing target cells allows selective outgrowth of even small minority populations, resulting in superior sensitivity over the genotypic predictors available to date. Initially, recombinant phenotypic assays were capable of detecting CXCR-4-using variants accounting for at least 5–10% of the population, but sensitivities have since improved; for example, the enhanced sensitivity version of the Trofile assay can now detect minority populations at levels of 0.3%. Second, the actual ability of the patient viral envelope to bind CCR-5 and/or CXCR-4 is tested rather than predicted, which is critical as not all of the molecular determinants of CXCR-4 use have been catalogued, and these sometimes reside outside the V3 loop (the region used in most genotypic predictors; see below). For these reasons, genotypic approaches are currently not recommended for screening patients considering CCR-5-inhibitor treatment. However, as with commercially available resistance tests, tropism testing generally requires a plasma sample with an HIV-1 level of ≥1,000 copies/ml. Thus, patients on suppressive treatment regimes who are considering CCR-5-inhibitor treatment for reasons other than failure cannot be tested.

Genotypic Co-receptor Prediction

Because the cost of recombinant phenotypic assays remains high, if they are locally available at all, investigators and clinicians have looked to virus sequence analysis to predict CU. Efforts have concentrated mainly on identifying sequence patterns in the V3 loop that are associated with the ability of the viruses to fuse to CD4 cells via the CCR-5 or CXCR-4 co-receptors. The most recent genotypic predictors of CU incorporate information from across the V3 region, along with genotypic correlates outside the V3 region. Some can also incorporate clinical data on the infected individual. Progress is also being made in terms of the inclusion of biomolecular structure information to assist CU prediction, as well as the ability to discriminate between X4 and R5X4 virus. The specificity and sensitivity of most CU predictors exceed 90 and 80%, respectively, in X4 prediction for cloned subtype B viruses. The fundamental technical concept behind the development of CU predictors is known as ‘supervised learning’, and corresponds to the virtual phenotype in drug resistance genotyping. The predictor essentially corresponds to the virus sequence being tested to known X4 and R5 virus sequences and yields a prediction based on whether features of the test sequence are more similar to known X4 or known R5 features. The set of sequences associated with biologically assayed phenotypes is often referred to as the ‘training set’. The mechanics of the comparison are more or less sophisticated depending on the learning algorithm used. The algorithms are able to identify amino acid residues at given positions that discriminate best between X4 and R5 viruses and to down-weight residues and/or positions that provide little discriminatory information. One remarkable finding is that as long as the entire V3 loop is used to create the predictor, the most sophisticated algorithms (e.g. support vector machines or random forest) gain relatively little discriminatory power over the less sophisticated algorithms (e.g. position-specific scoring matrices [PSSM]). However, because the prevalence of X4 virus is not high, small increments in the quality of prediction are likely to lead to large improvements in the predictive value of the tests, the most important measure of quality in the clinic.

Table 1 provides an overview of CU predictors freely available online or by request.

Factors Influencing the Success of Genotypic Predictors

While there are some basic similarities in the bioinformatic methods, there are important differences in the biological problem being tested between typical drug resistance genotyping and CU prediction. Table 2 summarises some of these differences. Each factor in the table represents a point at which uncertainty in the prediction and its interpretation is introduced. The most important factor influencing the success of a predictor may be the availability of training data. The clinical and financial importance of antiretroviral therapy and the speed at which resistance to drugs evolves have driven a research and clinical enterprise yielding massive amounts of validated resistance and sequence data, detailed accounts of resistance mechanisms and continual updates of genotype-based resistance
prediction. While co-receptor usage has been studied for longer, validated data sets remain much smaller and more difficult to compile, at least from current data in the public domain. In addition, the connection between the assay outcome (e.g. fusion of recombinant env to CXCR-4-expressing indicator cells) and the disease outcome (e.g. more rapid decline in CD4 count) influences accuracy and interpretation. The physician requires a prediction of clinical outcome, but the predictor directly predicts only the likelihood of the assay outcome. There is some evidence that the high expression levels of CXCR-4 on the indicator cell lines used in recombinant phenotypic assays allow the replication of viruses that are not capable of using CXCR-4 on primary cells. However, in longitudinal follow-up studies, these types of virus have been observed only in individuals showing subsequent emergence of viruses capable of using CXCR-4 on primary cells, and never in individuals progressing to AIDS with R5 virus only.38,108

