Viral Correlates of HIV-1 Disease

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Abstract: The transmission of HIV and the progression of HIV disease are influenced not only by a large number of human host factors, but also by certain correlates of the ever fluctuating virus quasispecies. The present review article aims at providing the current state of knowledge as well as an in-depth critical discussion of recent developments on the potential effects of HIV subtype, phenotype and attenuation on HIV disease. Despite the extensive research, several questions regarding the precise role that each of these correlates plays in human AIDS pathogenesis remain unanswered. Unraveling these roles is expected to aid the continued quest for truly effective antiretroviral drug regimens and preventive vaccines.

Keywords: Subtype; clade; biological phenotype; tropism; attenuation; transmission; disease progression

VIRAL SUBTYPE: HIV VARIABILITY FROM AN EVOLUTIONARY PERSPECTIVE

Two types of the etiologic agent of acquired immune deficiency syndrome (AIDS) have been described: human immunodeficiency virus type 1 and 2, HIV-1 [23, 131, 142, 248, 304, 335] and HIV-2 [70, 71], respectively. Both types of HIV apparently originated in Central Africa and evolved from lentiviruses that naturally infected other primate species {most recently reviewed in [59, 406]}. In particular, current research indicates that the introduction of HIV-1 and HIV-2 to humans represents separate cross-species (zoonotic) transmission events from simian immunodeficiency virus (SIV) $_{cpz}$-infected chimpanzees (subspecies *Pan troglodytes troglodytes*) [132, 370] and SIV_{smg}-infected sooty mangabey monkeys (*Cercocebus atys atys*) [170], correspondingly [160]. Although HIV-2 isolates exhibit similar functional genomic complexity to HIV-1, except for the presence of regulatory gene *vpx* in HIV-2 *in lieu* of *vpu*, HIV-2 viruses are less pathogenic and have been geographically restricted, mainly to West Africa [88, 263]; in contrast, the more virulent HIV-1 strains have spread around the globe, causing the AIDS pandemic.

Based on phylogenetic analyses, initially of partial nucleotide sequences coding for structural proteins of the virus (*env* and/or *gag*) [252, 364] and later of full-length sequences [367], HIV-1 isolates are broadly classified into three groups: M (for major or main, since it accounts for the vast majority of reported HIV-1 cases), O (for outlier) and N (for new or non-M, non-O). No subtypes have been clearly defined for the few group N isolates that have been identified so far. On the contrary, group O viruses may be distinguished into three phylogenetic clusters [355], but with a rather weak distinction compared to group M that is subdivided into at least nine, associated but distinct, genetic subtypes or clades, A-D, F-H, J, and K, and fifteen circulating recombinant forms (CRF01-CRF15) at present [1, 51, 133, 159, 181, 190, 251, 252, 271, 398, 422, 432]. Subtypes A and F are further distinguished into two sub-subtypes each, designated as A1 and A2 [136], and F1 and F2 [421, 422], respectively.

Separate chimpanzee-to-human transmission events are thought to have brought about the generation of each HIV-1 group [78, 132]. The date of the last common ancestor of group M, which was probably present in a human host, thereby rendering highly improbable the theory that oral poliovirus vaccines were the initial source of HIV [340, 444], was estimated to be towards the first half of the 20th century, around 1931 [226]. Group M viruses are thus perceived to have radiated out of the territory within or near the Democratic Republic of Congo {(DR Congo), former Zaire}, where the first documented HIV seropositive sample was collected in 1959 [304, 468] and the highest degree of diversity has been detected [298, 438]. In an analogous evolutionary mode, group O strains seem to have diverged out of the region of Cameroon [355], the country where the few infections with group N viruses have also been documented [19, 398]. Given their mosaic pattern that is formed by SIVcpzUS- and HIV-1-related sequences, group N strains presumably arose from an ancestral recombination event in a chimpanzee host [132].

Non-B HIV-1 subtypes that prevail in Africa and Asia bear more significance than subtype B, the predominant clade in Western and Central Europe, North America, and Australia, in the molecular epidemiology of the pandemic {reviewed in [6, 113, 328, 405]}. In the year 2000, the largest proportion of all HIV-1 infections worldwide was due to subtype C strains (47.2%), while subtype A/CRF02_AG constituted the second leading cause of all infections (27%), followed by subtype B strains (12.3%) [319]. The same analysis that attempted to monitor the dynamics of the global spread of HIV-1 confirmed an increasingly important role of CRFs in the pandemic. The effects of recombination and superinfection with multiple strains or subtypes of HIV and SIV viruses have been reviewed in detail elsewhere [40]. A few months ago, however, the evolution of a second generation, inter-CRF recombinant between CRF07_BC and CRF08_BC was described in Yunnan Province of China [453], thereby justifying the characterization of the area of Southeast Asia that includes West Yunnan and Central

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Myanmar (Burma) as a "melting pot" of recombination for HIV-1 [411]. It should be noted that such findings may reflect a selective advantage of new recombinant forms, some of which exhibit "pseudotype" virion structures with the external sections of their envelope glycoproteins exchanged with strains of different lineages over simpler parental strains as observed before [100], hence posing an additional challenge to researchers for the development of effective vaccines and therapies.

Previously referred to as lymphoadenopathy-associated virus type 2, HIV-2 is endemic in the Northwestern part of Africa, especially in the Ivory Coast (Côte d'Ivoire) and Guinea-Bissau, and to a lesser extent in neighboring countries {reviewed in [342, 375]}. Infections with HIV-2 are also found in Brazil [80] and India [360], while the highest incidence of this viral type in Europe is encountered in Portugal, with HIV-2 accounting for 4.5% of documented AIDS cases in the country [401]. Concurrently, the highest diversity of HIV-2 strains is encountered in Sierra Leone that was found to display a very low seroprevalence of HIV-2 infection (0.021%) [62]. In this small West African country, there exist both free-living and household pet sooty mangabeys that evidently harbor highly divergent variants of SIV_{smg} viruses.

Independent introductions of such genetically diverse SIV_{smg} strains into humans presumably gave rise to the seven distinct and roughly equidistant subtypes, A-G, that have been described for HIV-2 [62, 135, 451]. The prototype HIV-2 strain that had been isolated from Cape Verde Islands AIDS patients was found to belong to subtype A [70]. In fact, subtype A variants constitute the main, and almost exclusive, cause of the HIV-2 epidemic [201, 310, 375]. Evidence showing a correlation between HIV-2 subtype and geographical origin of infecting strains in patients living in France has been presented; more specifically, subtype A-infected subjects were epidemiologically related to Cape Verde Islands and Guinea-Bissau, whilst the majority of subtype B-infected individuals were linked to the Ivory Coast or Mali [86]. The most recent common ancestors for HIV-2 subtypes A and B arose around 1940 and 1945, respectively [247]. Although very few representative members have been characterized for most of these rare HIV-2 subtypes, an intersubtype variation up to 25% in *gag*, *pol* and *env* regions, that is comparable to that calculated for HIV-1 groups, has been documented for HIV-2 subtypes [135]; accordingly, the formerly known A-G subtypes of HIV-2 are currently termed HIV-2 groups {reviewed in [406]}.

HIV exhibits remarkable variability not only among infected individuals (interhost variability), but also within each infected individual (intrahost variation) over time {reviewed in [256, 313]} and among different compartments {i.e. [103, 369, 395]}. Cell types or tissues with a restricted virus flow between them are termed virologic compartments and, as suggested recently [308], they may be differentiated from virologic reservoirs (that imply a relative suppression in viral replication among cell types or tissues), using phylogenetic criteria. The extensive variation of HIV, which provides the virus quasispecies with the capacity to adjust to immunologic, pharmacologic or other microenvironmental selection pressures, stems mainly from the inherent infidelity of the reverse transcriptase enzyme in conjunction with the lack of proofreading mechanisms, high turnover of virions (although many progeny viruses may be defective in their replicative abilities), and propensity for recombination [172, 183, 338, 351].

