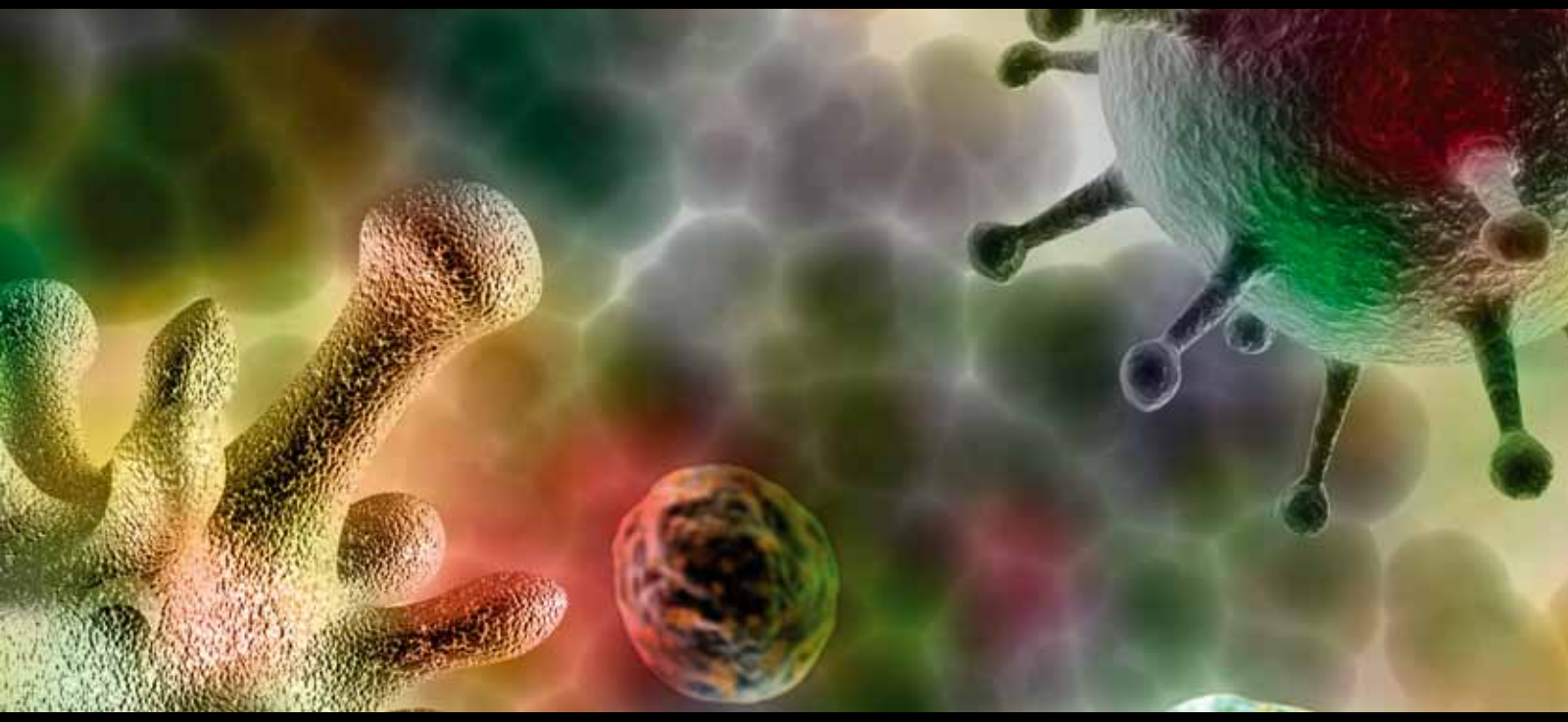
Advances in Virology

# Virus Variability and Its Impact on HIV and Hepatitis Therapy

Guest Editors: Domenico Genovese, Christoph Boesecke, Nicola Coppola, and Stefano Vella



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# Editorial Board

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*Editorial*

# Virus Variability and Its Impact on HIV and Hepatitis Therapy

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Human immunodeficiency virus (HIV) and hepatitis C virus (HCV) RNA genome, as well as hepatitis B virus (HBV) DNA genome, introduce several nucleotide substitution due both to the lack of proof reading activity of viral polymerase and to the high replication activity. As a consequence, viral populations are extremely heterogeneous and have been labeled molecular quasispecies. The virus population structure has numerous implications for the biology and associated pathology. This variability could be utilized by the virus to adapt itself to the different host conditions as immunitary system efficiency and presence or absence of antiviral therapy.

The knowledge of viral population variability molecular markers could be utilized to modify the classic approach to antiviral therapy based on generalized protocols. From such studies, it will become possible to estimate genetic diversity within a virus and its known strains, estimate patterns and rates of mutation for each viral gene or noncoding region, identify causal strains associated with pathological features and with specific antiviral response.

Due to their shared routes of transmission, hepatitis viruses infection is common among persons infected with HIV. Chronic coinfection with hepatitis C virus (HCV) is documented in one-third of all HIV-infected persons in the United States, especially in those with a history of intravenous drug use; it is associated with an increased liver-diseases-related morbidity and mortality compared with HCV or HIV monoinfection due to the prolonged HIV survival with the advances in ART therapy and to a more severe liver disease.

This special issue addressed a variety of aspects of the research against HCV and HIV infection with particular regard to specific aspects of the viral variability in relation to treatment and to the response of the immune system.