For drug resistance, there is an often a strong relationship between in vitro resistance and treatment failure, for two reasons. Because drugs eliminate competition by susceptible strains, resistant strains in the presence of drug rapidly outgrow and become dominant, and so most viruses sampled from individuals who have failed treatment are resistant in vivo and in vitro. Also, protease or reverse transcriptase (RT) inhibitors interfere with the action of virus gene products by disrupting the function of particular sites on the virus protein. Single amino acid changes in these sites often significantly reduce the affinity of the drug for those sites while preserving the native function of the protein, and therefore confer measurable drug resistance. With the strong links between sequence, assay outcome and patient response, genotypic drug resistance predictors are quite successful and have become indispensable as clinical tools.102 In the case of co-receptor usage, the biological problem is somewhat more subtle. Even though many of the molecular details of the envelope–co-receptor interaction are understood, the population biology of tropism variants remains elusive. As outlined above, the selection pressures that lead to the outgrowth of X4 virus are variable and complex. X4 virus that threatens the efficacy of a co-receptor blocker regimen may co-exist with the predominant R5 virus, but remain rare enough to go undetected by sequencing assays through most of the pre-therapy period.109 Searching for pre-existing X4 genotypes is also complicated by the fact that the region of the envelope gene with the greatest influence on tropism also has the highest genetic diversity in the HIV genome: approximately two to three times higher than that in pol.109 This complicates the interpretation of sequences, especially when so-called bulk sequencing is employed. This method captures only the sequences present in the highest (>25%) proportions, and the resulting sequence reads are difficult to interpret when many genetic variants are present. An additional complication exists at the level of relating the phenotype to disease or treatment outcome. Current commercial phenotype assays generally test whether a given env can mediate fusion via a particular co-receptor molecule; that is, whether the sample env is R5 or X4. While these assays are rapid and reproducible, epidemiological studies have shown that genotypic similarity of patient virus to known SI virus is a better predictor of subsequent treatment success than similarity to CXCR-4-tropic viruses.110 Since a CU predictor formally predicts assay outcome only, the investigator using a given predictor needs to be aware of the nature of the samples in the training set and the correlation between the CU properties of those samples and the clinical outcome of interest.

### Prognosis for Genotypic Co-receptor Usage Prediction

Because these factors combine to obscure the relationship between CU prediction and treatment performance or disease progression,111 the latest clinical recommendations do not support the use of sequence-based tropism testing.102 Nevertheless, there are good reasons to believe that CU prediction will eventually come into its own. As co-receptor blocker treatment becomes more widespread, resistant strains are likely to develop and become regularly detectable in patients. These strains will be subjected to study and contribute to new predictors. UDS also promises to change the terms on which CU prediction is performed. UDS offers the potential to sequence thousands of virus clones separately in a single read at low per-sequence cost. Minority drug-resistant sequences at 5% or less can be reproducibly detected with this method.112 Use of UDS in epidemiological studies would also lead to improved estimates of X4 prevalence. These estimates are likely to increase with increased sensitivity of detection and lead to improved estimates of positive predictive value for CU predictors.

Increased effort towards characterising tropism of non-B subtypes will also be key to expanding the utility and improving the interpretation of CU predictions. Most CU predictors have been based on subtype B training sets, for which publicly available data are most abundant. However, the V3 loop in different subtypes has evolved configurations that perform similar functions in apparently different ways. For example, while the mere
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presence of positively charged amino acids at V3 sites 11 or 25 is an excellent predictor of CXCR4 usage in subtype B, only half of a set of 51 unique, known X4 viruses from subtype C possessed positive 11/25 sites. Existing predictors can be optimised for use on non-B subtypes, but the best solution is to create predictors using validated phenotypic data for the desired subtype. A CU predictor based on PSSM B subtypes, but the best solution is to create predictors using validated PSSM in both R5/X4 and SI/NSI prediction for subtype C and other non-B subtypes.

Conclusions

The problem of HIV-1 tropism has fascinated HIV biologists and clinicians for nearly 20 years. The search for solutions has recently increased in urgency, with the advent of new antiretroviral agents that target the interaction between HIV and its co-receptors and the growing capability and political will to treat non-B subtype infections in the developing world. These driving forces have led to a flurry of epidemiological studies of tropism, pathogenesis and treatment that are bridging the gap between basic science and clinical application. Because of the complex nature of the molecular and population biology of tropism, we expect that assays of co-receptor blocker resistance will be at least as challenging to perform and interpret as current drug resistance assays. Nevertheless, significant progress has been made on both the phenotypic and genotypic fronts. The genetic determinants of CU are clearly concentrated in the V3 loop, but we now know that other factors, including other regions of env and host factors, also play a role in shaping the tropism composition of virus populations in any individual. If X4 use has an impact on disease progression, it is likely that it does so by reducing the future complement of T cells rather than through a vigorous attack on existing cells. However, X4 virus is not required for disease progression, particularly in subtype C. In fact, much of the evidence now suggests that X4 virus is less fit than R5 virus in the host environment early in infection, and that immune system decline may pave the way for X4 emergence in many cases. However, for all the progress we have made, the basic questions remain: what is the precise role of X4 virus in HIV-1 pathogenesis, and what pressures account for its emergence and overall dynamics within the infected individual?

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