SUBTYPE DIVERSITY AND BIOLOGICAL PROPERTIES OF HIV: VIRAL PHENOTYPE, CHEMOKINE RECEPTOR USAGE AND CELL TROPISM

Based on *in vitro* replicative characteristics and cytopathic effects that determine their biological phenotype, HIV-1 strains may be classified as either rapid/high (R/H), syncytium-inducing (SI) or slow/low (S/L), non-syncytium-inducing (NSI) [17, 123, 414, 448]. A characteristic difference between the two phenotypes is that established $CD4^+$ T-cell lines, including MT-2, can be readily infected with SI, but not with NSI viruses. The principal coreceptors utilized by NSI and SI viruses for infection of $CD4^+$ cells are the beta-chemokine coreceptor CCR5 and the alpha-chemokine coreceptor CXCR4, respectively [37, 67, 102, 107, 110, 118]. In the acute and early asymptomatic stages of the infection, HIV-1 strains of most clades are typically monocytotropic, NSI variants using CCR5 as coreceptor {"macrophage" (M)-tropic or R5 isolates}; later into the course of infection, in approximately 50% of patients {although this percentage could be significantly higher [383]}, R5 isolates are replaced by SI variants with altered tropism that expand to utilize CXCR4 ["T-cell line" (T)-tropic or X4 isolates] or both CCR5 and CXCR4 (R5X4 isolates) [11, 17, 18, 63, 77, 79, 123, 225, 278, 341, 374, 379, 380, 396, 414, 466, 469].

A similar capacity for replication within cells of the monocyte-macrophage lineage has been found for HIV-1 strains belonging to different genetic subtypes, with NSI biological clones replicating more efficiently than SI clones [202]. Emergence of the SI phenotype is associated with an accelerated decline of $CD4^+$ T cells and a more rapid progression towards AIDS in HIV-1-infected subjects harboring not only subtype B [63, 75, 76, 203, 213, 223, 280, 348, 354, 379, 404, 415, 416, 439], but also other genetic subtypes {reviewed in [120]}, such as subtype E [456], and even CRF02_AG, which prevails in West and Central Africa, as described recently [436]. In HIV-1-infected children, presumably with subtype B, the change in coreceptor use has also been connected to disease progression; indeed, a significant association with decreased $CD4^+$ cell levels and severity of disease has been shown [54], though, interestingly, the coreceptor change appeared months or even years after the beginning of the immunological deterioration in pediatric patients infected with subtypes A, D, or CRF01_AE, thus suggesting that CXCR4-using virus may emerge as a possible consequence of immune deficiency. In adults, the frequency of SI variants has been suggested to differ according to subtype [361], yet an expanded coreceptor repertoire appears not to be a prerequisite for a progressive clinical course of HIV-1 infection [95].

In contrast to HIV-1, many primary HIV-2 strains have the ability to enter susceptible cells in a CD4-independent

way [343], utilizing a broad range of coreceptors [292, 400, 465], including CCR5 and CXCR4 {reviewed in [112, 342]}; furthermore, a clear R5 to X4 evolution does not seem to occur during the course of HIV-2 infection {[48, 158, 272, 343] as cited by [342]}, even though expanded coreceptor usage by HIV-2 may correlate with disease progression [321]. Most HIV-2 isolates appear to have the ability to replicate in MT-2 cells [292], without inducing syncytia [119]. Still, the molecular features underlying the biological phenotypes of HIV-2 and HIV-1 exhibit a high degree of similarity [10], including similar cytopathicity [377]. Recently presented data from a nonetheless limited study indicated that HIV-2 viruses from some asymptomatic individuals are unable to use the major coreceptors to infect peripheral blood mononuclear cells (PBMCs); the authors suggest that the observed reduced replicative fitness of HIV-2 isolates may be associated with the longer asymptomatic stage and lower pathogenic potential of HIV-2 compared to HIV-1 [21].

Soon after the identification of CXCR4 and CCR5, the question whether coreceptor usage that is the major determinant of viral tropism, differs across HIV-1 subtypes was addressed. No such differences across HIV-1 subtypes (A, C, D, E, and group O) were identified by this first study, which suggested, instead, that biological phenotype played a more important role than genetic subtype in the choice of coreceptor [461]. A subsequent investigation confirmed that coreceptor usage was closely associated with the biological phenotype of the virus, but not with its genetic subtype [37]. Next, an important study that examined 81 isolates representing 9 different subtypes confirmed the notion that use of CXCR4 correlated with observed biological phenotype across all subtypes, including subtypes F, G, H and J that had not been investigated before, since the CXCR4-expressing cell line could be infected by all isolates bearing a rapid/high SI phenotype and none of the isolates displaying the slow/low NSI phenotype [425]. Interestingly, however, subtype-dependent differences in coreceptor usage that could not be attributed to differences in clinical status, CD4 counts, or treatment were also identified; namely, dual tropism for both CXCR4 and CCR5 was rare among subtype D isolates, possibly reflecting a transition stage between CCR5 and CXCR4 monotropism, whereas the CXCR4-positive/SI phenotype that correlates with faster disease progression was found to be rare among subtype C isolates [425]. Studies by other groups corroborated this latter finding [38, 57, 293, 325, 330, 418, 420], even in hospitalized patients with AIDS [2].

The reason why SI viruses may be phenotypically uncommon among the most prevalent worldwide subtype C strains is still not clear. It has been speculated that the substantial immune activation due to concurrent infections such as that of the African context, could result in an over-expression of CCR5, and, thus, in a reduction in selective pressure for switching away from the NSI phenotype [3, 26, 27, 274, 349]. Expression of CCR5 is indeed favored on activated and memory T cells, while expression of CXCR4 is favored on naive T cells [42]. However, the spread of subtype C strains in so many countries with evidently diverse immunological backgrounds of inhabitants renders this explanation rather unlikely {reviewed in [405]}. Besides, the rarity of the SI phenotype has also been shown in Caucasians who contracted subtype C HIV-1 strains during deployment in Djibouti, East Africa [325]. Moreover, a delayed conversion rate to the SI phenotype has been observed in subtype B-infected, CCR5-delta32 heterozygotes who exhibit lower CCR5 expression levels [94].

Another explanation for the rarity of the SI phenotype in clade C isolates could stem from the different molecular architecture of the long terminal repeat (LTR) region that encodes for the transcriptional promoter of HIV-1; subtype C strains have, though not consistently [352], extra nuclear factor-kappa B (NF-κB) enhancer sites upstream of the usual two sites present in subtype B strains [193, 284, 366], possibly resulting in the more efficient transcription of mRNA without displaying the SI phenotype [195, 312]. Nevertheless, a more recent study suggested that the extra NF-κB sites may be biologically inactive [353]. Bjorndal *et al*. had expressed the view that the pathogenic potential of subtype C isolates may be governed by factors other than viral phenotype [38].

In the meantime, the presence of subtype C SI strains was demonstrated in 3 of the 24 studied male recent seroconverters from Zimbabwe, implying that SI viruses were actually transmitted or dominated early in the course of infection with subtype C strains [24]. Conversely, more SI than NSI subtype C strains with broadened coreceptor usage were isolated from South African patients at later disease stages whose CD4 counts were low [430]. In accordance to this report, SI viruses capable of utilizing CCR5, CXCR4, or both CXCR4 and CCR5 for entry and sensitive to specific entry inhibitors via these coreceptors, were identified in subtype C-infected patients with advanced AIDS [68]. Significant genetic changes were also observed in the V3 loop of these SI strains compared to the NSI viruses from the same cohort, suggesting that the prerequisites of the interaction of gp120 proteins with CXCR4 pertain to all subtypes. Compared to subtypes A and B, a more active protease may be encoded by subtype C isolates [435], which, nonetheless, may be less fit following initial infection and may thereby lead to slower disease progression in comparison to subtype B strains, as shown by competition experiments that compared the *ex vivo* fitness, or relative replication efficiencies, of subtype B and C isolates [22].

In contrast to subtype C, investigation of the biological characteristics of CRF14_BG from Galicia, Spain revealed an SI phenotype for these strains, even though they were obtained from a patient in the early A1 clinical stage; in addition, utilization of CCR2b, CCR3, CCR5 or CXCR4 as coreceptors as well as a characteristic pattern of mutations in V3 were also discovered [328]. On the contrary, unique recombinant forms co-circulating in the area have been found to display a NSI/CCR5 phenotype that may provide these strains with a selective advantage for heterosexual transmission [328]. According to Spira *et al*. subtype A viruses also tend to favor utilization of CCR5, even at later stages of the disease course [405]. Clade A-infected women near seroconversion have been reported to harbor viruses with distinct envelope genotypes that confer unique phenotypes which may be due, in part, to different requirements for relative configuration of CD4 and CCR5 on infected cells and which, in turn, affect viral fitness [322].