From 10 submissions, 7 papers are published in this special issue. Each was reviewed by at least two reviewers and revised according to review comments. The papers cover the following arguments: impact of HIV-1 genetic variation on AIDS; role of antibodies in the development of HIV infection outcome; immunology of HIV and HCV; role of HCV variability in therapy.

HIV-1 encodes a transactivating regulatory protein (Tat), which is essential for efficient transcription of the viral genome. Tat acts by binding to an RNA stem-loop structure, the trans-activating response element (TAR), found at the 5′-ends of nascent HIV-1 transcripts, recruiting a positive transcription elongation complex and increasing the production of full-length viral RNA. Tat protein also associates with RNA polymerase II complexes during early transcription elongation. All of these studies have motivated a number of researchers to use Tat as an important target in combination antiretroviral therapies, but to date none of these have materialized into clinically effective antiviral agents. The paper of L. Li et al. describes the different functions of this intriguing protein observing that the genetic variability within the Tat gene may impact the molecular architecture of functional domains of the protein and as a consequence afflict HIV pathogenesis and disease. Tat as a therapeutic target for anti-HIV drugs has also been discussed. The isolation of mutant sequences of Tat from different sites can also be used to identify tissue-specific functions of Tat that may have great bearing on long-term use of Tat inhibitors. We must also consider that the eradication of latent reservoirs by irreversibly blocking LTR

activation or Tat transactivation activity could be a major step in controlling the HIV-1 pandemic.

The strains of HIV-1 can be classified into four groups: the "major" group M, the "outlier" group O, and two new groups, N and P. Within group M, there are known to be at least nine genetically distinct subtypes (or clades) of HIV-1. These are subtypes A, B, C, D, F, G, H, J, and K. Occasionally, two viruses of different subtypes can meet in the cell of an infected person and mix together their genetic material to create a new hybrid virus. Many of these new strains do not survive for long, but those that infect more than one person are known as "circulating recombinant forms" or CRFs. The classification of HIV strains into subtypes and CRFs is a complex issue and the definitions are subject to change as new discoveries are made. In the paper of R. Sampathkomar et al. the origin and development of quasispecies and CRFs has been deeply described as well as the role of antiviral and immunological response in HIV genetic makeup and the ability of host genetic factors to influence HIV-1 evolution.

Integrase inhibitors are a class of antiretroviral drug designed to block the action of integrase, a viral enzyme that inserts the viral genome into the DNA of the host cell. Since integration is a vital step in retroviral replication, blocking it can halt further spread of the virus. The paper of X. Ni et al. describes the susceptibility of different HIV-1 strains to integrase inhibitors. This paper demonstrates by in-silico and in-vitro studies that the differences in the sequences of integrase gene from different subtypes do not affect the susceptibility to integrase inhibitors.

The V3 loop of the HIV-1gp120 glycoprotein presenting 35-residue-long, frequently glycosylated, highly variable, and disulfide bonded structure plays the central role in the virus biology and forms the principal target for neutralizing antibodies and the major viral determinant for coreceptor binding. HIV-1's subtype C V3 loop consensus sequence exhibits increased resistance to anti-V3 antibody-mediated neutralization as compared to the subtype B consensus sequence. D. Almond et al. in their paper describe the differences in the three-dimensional V3 loop structures between subtypes B and C. Differences in the flexibility in specific $\beta$-strand structure could be responsible for the diverse binding activity of anti-V3 antibody. The different susceptibility to human antibody-mediated neutralization could also result in different extents of global spread between different subtypes.

Effective immunity to HIV is poorly understood. Cytotoxic T lymphocytes and neutralizing antibodies readily select for immune escape HIV variants. It is now also clear that NK cells can also select for immune escape through both direct Killer-immunoglobulin-like-receptor- (KIR-) mediated killing as well as through facilitating antibody-dependent cellular cytotoxicity (ADCC). Viral fitness is a complex parameter illustrating the overall contribution of all mutation-related advantages and losses. Even though the evasion of immune responses presented by escape mutations presents a definite fitness benefit to the virus, the HIV-1 proteome is not infinitely malleable hence the same mutations can result in fitness costs. Some immune escape variants have reduced replicative capacity of the virus (reduced "fitness") that slows the progression of disease. There is an urgent need to identify effective immunity to HIV and to use this information to design efficacious vaccines and immunotherapies, and the paper of Isitman et al., contributes efficiently to this knowledge, reviewing the introduction and effects of immune escape driven by CTL, neutralizing antibodies and NK cells.

Viral immunology is a rapidly evolving field. Major strides have been made in our understanding of innate and adaptive immune responses to viruses, largely based on highly reductionistic animal infection models, but more recently in humans, with validation that fundamental immunological concepts do in fact translate into clinical science well. From these studies has been emerging an appreciation of the enormous complexity of the immune response to viral infections as well as the diverse array of strategies developed by viruses to deal with immune detection. HIV and HCV markedly differ in their virological properties and in their pathogenesis but share many common features in their immune escape and survival strategy. Both viruses have developed sophisticated ways to subvert and antagonize host innate and adaptive immune responses. In their interesting paper M. G. Quaranta et al. describe current knowledge on innate and adaptive immune recognition and activation during HIV and HCV monoinfections and evasion strategies as well as the genetic associations between components of the immune system, the course of infection, and the outcome of the therapies.