Key elements that determine usage of chemokine coreceptors, biological phenotype, and viral tropism are located in the third hypervariable domain of the gp 120 envelope glycoprotein of HIV-1 [15, 35, 49, 60, 64, 65, 72, 92, 126, 127, 174, 186, 188, 314, 350, 387-389, 403, 423, 424, 445, 446, 449, 450, 454]. Other regions of the envelope glycoprotein also affect these biological attributes within the sequence context of the V3 loop [44, 52, 66, 152, 153, 220, 221, 358, 378]. Complementing previous observations, a more recent study also predicted specific positions that contribute to a functional relationship between V3, V2, and C4 and confirmed that CXCR4 usage is associated with increased positive charge in the V2 hypervariable region [173]. In general, higher net charges in the V3 loop ($\geq +5$) characterize SI variants compared to the lower V3 loop net charges of NSI variants ($\leq +4$), typically associated with acute infection {reviewed in [328]}; nevertheless, the V3-loop charge alone cannot be used as a marker for phenotype prediction [436]. The increase of the overall positive charge of the V3 loop that may switch the viral phenotype from NSI to SI results from the introduction of basic amino acid residues at one of the two positions 11 and/or 25 (counting from the amino-terminal of the cysteine residue) of the V3 loop (HXB gp160 306 and 322, respectively) [92, 125, 126, 347].

Positively charged amino acids were thus found to occupy these positions in the V3 loop of SI isolates, whereas one or both amino acid residues were reported to be neutral or negatively charged in NSI strains [92, 126, 281]. The association between the positively charged residue(s) in the V3 loop and the SI biological phenotype was shown to hold not only for subtype B [126, 467], but also for subtypes A, C, D, E, F, G, and H of group M as well as for group O isolates [91, 96, 202, 245, 456, 467]. Subtype D variants, in particular, frequently displayed not only a high number of positively charged amino acid substitutions in the V3 loop, but also heterogeneity and length polymorphism [96, 317]. Less variation was observed in subtype C variants that lacked the highly conserved across the other subtypes N-linked glycosylation site {[318] as cited by [405]}. Consistent with restricted coreceptor usage and a NSI phenotype, the overall positive charge of the V3 region was found to be low in subtype C strains [137].

Other studies revealed a lack of correlation between the V3 loop amino acid sequence and the *in vitro* SI formation capacity of some HIV-1 strains across different subtypes {reviewed in [362]}, including subtype B [325], subtype F [178], and CRF02_AG recently [436]. Instead of positively charged amino acids, Leitner *et al*. had discovered specific neutral residues at these positions in the direct PBMC sequences from individuals (most of whom were infected with subtype B) with MT-2-positive isolates; hence, they argued that predicting the biological phenotype of HIV-1 may be more difficult using sequences from uncultured PBMCs rather than from virus isolates [246]. Tscherning *et al*. also found a weak correlation between the V3 genotype of isolates obtained from uncultured PBMCs and biological phenotype [425]. The preferential selection of SI over NSI clones from the population of HIV-1 viral quasispecies present in PMBC *in vivo* [154, 275, 379] by the *in vitro* isolation procedure may account for this discrepancy [176]. It should be stressed, however, that the inevitable selection during the isolation process does not render invalid the utility of biological phenotyping, which has been shown to be an independent marker of HIV-1 disease progression [203, 223].

Subtype E viruses have been suggested to be more readily transmitted heterosexually than subtype B strains by studies of discordant couples in Thailand [231], perhaps due to a greater tropism for Langerhans cells, maturing epidermal dendritic cells which line the vagina, cervix, and penile foreskin, and which, therefore, represent early cellular targets during heterosexual transmission [114, 402, 459]. Other studies did not confirm this hypothesis [105, 333]. The first report providing biological, serological, and genetic characterization of HIV-1 strains from Thailand dates from 1994 [187]. In HIV-1 subtype E env V3 loop, specific combinations of amino acid changes have been shown to be critical for determining viral coreceptor usage; however, cellular tropism was not dictated solely by viral coreceptor utilization. Position 11 of the V3 loop was indeed found to be pivotal for utilization of CCR5, while CXCR4 usage was conferred by a minimum of two arginine substitutions, regardless of combination. Additionally, arginine substitutions at positions 8 and 11 were required for T-cell line tropism, while macrophage tropism was not conferred by the V3 loop of R5 strain *per se* [206]. Analysis of the V3 sequences from the same intrafamilial subtype E infection case indicated that NSI/R5 variants were more resistant to positive selection pressure for amino acid diversification than those of the SI/X4 variants [386].

No such correlation between signature amino acids and syncytium formation in MT-2 cells was reported for subtype F viruses from Romania, although a tendency toward a more positive net charge in the V3 loop of SI isolates was noted [178]. A more recently published study showed that the V3 loop of subtype F isolates from Romania indeed harbors the determinants of MT-2 tropism and that induction of syncytium formation occurs in the presence or absence of positively charged amino acids at positions 306, 320, and/or 324; additionally, the net positive charge of V3 loop sequences derived from SI viruses was found to be higher than that of NSI isolates [177].

According to Hu *et al*. the lack of consistency of the observed associations between biological correlates and viral subtype diversity indicates that they may be due to chance alone or that they are affected by other unknown parameters; additionally, Hu *et al*. caution that these associations have been noted mostly from *in vitro* studies, which may not reflect accurately the *in vivo* conditions [180]. Still, the possibility that HIV-1 subtypes may indeed differ in their biological attributes and, as a consequence, possibly in their pathogenic potential as well, cannot be excluded and has to be explored further. Construction of infectious clones that are capable of producing replication-competent viruses following transfection of permissive target cells facilitates the characterization of the molecular biological attributes of HIV viruses. In this respect, clones belonging not only to subtype B, with which replication studies were almost exclusively conducted, but also to subtypes C, D, and CRF01_AE and CRF02_AG recombinant forms, have been generated [133, 233, 282, 296, 306, 311, 365, 410]. Notably, the first non-subtype B infectious molecular clone

of a fast replicating, high producer, X4-tropic primary HIV-1 isolate that clusters with CRF02_AG strains has even been constructed [413]. Characterization of the biological properties of HIV strains through such means is expected to aid in assessment studies of vaccines and antiretroviral agents.

DIFFERENCES IN TRANSCRIPTIONAL PROMOTERS AMONG HIV-1 SUBTYPES

The promoter and transcriptional start site of the integrated HIV-1 provirus are located in the 5′ LTR, a region with remarkable nucleotide sequence variation among group M subtypes [193, 283, 285, 437, 457]. In the absence of stimulation, the integrated viral promoter remains transcriptionally silent in the chromatin-bound conformation. Transcription is stimulated by the HIV-1 *trans*- activator (Tat) protein, which also displays divergence among clades that may affect its functional properties [283, 285]. The viral Tat protein binds the *trans*-activation responsive region (TAR) element [31, 104], a stem-loop structure at the 5' end of viral mRNA {reviewed in [138, 205]}, thus inducing a remodeling of the nucleosome arrangement downstream of the transcription-initiation site. Transcription elongation is stimulated by the formed Tat-TAR RNA complex through the recruitment to the promoter of a cyclin-cyclin-dependent kinase complex that phosphorylates the C-terminal domain (CTD) of the largest subunit of RNA II polymerase [192, 196, 441].

Activation of the integrated HIV-1 LTR from the chromatin context is aided by Tat-associated histone acetyltransferases (TAHs), including p300 and p300/CBP-associating factor (PCAF), presumably *via* the acetylation of histones [25, 264]. In brief, two discrete and functionally critical steps in transcription appear to be regulated by the acetylation of Tat: acetylation at Lys28 by PCAF enhances Tat binding to an RNA II polymerase CTD-kinase, while acetylation by p300 at Lys50 of Tat promotes the release of Tat from TAR RNA during early transcription elongation [212]. Histone deacetylase inhibitor trichostatin A was found to synergize with Tat in the transcriptional activation of the HIV-1 LTR, thereby providing support for a functional role of acetylation *in vivo*. Preliminary evidence further suggests that a correlation may exist between the acetylation of subtype-specific Tat proteins and their transactivation efficiency. Clade E Tat protein was actually reported to possess the greatest transactivation capacity compared to Tat B or C, regardless of LTR context [353]. This report is in accordance to the results of Verhoef *et al*. who found that a molecular clone of a clade E-replaced LTR strain replicated more efficiently in comparison to a wild-type clade B HIV-1 strain (LAI) [437], thus demonstrating that such recombination events may increase viral fitness.