HCV infection therapy is based on a combination of pegylated interferon and ribavirin. This therapy is, unfortunately, very little effective (only a percentage of around 50% of the subjects respond to drugs and, moreover, a part of these is subsequently reinfected with the virus) and is associated with major side effects. For this reason, taking into account the experience with the anti-HIV therapy, new HCV direct antiviral drugs (STAT-C, including protease inhibitors and polymerase inhibitors) are already at an advanced stage of experimentation. Telaprevir and Boceprevir (two protease inhibitors) have been introduced in the USA guidelines for HCV genotype 1 therapy. The new therapeutic options are able to significantly increase the sustained virological response (SVR) compared to the current standard therapy, and potentially reducing the overall duration of the treatment. However, the occurrence of side effects requiring discontinuation of treatment makes it critical to optimize the therapy. In their paper, C. Strahotin and M. Babich summarize the current knowledge on the pathogenesis of HCV therapy with particular reference to therapy and introduce the problem of resistance to the new protease inhibitors. This is a highly relevant and interesting topic because new upcoming compounds are likely to be much more effective than the present. Probably, a highly effective interferon-free regimen for the treatment of HCV infection could be soon available, and the problem of resistant mutants could be a focal point of anti-HCV therapy research.

These papers represent an exciting, insightful observation into the state of the art, as well as emerging future topics in a moment when strategies for the fight against HIV and HCV are at a critical point. For HIV we start to talk about

HIV eradication strategies, while the development of new HCV therapies, for the first time, gives us the opportunity to finally defeat this disease and its important and serious sequelae. We hope that this special issue would attract a major attention of the peers. We would like to express our appreciation to all the authors and reviewers.

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*Review Article*

# Impact of Tat Genetic Variation on HIV-1 Disease

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The human immunodeficiency virus type 1 (HIV-1) promoter or long-terminal repeat (LTR) regulates viral gene expression by interacting with multiple viral and host factors. The viral transactivator protein Tat plays an important role in transcriptional activation of HIV-1 gene expression. Functional domains of Tat and its interaction with transactivation response element RNA and cellular transcription factors have been examined. Genetic variation within *tat* of different HIV-1 subtypes has been shown to affect the interaction of the viral transactivator with cellular and/or viral proteins, influencing the overall level of transcriptional activation as well as its action as a neurotoxic protein. Consequently, the genetic variability within *tat* may impact the molecular architecture of functional domains of the Tat protein that may impact HIV pathogenesis and disease. Tat as a therapeutic target for anti-HIV drugs has also been discussed.

## 1. Introduction

The human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). The HIV-1 genome is about 9.8 kb in length, including two viral long-terminal repeats (LTRs) located at both ends when integrated into the host genome. The genome also includes genes that encode for the structural proteins ([Gag], [Pol], and [Env]), regulatory proteins (Tat and [Rev]), and accessory proteins ([Vpu], [Vpr], [Vif], and [Nef]). The HIV-1 transactivator of transcription (Tat) protein is an early regulatory protein containing from 86 to 106 amino acids in length with a molecular weight of approximately 14 to 16 kDa. Tat is a multifunctional protein that has been proposed to contribute to several pathological consequences of HIV-1 infection. Tat not only plays an important role in viral transcription and replication, it is also capable of inducing the expression of a variety of cellular genes as well as acting as a neurotoxic protein. In this review, the functions of Tat and molecular diversity in Tat are addressed. Moreover, the interaction of Tat with the viral LTR and cellular factors are documented and discussed. Because of its pivotal role in viral replication and disease pathogenesis, Tat and the cellular pathways targeted by Tat could be potential targets for new anti-HIV drugs. Therapeutic strategies that have focused on this topic are also reviewed.

## 2. Functional Domains of the Transactivator Protein Tat

Tat is a 14 to 16 kDa nuclear protein. It is a multifunctional protein, which is essential for the productive and processive transcription driven from the HIV-1 LTR promoter, and is required for overall productive viral replication [1, 2]. It is a 101-amino acid protein encoded by two exons: the first exon encodes amino acids 1 to 72; the second encodes residues from 73 to 101 (Figure 1) [3]. Most clinical HIV-1 isolates of Tat include 101 amino acids, whereas a few isolates contain from 86 to 106 amino acids, with the second exon coding from 14 to 34 residues at the C terminus of the protein [4]. The HIV-1 IIIB Tat used in many *in vitro* experiments contains 86 amino acids, corresponding to HIV-1

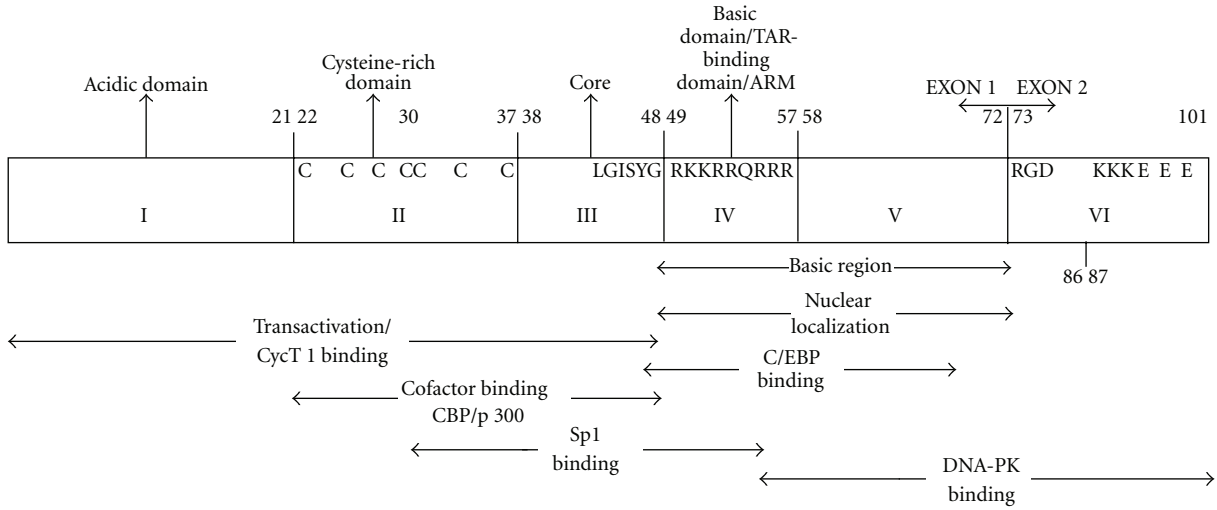


Figure 1: Schematic representation of HIV-1 Tat with locations of the six main domains indicated. Within each domain, important amino acid residues are designated. In addition, known functions of the domains or interactions with the protein involved in transcription are also highlighted.

(strain BRU) or a closely related sequence from the HXB2 HIV-1 infectious molecular clone [5, 6]. This 86-amino acid configuration of Tat is the most frequently used form for laboratory investigations; however, it must be noted that it represents a truncated protein when compared to Tat from many clinical isolates. Several studies have established that HIV-1 Tat maintains the 101-amino acid composition as previously reviewed [7]. The more truncated 86-amino acid version of Tat appears to be functional [4], but functions like modulation of host cell cytoskeleton modifications [8] and perhaps optimal replication in cells of the monocyte-macrophage lineage have been attributed to the second exon. Also, the fact that most clinical isolates preserve the full 101-amino acid form is indicative of the functional relevance of the second exon in an *in vivo* setting.

Tat has been divided into six different functional domains (Figure 1) [3, 4, 9]. The N-terminal domain (residues 1–21, also known as the acidic domain) is a proline-rich region containing a conserved tryptophan residue and a number of acidic amino acids. This region is able to form an $\alpha$-helix and is tolerant of numerous single-residue changes without severe compromise in protein function. The second domain (residues 21–37, also referred to as the cysteine-rich domain) contains a highly conserved cysteine-rich tract including seven cysteines at positions 22, 25, 27, 30, 31, 34, and 37, four of which are responsible for the formation of disulfide bridges; changes in any one of six of the seven cysteines significantly affect Tat function [10]. The third domain (residues 38–48) has a hydrophobic core sequence: $_{43}$LGISYG$_{48}$. The first three domains (amino acid 1–48) comprise the minimal region for Tat transactivation capability. Within this region, genetic variation resulting in changes in amino acids from 1 to 21 is typically tolerated; however, changes in residues from 22 to 40 are deleterious with respect to transactivation. The fourth domain (residues 49–57, the basic domain) is a positively charged region composed of a well-conserved $_{49}$RKKRRQRRR$_{57}$ motif, also known as the arginine-rich motif, or transactivation response element-(TAR) binding domain. This region is necessary for Tat nuclear localization, binding to the HIV-1 leader RNA TAR [11–13], and uptaken by other cells [14, 15]. Studies have also demonstrated that Tat utilizes the basic domain residues from 48 to 60 for the functional internalization into cells [16, 17]. The fifth domain (residues 58–72) is a glutamine-rich region shown to exhibit the greatest degree of genetic variability. The fourth and fifth domains together (residues 49–72) are referred to as the basic region. The sixth domain (amino acid 73–101) encoded by the second exon has been less well characterized but may contribute to viral infectivity and binding to cell-surface integrins [18–20]. Two short motifs have been identified in the C terminus of Tat: the RGD (arg-gly-asp) motif, which is a ligand for several integrins, and the highly conserved ESKKKVE motif, which may be related to optimal HIV-1 replication *in vivo* [18, 21]. Although the transactivation domain has been localized to Tat exon I, Tat exon II also plays a role in kappa-light-chain-enhancer of activated B cell-(NF-$\kappa$B) dependent control of HIV-1 transcription in T cells [22]. The glutamic acid residues 92, 94, and 96 or lysine residues 88, 89, and 90 within the Tat exon II exhibit a critical role in activating NF-$\kappa$B, transactivating the HIV-1 LTR, and enhancing HIV-1 replication in T cells.

## 3. Basal and Stimulated Transactivation Driven by the HIV-1 LTR

The HIV-1 LTRs are generated during the process of reverse transcription and located on each end of the proviral DNA when the provirus is integrated into the host genome. The LTR is approximately 640 base pairs in length and divided into the unique 5′ (U5) and 3′ (U3) regions as well as the repeat region (Figure 2). LTR sequences include four