HIV-1 transcription in infected cells is regulated not only by the viral Tat protein, but also by cellular factors that bind to enhancer elements in the U3 region of the LTR, such as the binding sites for the Sp1 and nuclear factor-kappa B (NF-κB) transcription factors {reviewed in [139, 214]}. Three tandem Sp1 sites are typically located upstream of a canonic TATA box and TAR element, yet this core promoter arrangement may be different in natural HIV-1 isolates; for example, viral strains containing four [222] or even five Sp1 sites [359] have been characterized. To regulate transcription through NF-κB binding sites in the LTR, HIV-1 utilizes the NF-κB/Rel proteins [238]. Merely a 10-bp stretch of DNA with the consensus sequence 5′ -GGGPuNNPyPyCC-3′ is recognized by NF-κB, a heterodimer composed of p50 and RelA subunits [391], the latter of which provides the transactivation domain [232]. Although both Sp1 and NF-κB are constitutively expressed, NF-κB is retained in the cytoplasm of unstimulated cells, coupled with inhibitory IκB proteins; many extracellular stimuli, including multiple cytokines, phorbol esters, and concurrent infection with some viruses, can induce the dissociation of the complex IκBα, resulting in the release and migration of active NF-κB into the nucleus [391, 417]. Stimulation of NF-κB transcription factors, which orchestrate the host inflammatory response and activate the LTR, can lead to high levels of viral gene expression and replication, thus affecting pathogenesis {reviewed in [101]}.

Transcription assays with luciferase reporter constructs showed that all subtype LTRs are functional promoters with a low basal transcriptional activity and a high activity in the presence of the viral Tat transcriptional activator protein; no major differences in the mechanism of Tat-mediated *trans* activation were identified among subtypes since all subtype LTRs responded equally well to the Tat *trans* activator protein of subtype B [193]. However, sequence analysis demonstrated a unique LTR enhancer-promoter configuration for each subtype, with alterations in the TATA box, NF-κB enhancer, TAR as well as in other modulatory elements such as Sp1, upstream stimulating factor (USF), and NF-AT binding sites [134, 193, 283, 285, 366], as discussed in a recently published review by Spira *et al*. [405].

Two NF-κB enhancer sites are thus present in subtype A, B, and D isolates, three to four in subtype C and only one in the promoter region of subtype E isolates [134, 283-285, 366]. The NF-κB enhancer configuration was reported to be correlated with responsiveness to the proinflammatory cytokine tumor necrosis factor–alpha (TNF-α) [193, 284]. The LTRs of subtype C were thereby activated more by TNF-α in comparison to subtypes A, B, D, F and G, with the lowest stimulation noted in subtype E that contains one NF-κB site [193]. Especially for the widely spread subtype C isolates, the extra NF-κB enhancer sites that displayed increased p65/RelA responsiveness [285] were initially thought to instill to these strains a gain-of-function in transcriptional activation, mediated *via* TNF-α; however, as discussed in the previous section, a subsequent study suggested that the duplicated κB sites of subtype C may in fact be biologically inactive, since they were found not to compete for NF-κB binding with the corresponding sites from subtype B or E strains [353].

The higher promoter/enhancer activity of subtype C viruses correlated not only with the presence of additional potential NF-κB sites, but also with an alternative core-negative regulatory element (NRE), position -174 to -163 [302, 303]. A characteristic E-box motif that constitutes a target for USF is contained in this site [150, 372, 373]. HIV-1 subtype B LTR-directed transcription may be affected both negatively [143, 254, 255] and positively [83, 260, 294, 392, 460] by USF, which may be involved in the

pathogenesis of AIDS [359, 394]. In fact, USF may be involved at two critical steps of HIV-1 replication, viral entry and viral expression, through an upregulation of the CXCR4 promoter activity [291]. Another study extended these findings to show that USF acts independently of the core-NRE USF-binding site, through an unidentified yet element conserved in subtypes A to E and G and in a cell-specific manner; hence, transcription directed from representative LTR sequences of all tested subtypes was found to be repressed in an epithelial cell line, and activated in a T-cell line, *in vivo* [300].

Intracellular expression of human high mobility group B 1 (HMGB1) protein, an abundant nuclear protein in all mammalian cells, was shown to affect HIV-1 LTR-directed transcription in both a cell- and promoter-specific manner [301]. More specifically, endogenous HMGB1 repressed HIV-1, but not simian virus 40 or cytomegalovirus, gene expression in monocytes and cultured epithelial cells through inhibition of LTR-mediated transcription; the HMGB1 inhibition of HIV-1 subtype C expression, in particular, was dependent on the number of NF-κB sites in the LTR region. The authors suggest that specific features in the LTR of subtype C and E isolates, such as the NF-κB sites, may enable them to overcome the inhibitory effect of HMGB1 in monocytes, since the HMGB1/NF-κB interaction may bring about a repression state of the chromatin at the LTR by recruiting other repressive proteins, thus offering another explanation for the global expansion of these subtypes. Functioning, therefore, as a homologue of the dorsal switch protein (DSP1) of *Drosophila* [244], constitutive HMGB1 mRNA expression in macrophages may play a role in the control of HIV-1 replication.

Subtype E strains contain an enhancer with only one functional NF-κB site, apparently without losing in promoter function [285], since they were also reported to contain an additional, mutated binding site that displayed reduced TNF-α responsiveness [283]. The gain of LTR function of clade E versus B viruses seems to be due, at least in part, to this conversion to a new binding specificity (NF-κB to GABP, an Ets transcription factor family member protein) [193, 437]. All 18 reported subtype E sequences were characterized by the presence of the NF-κB to GABP switch [437]. In contrast, lower TNF-α responsiveness *in vitro* has been demonstrated for the LTR of HIV-2 that contains one NF-κB site [162]. The importance of sequence variations of NF-κB enhancer sites is also made evident by fact that the function of Tat is dependant upon κB-responsive elements within the LTR in resting $CD4^+$ T lymphocytes [171].

Other differences among subtypes have been noted in further upstream motifs, such as the *nef*-overlapping USF transcription factor that is present only in clade B, and the AP-1 transcriptional factor binding site that is present at two sites in clades A and F, one site in clades C, E, and G, and not present in clades B or D [193]. Furthermore, a specific motif for the NF-IL6 transcriptional factor (C/EBP-β) that is contained in the -170 region of U3 and that transactivates the LTR of HIV-1 in cells of monocytic origin [169], has been found only in subtype B and not in subtypes A, C, D, or group O viruses [457].

Not surprisingly, therefore, different responses are elicited by HIV-1 subtypes to different transcription factors in different cell types. For instance, compared to subtype B or E, subtype C strains are stimulated to a greater extent by the NF-κB binding factor, Rel-p65 and nuclear HeLa cell extract [285, 303]. Although, the LTR is also considered important for cellular tropism [356], further studies are needed to elucidate the role that these structural and functional differences among subtypes may play in gene expression and replication kinetics of HIV-1. According to Jeeninga *et al*. the differences among subtypes may be based on cell type-specific variation in the concentration and activity of nuclear transcription factors interacting with the LTR [193]. Virus function is certainly influenced by the redundancy and context of promoter/enhancer elements, as also shown by the observation *in vitro* of cell-type-dependent compensatory and dosage effects [61, 357, 463].

IMPACT OF HIV DIVERSITY ON TRANSMISSION AND VIRULENCE

The rate of HIV-1 transmission and disease progression may be influenced not only by the known multitude of host genetic factors {recently reviewed in [13]}, but also by such viral factors as infecting subtype. Given that HIV-2 is less infectious and virulent compared to HIV-1 [200, 263] and, moreover, that the different HIV-1 groups and subtypes are not distributed evenly throughout the globe, the association of viral subtypes with differing transmissibility and pathogenicity seems plausible, although such epidemiologic studies are obviously difficult to perform and to interpret {reviewed in [6, 175, 180, 328]}. No evidence to suggest that group O and group N viruses are less efficiently transmitted or less virulent has been presented; a stochastic or chance process may account for the disproportional global spread of group M viruses.

Different group M subtypes have nonetheless been associated with different modes of transmission. For instance, in South Africa subtype B and subtype C viruses were typically associated with male homosexual transmission and with heterosexual transmission, respectively [429], although subtype B has also been isolated from heterosexual patients in the country recently [430]. Intravenous drug users may have a worse prognosis for progression towards AIDS and death than homo-/bisexual men, possibly because the transmission route may determine the seemingly critical, initial immune response [326]. Yet, Spijkerman *et al*. had reported a lower prevalence and incidence of the SI phenotype among injecting drug users compared with homosexual men [404]. Other epidemiological studies do not support that the rate of disease progression is influenced by the route of infection [258]. In countries with poor resources, progression rates to AIDS were similar to those in developed countries for homosexual cohorts and greater for cohorts infected by other modes of transmission, before the introduction of antiretroviral therapy [290]. In general, circulation of subtype B strains in Europe and the Americas has been associated with transmissions among homosexuals and intravenous drug abusers, whereas the predominant non-B subtypes in Africa and Asia have been associated primarily with

transmissions among heterosexuals [405]. Irrespective of viral subtype, however, similar transmission frequencies have been reported for heterosexual and vertical transmissions [265, 382]. Data on the potential effect of genetic subtype on the risk for both of these categories of viral transmission are controversial.