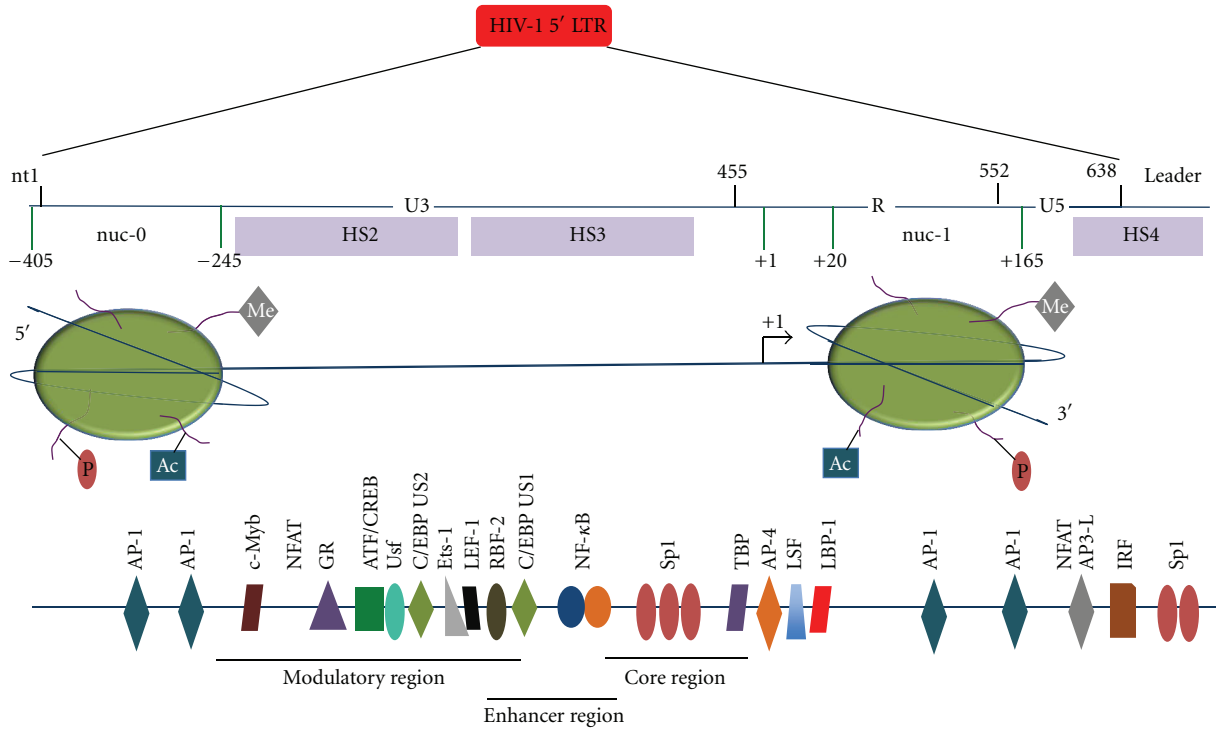


FIGURE 2: Molecular architecture of the HIV-1 long-terminal repeat. The viral promoter, the long-terminal repeat (LTR), can be divided into the U3, R, and U5 regions. Upon integration, the LTR presents stretches of DNase I hypersensitivity sites (shown as HS2, HS3, and HS4) as a result of the well-defined, conversed positioning of the two nucleosomes; nuc-0 and nuc-1. This architecture results in exposure of stretches of DNA extremely rich in transcription factor-binding sites that include different regulatory proteins in the process of HIV-1 transcription. These factors respond to various extracellular and intracellular stimuli, resulting in upregulation/downregulation of specific downstream transcription factors that act via binding to their respective binding sites in the LTR. Also, positioning of nuc-1 is crucial because it is present immediately downstream of the start site (+1); this nucleosome needs to be remodeled for active processive transcription to ensue from the LTR. Moreover, modifications like acetylation, phosphorylation, and methylation of histone tails regulate LTR-directed transcription. The HIV-1 Tat protein regulates the chromatin environment via interactions with several components including methyltransferases, acetyltransferases, and a number of transcription factors in addition to binding to the TAR element in nascent HIV-1 RNA.

functional regulatory regions with respect to the control of HIV-1 transcription: TAR element, core promoter, enhancer region, and modulatory region [47]. A multitude of HIV-1 promoter regulatory elements are located within the U3 region of the 5′ LTR and drive the production of HIV-1 mRNA that codes for proteins involved in regulating viral replication as well as the assembly and release of infectious progeny virus. The core region of the LTR is composed of the TATAA box, which is located 29–24 nucleotides upstream of the transcriptional start site, and specificity protein (Sp) binding sites, which are three tandem GC-rich binding sites (−45 to −77) interacting with transcription factors Sp1 through Sp4. The TATAA box binds TATAA-binding protein in association with a number of other proteins that comprise the RNA polymerase II (pol II) transcription complex for transcription initiation and elongation [48–50]. The enhancer element is primarily composed of two copies of 10-base pair binding sites for NF-κBs and related proteins [51]. The modulatory region, which is in the 5′ end of the U3 region, contains binding sites for many factors, including CCAAT/enhancer-binding protein (C/EBP) factors [52], activating transcription factor/cyclic AMP response element-binding protein (ATF/CREB) [53], nuclear factor of activated T cells (NFAT) [54], and a number of other proteins, depending on cell phenotype, differentiation status, and state of activation (Figure 2) [55–58].

The integrated proviral DNA interfaces with the normal molecular architecture of the host chromatin, which is assembled into nucleosomes. Each nucleosome contains a protein core made of eight histone molecules (H2A, H2B, H3, and H4) and 146 nucleotide-long double-stranded DNA wrapped around it [59]. Independent of the integration site, two nucleosomes (designated nuc-0 and nuc-1) are precisely organized on the HIV-1 viral promoter DNA (Figure 2) [60, 61]. In a transcriptionally quiescent state, nuc-0 is positioned at nucleotide from −405 to −245 relative to the transcriptional start site, and nuc-1 is positioned at nucleotide from +20 to +165 relative to the transcriptional start site (Figure 2). These wrapped regions define two open nucleosome-free regions in the viral DNA, extending from −244 to +19 and +166 to +256 relative to the transcription start site (Figure 2). These open regions include the HIV-1 LTR modulatory, enhancer/core region, transcription factor-binding sites for AP3-L, Sp1 [60], and upstream regulatory

factor (USF) [62], and a region overlapping the primer-binding site immediately downstream of the 5′ LTR [63]. It has been proposed and shown that displacement of nuc-1 is a prerequisite for HIV-1 transcription as observed in response to T cell activation stimuli [60]. Conformation of the nucleosomes is modulated in two ways: (1) posttranslational modifications of N-terminal tails of histones, namely acetylation, phosphorylation, and methylation through factors like histone acetyl transferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and kinases and (2) ATP-dependent chromatin remodeling complexes such as P300/CBP-associated factor (PCAF) and the SWI/SNF family [64].