Subtype E viruses have been suggested to be more readily transmitted heterosexually than subtype B strains by studies of discordant couples in Thailand [231], perhaps due to a greater tropism for Langerhans cells that represent early cellular targets during heterosexual transmission [114, 402, 459]. In support of this hypothesis, the estimated probability of female-to-male HIV-1 transmission per sexual contact in Thailand was found to substantially higher than analogous estimates formulated in North America where subtype B strains prevail [266]. These studies reinforced the notion that the biological properties of some non-B subtypes facilitated their heterosexual transmission, thus contributing to the rapid expansion of HIV-1 in Asia and Africa. Nevertheless, this hypothesis was not confirmed by other studies [105, 333, 334]. No association between the pattern of coreceptor usage and transmissibility of subtype E isolates was found by a study of Thai men with HIV-1-infected and uninfected spouses either [426]. In addition to subtype E, subtypes A and C have also been associated with an increased risk for heterosexual transmission, possibly due to molecular evolutionary changes that include receptor affinity mediated by the env gene, and increased transcriptional activation mediated by changes in the LTR and *tat* [113].

Women infected with subtype A, C or intersubtype recombinant viruses were found to be more likely to transmit HIV-1 to their infants than mothers infected with HIV-1 subtype D, according to a study of 51 matched transmitting and nontransmitting mothers from Tanzania [346]. Independently of maternal CD4 counts at enrolment, viruses containing subtype C LTRs, in particular, were 6.1 times more likely to be transmitted from infected mothers to infants in Tanzania than those with subtype D LTRs [41]. Specific patterns among the transmitted recombinant genomes that may confer an advantage for transmission were identified as well [228]. Furthermore, as shown by a recently published report examining the same cohort, the observed differences in perinatal transmission rates among subtypes could not be associated with the likelihood for multiple quasispecies transmitted independently during in-utero infections [345].

Hypermutated HIV-1 genomes, which have been associated with slower disease progression, were isolated from infants soon after vertical transmission as well, suggesting that they were generated in-utero and soon after birth, and/or that they were vertically transmitted; no relationship between the percent hypermutated Gs and viral subtype or transmission status of the mother was nonetheless identified, although the extent of hypermutation was lower in infants than in the mothers [227]. Other studies also did not reveal an association of a particular maternal infecting viral subtype with increased risk for mother-to-child transmission rate. No significant differences there thus found between maternal clades A and D and either frequency or mode of vertical transmission in Nairobi, Kenya [299], or between the transmissibility of clade E and B viruses from Japanese mothers to their babies [455]. A recently published study did not show any significant differences in the mother-to-child transmissibility of subtypes A, C, D and recombinant forms either [412]. On the contrary, evidence for significantly higher rates of mother-to-child transmission among mothers infected with subtype D compared with subtype A has been recently presented from a large perinatal transmission study in Kenya [452].

The markedly different global epidemiology of the pandemic with increasing prevalence rates for HIV-1 compared to the endemic at stabilized rates HIV-2 [344], despite the similar modes of transmission of the two types of viruses {reviewed in [375]}, may be accounted for by differences in viral load levels throughout the natural history of infections that appear to correlate with lower transmissibility of HIV-2 than HIV-1 [56, 88, 144, 336, 397]. Compared to HIV-1, a 5- to 9-fold and a 10- to 20-fold reduction characterizes HIV-2 heterosexual [200] and perinatal transmissions [4, 267, 315], respectively {reviewed in [342]}. A protective effect of HIV-2 infection on incident HIV-1 infection is not supported by the currently available epidemiological data {reviewed in [148]}.

The observed differences in the natural history of HIV-1 and HIV-2 infections that reflect the lower HIV-2 virion expression levels *in vivo* are also related to differences in vilurence and clinical progression of the two infections; accordingly, incubation periods and survival time with AIDS are longer for HIV-2 infections [14, 34, 89, 268, 327]. In agreement with the estimated dates for the most recent common ancestors for HIV-2 subtypes A and B, Gomes *et al*. argue that the principal route that sparked the HIV-2 epidemic during the independence war in Guinea-Bissau was parenteral transmission [145]. No significant clinical differences between HIV-2 patients infected either with subtype A or with subtype B, have been identified [86].

Patients infected with closely related HIV-1 serotypes may differ in the rate of disease progression; accordingly, patients infected with serotype B (GPGR) tended to progress faster to AIDS compared to patients infected with serotype B-Br (GWGR) in Brazil [55, 371]. Meanwhile, a cross-sectional study of HIV-1-infected women in Kenya showed that subtype C-infected women had the highest plasma viral RNA levels and significantly lower CD4 lymphocyte levels than women infected with subtypes A, D or G [307]; hence, based on these prognostic markers, women infected with subtype C were at more advanced stages of immuno-suppression, thereby suggesting that subtype C is associated with a more rapid disease progression, or, alternatively, that subtype C represents an older epidemic in Kenya. Data from a prospective study of registered female sex workers in Senegal also suggested that HIV-1 subtypes may differ in rates of progression to AIDS, since women infected with a non-A subtype (namely C, D, or G) were eight times more likely to develop AIDS than those who were infected with subtype A [199].

This paper was received with skepticism by some researchers since all samples were collected from a single geographical region where subtype A predominates (without discriminating subtype A from AG recombinants), thus introducing bias to the study [6]. Yet, a similar effect was

demonstrated by a study of 1045 adults in Uganda, corroborating previous findings which had not nevertheless reached statistical significance for most indicators of progression [198]. This study revealed an association of subtype D with faster progression to death and with a lower CD4 cell count during follow-up with respect to subtype A, after adjusting for CD4 cell count at enrollment [197]. In support of these results, HIV-1 RNA levels were found to be significantly higher in subtype D- compared to subtype A-infected persons in Uganda [289]. Several studies infer that HIV-1 infection among infected people in Africa is characterized by faster disease progression rates compared to infections of patients in the West.

Other studies did not attribute differences in disease progression rates between Africans and Caucasians either to the genetic subtype of the infecting virus, or to the ethnicity of the host, but rather to the quality of available health care and possibly to concomitant exposure to other pathogens. No differences in the rate of CD4 cell decline, clinical progression or plasma HIV-1 RNA levels were thus recorded between individuals infected with subtypes A, B, C or D, or between Africans and Swedes [7]. Although seroconversion dates were unknown for most of these cases, other parameters that could introduce bias were controlled for; more specifically, study subjects were infected with HIV variants originating from different countries in and outside Africa, had access to same quality medical care and all were treatment naive. The outcome of this study is in accordance with previous reports that concluded not to have found differences in disease progression or survival associated with race or subtype (assumed from the origin of the patients and not actually tested for) among HIV-infected persons who received medical care from a single center in Baltimore, USA [58], or among infected Africans and non-Africans in London, UK [99, 253].

Likewise, subtype B-infected Israeli men and subtype C-infected immigrants from Ethiopia have been reported to exhibit similar rates of $CD4^+$ lymphocyte decline [130] and similar immune activation profiles at all stages of the infection [442]. In addition, patients from Thailand infected with HIV-1 subtypes B' or E (mainly injecting drug users and sexually-infected individuals, respectively) displayed similar spectrums of levels of immunosuppression, opportunistic infections, and in-hospital mortality rates [12]. Close to seroconversion, three times higher viral loads have been associated with subtype E compared to subtype B, but this difference faded out later in the course of infection, such that viral loads were similar at 12, 18, and 24 months following seroconversion [182]. Finally, a prospective multicenter cohort study conducted in Cameroon and Senegal revealed no differences between patients infected by CRF02_AG and those infected by other viral strains in terms of survival, clinical disease progression, or CD4 cell decline [240].

Thus, it becomes apparent that finding consistent associations between HIV-1 subtype and correlates of transmission or of pathogenesis is extremely difficult since the answer to this question appears to a matter of degree rather an absolute dichotomous outcome [180]. To elucidate the relation between infecting viral subtype and disease progression, long-term prospective studies of recent seroconverters would be necessary [328]. Identification of such differences could shape differently the future of the pandemic.