Acetylation of specific lysine residues within the N-termini of selective core histones by HATs neutralizes positive charges on these amino acids, thereby weakening histone-DNA interactions and making the DNA more "open" or accessible to the transcriptional machinery [65, 66]. In contrast, recruitment of HDACs results in transcriptional repression. For example, recruitment of histone deacetylase-1 (HDAC1) by NF-$\kappa$B p50 was found to constitutively maintain nuc-0 and nuc-1 in a deacetylated state, thus keeping the chromatin in a condensed state, impairing RNA pol II recruitment and transcriptional initiation (Figure 3) [67]. The importance of HDAC1 has been shown by studies that have proposed a dynamic model for LTR regulation in T cells by two cellular transcriptional regulators YY1 and LSF [68]. They form a trimeric complex with HDAC1 at a region-spanning nucleotides from −10 to +27 of the HIV-1 LTR. Whereas LSF-1 binds to DNA, YY1 serves as an intermolecular bridge to anchor HDAC1 to this region [68].

Histone methylation can also have a number of effects on transcription. For example, methylation of lysine-9 (K9) on histone-3 (H3) by HMTs has been shown to be linked with transcriptional silencing, just as methylation of K4 that is associated with activation [69]. Accordingly, maintenance of the heterochromatic state at the integrated HIV-1 promoter has been shown to be mediated by the methyltransferase Suv39H1, which specifically mediates H3-K9 trimethylation, and the heterochromatin protein-1$\gamma$, which has been shown to recruit HMT [70–72]. Apart from methylation of histone, hypermethylation of CpG sites found within the HIV-1 LTR has also been shown to repress basal and activation-induced promoter activity, thereby inducing a state of latency [73].

Activation of HIV-1 transcription is mediated by host cell transcription factors and viral proteins via interactions with the cis-regulating elements in the LTR, along with protein-protein interactions in the regulatory pathway(s) [3]. LTR-basal transcription is driven primarily through cellular transcription factors such as Sp1 and NF-$\kappa$B, which help recruit the RNA pol II complex to the transcriptional start site. This process can be enhanced during cell activation stimulated by a number of cytokines [74]. The availability of host cell transcription factors and viral proteins regulates HIV-1 gene expression in the context of specific cell types, cell-cycle regulation, cellular differentiation, and cellular activation [75]. Sequestration of two critical transcription factors, NF-$\kappa$B and NFAT, in the cytoplasm of resting $CD4^+$ T cells contributes to the repressive state of the HIV-1 LTR in these cells [76]. The paucity of these factors within the nucleus is reversed in response to activation signals. T cell receptor (TCR) cross-linking, cytokine stimulation (e.g., TNF-$\alpha$, IL-7), or mitogens (e.g., protein kinase C activators like the phorbol ester PMA and prostratin) lead to nuclear translocation of these molecules and subsequent binding to overlapping cognate sites in the HIV-1 LTR, thereby upregulating basal as well as Tat-mediated promoter activity (Figure 3). TCR ligation also induces transcription and heterodimerization of the c-jun/c-fos complex AP-1, which is absent in resting T cells [77] and synergizes with NFAT and NF-$\kappa$B to promote HIV-1 gene expression. Basal and stimulated transcription produces predominantly short RNA as a result of the hypophosphorylated state of RNA pol II. However, an increasing number of longer transcripts encode for a pool of viral regulatory proteins, especially Tat, that eventually feedback to enhance the next stage of viral transcription, designated Tat-mediated transcription.

## 4. Tat-Mediated Transactivation of the HIV-1 LTR through Cyclin-Dependent Kinase 9 and Cyclin T1

HIV-1 transcription involves an early, Tat-independent phase and a late, Tat-dependent phase, and transactivation of the viral genome is a critical step in the viral replication cycle [3, 78]. In the presence of Tat, LTR-mediated transcriptional activity can be enhanced tens or hundreds of fold [79–82], whereas viral replication falls to nearly undetectable levels in the absence of Tat, and short transcripts (30–50 nucleotides) predominate [83–85]. Tat is a unique transcription factor in that it binds to the "UCU" bulge of the TAR, a cis-acting RNA enhancer element within the 5′ end of all viral transcripts. The TAR is located immediately downstream of the transcriptional start site in the HIV-1 LTR, encompassing nucleotides from +1 to +59 [86, 87], and is required for the function of the viral transactivator protein Tat. The Tat-TAR interaction acts to tether Tat and allow its interaction with the basal transcriptional machinery, thus increasing viral transcription and elongation [88, 89]. In a mature transcript, TAR adopts a hairpin structure including a six-nucleotide loop, a trinucleotide pyrimidine bulge, and an extensive duplex structure [86]. U23, in the bulge, is critical for Tat binding [2, 13, 90]; the other two neighboring residues C24 and U25 can be replaced by other nucleotides without affecting Tat binding. Another two regions above the bulge (G26-C39 and A27-U38) and one region below (A22-U40) also contribute to Tat binding [2, 13, 90]. Although the loop structure does not appear to be required for Tat binding, the residues in the loop have been shown to be required for Tat transactivation activity [90].

Specifically, HIV-1 Tat has been shown to associate with the P-TEFb, which is composed of cyclin T1 and cyclin-dependent kinase 9 (CDK9) [24, 91, 92]. This association occurs in a sequential manner. Once bound, CDK9 has been shown to phosphorylate the carboxy-terminal domain (CTD) of RNA pol II and promote transcription elongation [24, 91, 93]. Therefore, the lack of HIV-1 gene expression in

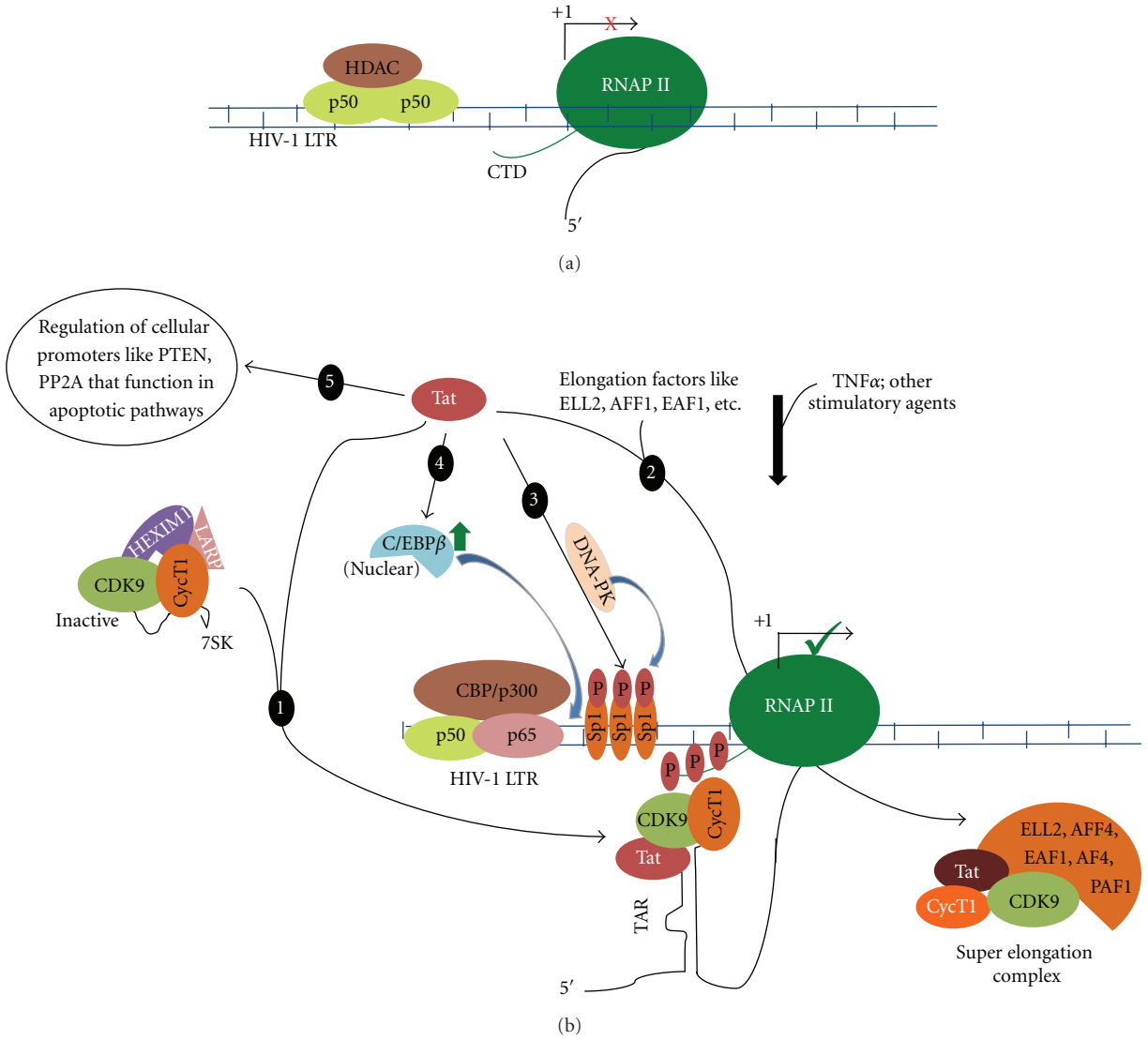


Figure 3: Functions of Tat. Tat plays a crucial role in synthesis of full-length HIV-1 mRNA transcripts. In the absence of Tat, the viral promoter remains latent with NF-$\kappa$B (p50 homodimers) and histone deacetylase complexes (HDACs) maintaining a repressive chromatin environment by deacetylating the histones (a). Thus, RNA polymerase-(RNAPII) driven transcription is not progressive- and full-length HIV-1 transcripts are not synthesized. (b) Under a cellular stimulation environment, the latent state is overcome by recruitment of CBP/p300 complex to the viral promoter, the LTR. The Tat protein translocates to the nucleus and triggers a release of P-TEFb (CDK9/CycT1) from an inactive complex with HEXIM1, LARP, and 7SK RNA (1). The active P-TEFb in complex with Tat then interacts with the stem-loop structure (TAR) in the nascent HIV-1 mRNA's 5′ end. This event triggers the phosphorylation of the C-terminal domain (CTD) of RNAPII by CDK9, resulting in productive transcription from the LTR (b). Tat also participates in formation of a "super elongation complex" with factors including ELL2, AFF1, EAF1, and others, which also aids in synthesis of full-length HIV-1 transcripts from the relieved template (2). Tat also recruits another kinase DNA-PK in a ternary complex with Sp1 transcription factor at the LTR (3). This results in phosphorylation of Sp1 and activation of Sp1-mediated LTR-directed transcription. Tat also interacts with C/EBP$\beta$ and triggers an increase in the nuclear levels of C/EBP$\beta$, again indirectly regulating the transcription of the HIV-1 genome (4). Moreover, Tat also regulates transcription of other cellular promoters of phosphatases like PTEN and PP2A. These play crucial regulatory roles in apoptosis of HIV-1-infected CD4$^+$ T cells (5).