IMPLICATIONS OF HIV GENETIC VARIABILITY

The genetic variability of HIV may affect the accuracy of initial diagnostic tests as well as of viral load assays that are important for the monitoring of disease progression and clinical management of infected subjects {reviewed in [6, 270, 328]}. For example, different performance characteristics in detecting newly acquired infections with subtypes B or E [324], or in the quantification of plasma HIV-1 RNA for non-B subtypes [8, 9, 16, 81, 82, 85, 250, 316, 323] have been reported. The currently recommended, fourth generation diagnostic assays are based on the detection of p24 antigen and anti-HIV antibodies. Concurrently, versions of the commercially available viral load assays display improved performance in the quantification of group M subtypes A to G [408]. The significance of HIV-1 genetic forms for vaccine design has been reviewed elsewhere [328]. HIV-1 subtype diversity in the context of sensitivity to antiretroviral drugs, drug resistance and viral fitness has also been reviewed elsewhere [328, 405].

VIRAL PHENOTYPE: CLASSIFICATION SYSTEMS OF HIV BIOLOGICAL PHENOTYPE

The biological phenotype of HIV-1, which may be determined using phenotypic assays [191, 225] or predicted by genotypic sequence data analysis {reviewed in [194]}, constitutes an independent prognostic indicator of AIDS progression {reviewed in [119, 122, 127, 175]}. Until recently, the phenotype of HIV-1 strains was defined in relation to the following properties: (i) preference for specific target cells (cellular tropism), (ii) cytopathology, and (iii) replicative capacity. Accordingly, three systems were used for the classification of the phenotype of HIV-1 variants, the determinants of which have been mapped to the gp120 subunit of the envelope glycoprotein, and particularly to the V3 domain, as discussed above [65, 92, 93, 126, 127, 186, 388, 389].

The first classification system distinguished between macrophage (M)-tropic and T-cell line (T)-tropic variants, both of which infect primary $CD4^+$ T lymphocytes, but only T-tropic variants grow in T-lymphoblastoid cell lines *in vitro* [74]; however, "dual-tropic" isolates that might grow in either macrophages or T-lymphoblastoid cell lines were also characterized [396, 427]. The second classification system distinguished between syncytium-inducing (SI) and non-syncytium-inducing (NSI) strains, based on the capacity or not to form syncytia (giant multinucleated cells) through cell fusion in the MT-2 cell line [225, 380, 414]. MT-2 cells express CXCR4 and not CCR5, yet NSI isolates can form syncytia with CCR5-positive cells too [379]. Additionally, in some cases, HIV-1 disease progression was shown not to follow the typical course during which the initially dominant M-tropic/NSI viruses are replaced by T-tropic/SI viruses, but, instead, M-tropic strains with SI characteristics might dominate in more advanced stages of the disease [73, 107, 385]. The third system mainly considered the *in vitro*

growth kinetics of viral strains in culture and distinguished between slow/low (S/L) and rapid/high viruses (R/H) [17, 123]. S/L viruses rarely induce syncytia, whereas extensive syncytia formation typifies R/H strains [121].

The interchangeable use of these three classification systems of HIV biological phenotype, which apparently are not synonymous, caused confusion. The present nomenclature that is based on the coreceptor used for viral entry into target cells in conjunction with CD4 [28, 106, 288] is more precise; therefore, isolates that principally use CCR5 and CXCR4 for cell entry (M-tropic/NSI and T-tropic/SI strains, respectively) are currently termed R5 and X4 viruses, correspondingly, while (T-tropic primary) viruses that can use either coreceptor with comparable efficiency [37, 66, 77, 229, 396, 399, 403] are termed R5X4 strains [29]. The ability of most of these viral strains to replicate in both CCR5- and CXCR4-positive cells may be due to their mixed composition of R5 and X4 viruses; therefore, a more appropriate designation for such isolates would be R5+ X4, but the conventional term R5X4 is still used because of the technical difficulties associated with distinguishing between truly dual-tropic and mixed-phenotypic variants {reviewed in [287]}. HIV-1 isolates that utilize CCR3 and/or CCR5 to enter microglia [168] are, by analogy, termed R3 or R3-R5 isolates. The same defining principle on the basis of coreceptor use may be applied to the categorization of the phenotype of HIV-2 and SIV strains as well [29].

HIV TROPISM, IMMUNE ACTIVATION, AND CD4$^+$ T CELL DEPLETION

The tropism of HIV isolates for specific coreceptors in conjunction with CD4 defines the cellular targets of the virus *in vivo*, as shown by studies of disease progression in humans [63, 151] and in nonhuman primates [179]. Most recently, the cellular and anatomical sites of HIV replication have been reviewed by Stebbing *et al*. [406], while other previously published articles also review in detail the cell tropism of HIV for immune and nonimmune tissues [30, 69, 98, 127, 210, 443]. CD4$^+$ T cells constitute the main target cells for HIV-1, possibly due to the high affinity of the CD4 receptor for the viral gp120 [84, 218, 376]. Nevertheless, all cells expressing CD4 and such coreceptors as CCR5 or CXCR4 may potentially be infected with HIV. Cells of the central nervous system (CNS), particularly the microglia and perivascular macrophages of the brain, may be infected too, causing the AIDS-associated dementia {reviewed in [129]}. The possibility that adipose cells might represent target cells for HIV-1 was investigated as well [165], but it was soon disproved [297].

Different CD4$^+$ T cell subpopulations are targeted by the phenotypic variants of HIV, R5 and X4 strains, which, as suggested recently by Moore *et al*. [287], may almost be viewed as two distinct lentiviruses; replication of R5 viruses is favored in activated, memory/effector T cells, whilst X4 viruses may additionally infect intrathymic T progenitor cells and naive T cells in the peripheral lymphoid system [32, 39, 236, 320, 407]. The direct effect of the switch in coreceptor usage [77, 374] at the peak of viral diversity [383], which expands the virus accessibility to circulating naive CD4$^+$ T cells, thereby also increasing viral burden [224], is the rapid CD4$^+$ T cell depletion [39, 76, 223, 320, 348, 379, 416]. Indirectly, however, the regenerative capacity of the immune system is also affected by the X4 emergence or predominance within patients, since chronic immune activation is then exacerbated, and the destruction precisely of the cells that are designed for the maintenance of the naive and memory CD4$^+$ T cell pools, is facilitated; this disruption of T cell homeostasis during HIV infection eventually leads to the collapse of the immune system {reviewed in [108, 117, 155, 166, 269]}.

The complex interplay between the phenotypic viral variants and the immune system, particularly the mechanisms responsible for T cell homeostasis and regeneration, have been reviewed in detail elsewhere [109]. The outlined above, complex model, which emphasizes the pivotal role of immune activation in the depletion of T lymphocytes during systemic HIV infection, is the one currently thought to hold [156]. Moreover, at present HIV infection is thought to be principally governed by the state of cellular activation and expression of chemokine receptors by specific subsets of CD4$^+$ T cells residing not in the peripheral blood or lymph nodes, but in mucosal lymphoid tissues {reviewed in [434]}. Indeed, the gut-associated lymphoid tissue (GALT) that contains almost half of the body's T cells [295], most of which are CCR5$^+$ and in an activated state, appears to play a significant role not only in combating food-borne pathogens, but also in HIV-1 pathogenesis {reviewed in [287, 406]}. Furthermore, recent data indicate that the accelerated naive CD4$^+$ T cell decline associated with the emergence of X4 variants may be mainly related to increased death and activation of naive T cells rather than to the impairment of thymic function [167].

UNANSWERED QUESTIONS REGARDING HIV PHENOTYPE

Several questions regarding the role of the biological phenotype of HIV in AIDS pathogenesis remain unanswered {reviewed in [287, 329]}. R5 strains seem to prevail in the vast majority (approximately 90%) of primary and early stage HIV-1 infection cases [433, 469], while the X4/SI phenotype that is associated with accelerated disease progression evolves *in vivo* in approximately half of infected subjects [45, 75, 223, 259, 348]. Hence, the progressive clinical course of HIV-1 infection does not necessarily depend upon an expanded coreceptor repertoire of HIV-1 [95]. Changes in coreceptor usage by the virus could constitute a continuous process that might lead to profound alteration in signalling at specific receptors [204]. The mechanism that generally prevents X4 strains from predominating *in vivo* is poorly understood. The results of a recently published study by van Rij *et al*. exclude target cell availability, at least at the peripheral blood, to act as a driving force for the R5-to-X4 virus phenotype evolution [431].

The majority of R5/NSI HIV-1-infected individuals do progress to AIDS too. R5 virus-mediated rapid disease progression was demonstrated to be associated with host, rather than viral, factors by one study [33]. However, in such cases the cytopathicity of HIV-1 R5 variants may increase

with progression to disease [235], possibly not independently of changes in coreceptor utilization [230]. On the contrary, in the rare cases of transmission of X4 strains, clinical progression is usually more rapid [124, 309]. In addition, the incomplete resistance to HIV-1 transmission conferred by homozygosity for the 32 base-pair deletion (Delta32/Delta32) in the CCR5 coreceptor gene is associated with acquisition of X4 variants, with differing pathogenic potential from late-stage X4 virus, or early X4 virus acquired by individuals with other CCR5 genotypes {reviewed in [384]}. The study of the persistence of dual-tropic HIV-1 in an individual homozygous for this deletion suggested that structural features of the viral envelope linked to CCR5 tropism could confer a selective advantage *in vivo* [146].

During the early stages of the infection, NSI viruses appear to predominate not as a result of limited availability of CXCR4-expressing cells within the mucosa, but because there seems to exist a fundamental requisite for CCR5-expressing cells at these stages, regardless of the route of transmission [447]. In sexual transmission, for example, the level of CCR5 on Langerhans cells has been suggested to play a decisive role in the preferential transmission of R5 strains [208]. Transmission of R5 viruses via the rectal route may also be favored by the preferential expression of CCR5, instead of CXCR4, on intestinal epithelial cells [43, 273]. Other studies nonetheless suggest that the transmission and propagation of X4 variants along mucosal membranes may be hindered by the downmodulation of CXCR4 on resident HIV target cells by extensive expression of stromal cell-derived factor-1 (SDF-1) [5]. The findings of Harouse *et al*. that infection of macaques with chimeric X4 simian-human immunodeficiency virus (SHIV) strains caused a profound loss in peripheral $CD4^+$ T cells that was not paralleled in the intestinal $CD4^+$ T cells as was the infection with R5 strains, offers support to this hypothesis [164].

An alternative explanation for the dominance of R5 over X4 strains in early years of the HIV-1 infection, despite the fact that there exist more cellular targets in the blood for the latter strains [42, 109], could be their selective tropism for the dendritic cell in the context of dendritic cell-T cell conjugates within lymphoid tissues {[128, 147, 207] as cited by [287]}. In a sense, the dendritic cell may function as a Troyan horse for HIV while fulfilling its duty in the initiation of adaptive immune responses by presenting pathogens or pathogenic antigens to T and B cells in regional lymph nodes [332]; the dendritic cell may indeed provide a vehicle of transportation for the virus to reach CCR5-expressing, activated $CD4^+$ T cells in the ideal, for rapid and efficient amplification, milieu of T cell-rich regions of the lymph nodes [141, 237, 332], since it can carry infectious HIV, internalized in intracellular endocytic vacuoles, for up to 5 days without being productively infected itself [237, 331].

Whichever of these hypotheses is correct, the fact remains that although both biological types of HIV may be successfully transmitted, R5 strains outcompete their X4 counterparts *in vivo*, once the virus has disseminated to the most active sites of replication. Thus, under conditions of primary HIV-1 infection, the X4 phenotype may be suppressed or it may even evolve into R5 {[79, 337] as cited by [287]}. Still, the possibility that undetectable levels of CXCR4-using viruses are always present cannot be excluded [69]. The precise mechanism(s) that allows for the cytopathic SI/X4 strains, all of which may not replicate more efficiently than NSI/R5 isolates [339], to typically prevail later into the course of infection is unclear. Also unclear is the selective mechanism(s) upon which the evolution of X4 variants is based: namely, whether disease progression is accelerated as a direct consequence of the emergence or predominance of X4 isolates (virological basis), and/or, conversely, whether the declining competence of the immune system of the host permits the growth of viral strains with increased replicative capacity (immunological basis). Other factors, such as viral subtype that was discussed in detail in the previous section, could influence the evolution of X4 variants in HIV-infected subjects as well.

Several arguments can be presented in favor of either one of these basal selective mechanisms. For instance, if similar relative "burst sizes" (virions released per infected cell) are assumed for R5-infected/producing activated memory cells and X4-infected/producing resting naive cells *in vivo*, then the greater burst size, by approximately an order of magnitude as measured in lymphoid tissue blocks *in vitro* [111], of R5 variants provides these strains with a virological advantage compared to their X4 cousins over multiple replication rounds {reviewed in [287]}. A number of immunologically-based mechanisms {i.e. [243, 381]} have also been proposed to account for the selection pressure against X4 strains, which are, after all, associated with low $CD4^+$ T cell numbers that reflect immunologic impairment [431].

Increased rates of SI variant acquisition in HIV-1-infected individuals in Japan were correlated with homozygosity of a polymorphism in the interleukin-4 (IL-4) promoter region, IL-4 -589T, which resulted in elevated IL-4 production and in the acceleration of the phenotypic switch from NSI to SI. [305]. This pleiotropic cytokine had been shown to increase the propagation of SI HIV-1 isolates, while inhibiting the propagation of NSI isolates through a dual effect: (a) by increasing CXCR4- expression and by decreasing CCR5-expression in primary $CD4^+$ T lymphocytes, and (b) by stimulating the expression of all viral isolates *via* a transcriptional activation mechanism [428]. In the Amsterdam Cohort of HIV-infected homosexual men, however, the IL-4 -589T promoter polymorphism was associated with a delayed acquisition of X4 variants, with no apparent effect on the rate of disease progression [234]. The possibility that plasma levels of another cytokine, interleukin-7 (IL-7), may be associated with the emergence of SI variants has been investigated as well [249].

Additionally, according to two early studies, acute phase SI variants were later suppressed in favor of NSI viruses [79] possibly via an immune-mediated mechanism [239]; then again, X4 and R5 primary viruses are equally resistant to neutralizing antibodies {reviewed in [69]}. Moreover, T cell epitopes on the envelope glycoprotein are few and unlikely to distinguish between the two phenotypic variants, thereby minimizing the possibility for T cell immunity to play a role {reviewed in [279] as cited by [69]}. Nevertheless, a recently published study of SHIV infection in the rhesus macaque model suggested that the selective outgrowth of R5

viruses may be due to the differential $CD8^+$ T cell-mediated control of X4- and R5-replication [163].

Therefore, it becomes apparent that finding the right answer to such a key question as why, or even if, the R5-to-X4 phenotypic switch occurs during HIV pathogenesis, is no easy task.

VIRAL ATTENUATION

Several, but nonetheless rare, instances of infection with attenuated forms of HIV-1 leading to considerably decelerated disease progression rates have been described in the literature in individual case reports or small patient cohorts {reviewed in [175, 219, 261]}. These patients who progress to AIDS very slowly or not at all -and are, hence, termed long-term survivors or non-progressors (LTNPs), respectively-, form "the end of the tail" of the normal distribution of HIV disease progression and they are perceived to withhold at least a key that opens the doors to preventive vaccine and treatment agents development [219].

DELETIONS IN NEGATIVE FACTOR (NEF) GENE

Kestler *et al*. first demonstrated that *nef*-defective SIV causes attenuated disease in primates; in particular, *nef* was shown to be required for maintaining high viral load and full pathologic potential during the course of persistent infection in rhesus monkeys *in vivo* [211]. Moreover, rhesus monkeys infected with *nef*-deleted live SIV were completely protected against challenge by intravenous inoculation of live, pathogenic SIV, thereby suggesting that deletion of this or other genetic elements from HIV might provide the means for creating an effective vaccine to protect against AIDS [87]. The crucial role of *nef* in the determination of the rate of disease progression towards immunodeficiency was also demonstrated in the mouse model that bears more similarity to pediatric human AIDS [161].

The best known example of slowly progressing subjects infected with attenuated virus is the Sydney Blood Bank Cohort that consists of eight unrelated blood or blood-product recipients from Sydney, Australia who acquired HIV-1 through transfusion from an infected donor [241]. All except one of the recipients and the donor remained clinically free of symptoms, with stable and normal CD4 T cell counts 10 to 14 years after infection. Genetic analysis of sequences from either virus isolates or patient PBMCs exhibited similar deletions in the nef gene and in the region of overlap of *nef* and the U3 region of the LTR [97].

However, follow-up analyses documented immunologic damage in three of the four subjects with detectable plasma HIV-1 RNA, and stable CD4 lymphocyte counts in three subjects with undetectable plasma RNA, thus suggesting that progression to immunosuppression may be significantly delayed, but not completely prevented in patients carrying attenuated viral quasispecies [242]. Another study of the same cohort further revealed that the maintenance of low levels of activated CD8 T cells and strong specific proliferative responses to HIV-1 p24 in the three LTNPs who harbored *nef*/LTR deleted strains was associated with control of viral replication [36]. Phagocytic efficiency of opportunistic pathogens, such as *Mycobacterium avium* complex and *Toxoplasma gondii*, appeared to be generally unimpaired in monocytes derived from members of this cohort. Yet, a recently published study [209] presented evidence of impairment in this function, coincident with further molecular deletions in the *nef*/LTR region of the viral genome, a finding that may herald the loss of attenuation of these strains.

Defects in *nef* sequences were detected in other adult [216, 262, 368, 440], and pediatric [53, 140] long-term survivors with slowly progressive HIV-1 infection or LTNPs. Nonetheless, a decline in $CD4^+$ T cells was also noted in later samples of the hemophilic LTNP with absence of intact *nef* sequences [149], whose case had been originally described by Kirchhoff *et al*. [216]. In fact, the presence of defective *nef* variants was shown to distinguish hemophiliacs with progressing or nonprogressing HIV-1 infection [46]. Detection of inefficient enhancement of HIV-1 infectivity and CD4 downregulation by HIV-1 *nef* alleles of Japanese LTNPs has also been demonstrated, raising the question, according to the authors, whether these characteristics of *nef* alleles are the cause or the consequence of the long-term nonprogression after HIV-1 infection [419].

These findings are not surprising given that *nef* functions not only as an amplifier of viral replication and infectivity, but also as a keeper of viral survival -that is pivotal for chronicity- through distinct immune evasion and anti-apoptotic mechanisms {reviewed in [20, 115]}. Interestingly, coevolution of defective and intact *nef* lineages, with the latter possibly supporting the survival of the biologically disadvantaged former as helper viruses, was documented in 10 of the 15 slow progressors whom Wang *et al*. studied [440]; a similar phenomenon, the relationship of which to HIV disease progression is not clear yet, has been described in the brain of patients with AIDS dementia complex [363]. Defective nef genes, nevertheless with deletions not confined to the U3 region as reported before for HIV-2 [458] or SIV [217], have also been described in HIV-2-infected asymptomatic subjects [409].

In contrast, other reports did not corroborate these results and concluded that the nef gene is not a common mediator of the rate of HIV disease progression in natural infection [277]. For instance, most isolated *nef* sequences (91.1%) of 10 long-term survivors contained a full-length and intact open reading frame (ORF), leading the authors to the conclusion that gross sequence abnormality or deletion within *nef* is not likely to be a common explanation for the well-being of long-term survivors of HIV-1 infection [185]. The same cohort of long-term survivors was also studied by Cao *et al*. who overall found orders of magnitude lower viral burden in the plasma and PBMC in these subjects than that typically found in subjects with progressive disease; in addition, no *in vitro* evidence of resistance by host $CD4^+$ lymphocytes to HIV-1 infection, but vigorous, virus-inhibitory $CD8^+$ lymphocyte and neutralizing-antibody responses were observed [50]. In addition, the kinetics of viral replication were consistent with the presence of a substantially attenuated strain of HIV-1 in two subjects. Therefore, a combination of strong virus-specific immune responses with some degree of attenuation of the virus may contribute to delayed HIV disease progression. Although a consensus has not been reached, specific sequence variations

in *nef* resulting in discrete amino acid substitutions (and not deletions or gross abnormalities) have been associated with different stages of HIV-1 infection; the frequency of these *nef* variations were strongly correlated with $CD4^+$ cell count and viral load [215].

ALTERATIONS IN OTHER GENES OR GENOMIC REGIONS

Infrequent mutations within the LTR [116, 462], *gag* [184], and the accessory genes (*vif*, *vpr*, *vpu*, *tat*, and *rev*) [189, 276] have also been described in LTNPs. Recently, a higher frequency of R77Q mutations in viral protein R (Vpr) that impaired the ability of the virus to induce T cell death, has been demonstrated in LTNPs (who were not homozygous for CCR5) than in patients with progressive disease [257]. Additionally, an elongation of the V2 region, which potentially restricts the capacity of the virus to replicate in macrophages and may thus lead to reduced viral load levels, has been observed in individuals with slow progression or non-progression of HIV-1 disease [390, 440]. The long-term survival of another patient was attributed to the known effects of the CCR5 +/Delta32 genotype as well as to changes in envelope sequences that conferred properties of viral attenuation [157]. On the contrary, no abnormalities were detected in *pol* [184], or in the accessory genes vif, vpr and vpu by other investigators [464].

COULD HIV BE EVOLVING TOWARDS ATTENUATION?

In a review published in 2001, Malim and Emerman discussed the issue of viral attenuation with respect to disease progression through a different prism that is worth noting here [261]. The question was therefore raised whether, given enough time -which could be translated to hundreds or thousands of years in evolutionary terms-, it would be possible for HIV-1 to eventually become attenuated, or, in other words, to evolve into a "molecular fossil" of what once constituted a deadly virus for its human hosts. De Groot *et al*. have suggested that a similar adaptive or coevolutionary phenomenon actually arose with SIV and its natural primate hosts; in particular, driven by the evidence they found for an ancient selective sweep in the major histocompatibility complex (MHC) class I gene repertoire of chimpanzees, the authors hypothesized that contemporary SIV-infected animals that remain asymptomatic may in fact represent the survivors of a retroviral pandemic that took place in the distant past [90].

Sooty mangabey monkeys that naturally harbor SIV can tolerate high levels of viral replication without developing an immunodeficiency syndrome [117, 160, 393]; on the contrary, transmission of SIV to humans or to such unnatural hosts as the rhesus macaque (*Macaca mulatta*) leads to a progressive depletion of circulating $CD4^+$ T lymphocytes and to an increased susceptibility to opportunistic infections {reviewed in [59, 406]}. The virus in both cases, which, nevertheless, differ in significant immunologic parameters, exhibits comparable replication dynamics. Apart from the reduction in $CD4^+$ T cell counts, generalized immune activation, manifested by increased numbers of cycling and apoptotic T cells, hyperplasic lymphoid tissues, and exacerbated immune responses, also characterizes the pathogenic SIV infection in macaques in sharp contrast to the asymptomatic SIV infection in mangabeys {reviewed in [59]}. The simian models therefore suggest that the critical determinants of retroviral pathogenicity do not lie so much with viral correlates, but within certain immune parameters of the host.

Akin to the asymptomatic SIV infection of chimpanzees and certain Old World monkey species, the continuous interplay between the diversifying virus and the adapting or coevolving immune system of humans may well convert the currently lethal HIV infection to a benign one eventually. Population-dependent genetic changes in the viral genome have already been noted in response to the immunologic pressure placed on the virus by its human hosts [47, 286].

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LIST OF ABBREVIATIONS

AIDS	=	acquired immune deficiency syndrome
HIV-1	=	human immunodeficiency virus type 1
HIV-2	=	human immunodeficiency virus type 2
SIV	=	simian immunodeficiency virus
CRFs	=	circulating recombinant forms
DR Congo	=	Democratic Republic of Congo (former Zaire)
R/H	=	rapid/high
SI	=	syncytium-inducing
S/L	=	slow/low
NSI	=	non-syncytium-inducing
(M)-tropic	=	"macrophage"-tropic
(T)-tropic	=	"T-cell line"-tropic
PBMCs	=	peripheral blood mononuclear cells
LTR	=	long terminal repeat
NF-κB	=	nuclear factor-kappa of B cells
Tat protein	=	*trans*- activator protein
TAR element	=	*trans*-activation responsive region element
CTD	=	C-terminal domain
TAHs	=	Tat-associated histone acetyltransferases
PCAF	=	p300/CBP-associating factor
USF	=	upstream stimulating factor
TNF-α	=	tumor necrosis factor–alpha
NRE	=	negative regulatory element
HMGB1 protein	=	human high mobility group B 1 protein

DSP1	=	dorsal switch protein 1
DCs	=	dendritic cells
CNS	=	central nervous system
GALT	=	gut-associated lymphoid tissue
SDF-1	=	stromal cell-derived factor-1
SHIV	=	simian-human immunodeficiency virus
IL-4	=	interleukin-4
IL-7	=	interleukin-7
LTNPs	=	long-term non-progressors
Nef gene	=	Negative Factor gene
ORF	=	open reading frame
Vpr	=	viral protein R
MHC	=	major histocompatibility complex

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