latently infected cells might not only arise due to the absence of Tat but also as a result of extremely low levels of CDK9 and cyclin T1, as observed in resting CD4$^+$ T cells [94]. In addition, mutations in the *tat* gene, the Tat-responsive element itself, might also contribute to the latent phenotype, as is evident from experiments performed in the U1 [95] and ACH-2 [96] cell lines, respectively. The cyclin T1 subunit of P-TEFb has been shown to interact with the activation domain of Tat and to bind to the central loop (+30 to +35) of TAR [92]. Once cyclin T1 binds to Tat, the CycT1-Tat complex has been shown to bind both the bulge and the loop regions of TAR with a higher affinity than Tat alone and to subsequently form the CycT1-Tat-TAR ternary complex [92, 97, 98].

With respect to sequence variation within the TAR region, different subtypes have been shown to have distinct TAR motifs, with most of the sequence variation occurring in the stem region [99]. Subtypes A/E and A contain a nucleotide deletion (T25) in the TAR bulge region (T23C24T25) leading to the formation of a two-nucleotide bulge [100–104]. This sequence alteration does not affect subtype B Tat binding to the subtype E TAR region. However, the studies concerning matched subtype A, A/E, or E TAR and Tat have not been reported [105]. A C-to-T change at position 24 in the bulge has been identified in subtypes C, D, F, most of G bulges, and 50% of subtype A bulges examined [104]. Additionally, a T-to-C change at position 2 of the loop structure has been consistently observed in subtype C [106]. Additional functional studies need to be performed to determine the impact of structural differences that may be important in the Tat-TAR-cyclin T1 interactions. Recent investigations have expanded the understanding of Tat-TAR-cycT1 interactions and have implicated the role of Tat acetylation in modulation of transcriptional elongation [107]. Moreover, mutation analysis of the N terminal of CycT1 [108] that impairs the transcriptional activity via compromising Tat-TAR-cycT1 interactions has laid the foundation for utilizing this aspect of HIV-1 transcriptional activation in novel therapeutic intervention strategies. These studies also add new aspects that extend our understanding of HIV-1-latency; however, adaptability of HIV-1 has hampered the use of these studies to any productive end. Another major issue is that most studies tend to examine these functional aspects of HIV-1 transcription in a subtype-specific manner and studies that encompass these variables are limited. Analyzing large databases of sequence information is still a tedious process, and development of more organized tools will encourage researchers to make better progress in this area.

## 5. Interaction of Tat with Other Proteins Involved in Transcription

The hypophosphorylated form of RNA pol II leads to the production of short RNA molecules (30–50 nucleotides in length including the entire length of the TAR sequence) as a result of premature termination of transcription. However, phosphorylation of the CTD of RNA pol II has been shown to prevent premature termination and promote the efficient elongation and production of full-length HIV-1 RNA transcripts [109]. Phosphorylation of the CTD of RNA pol II has also been shown to be important for the clearance of mediators from RNA pol II [110] (Figure 3). Transcription factor II H (TFIIH) is a part of the preinitiation complex involved in transcription, and a number of studies have shown that there are combinatorial networks of transcription factors and cofactors, such as P-TEFb, utilized by Tat to activate and repress gene expression [107, 111]. Tat and P-TEFb are recruited to viral preinitiation complexes prior to RNA transcription and are subsequently transferred to nascent RNA after initiation. In addition to regulating HIV-1 gene expression, Tat is known to be involved in dysregulating cellular function and altering cellular gene expression profiles; however, the mechanism by which Tat affects infected cells continues to be explored.

Tat also functions as a coactivator to recruit histone acetyltransferases, including CBP/p300 and PCAF to the LTR [112]. Tat-recruited HATs presumably acetylate histones in LTR-proximal nucleosomes, remodeling nuc-1, and potentiating transcription [60, 113]. In addition, Tat itself has been shown to be a substrate for the HAT enzyme activity associated with CBP/p300 and PCAF [114–118]. The HAT activity of CBP/p300 can also influence the activity of the NF-$\kappa$B p50 subunit. The increased acetylation of p50 leads to an increase in p50 DNA binding and the concomitant transcriptional activation of the HIV-1 promoter [119].

The SWI/SNF complex is another family of proteins that interacts with Tat and plays a role in the regulation of the HIV-1 promoter. The SWI/SNF proteins are integral components of the RNA pol II holoenzyme [120] and have the ability to disrupt DNA-histone contacts and allow access to transcriptional activators [120, 121]. In the context of HIV-1 transcription, SWI/SNF proteins are required for the transactivation ability of Tat and generation of mature full-length transcripts. Physical interaction between Tat and the remodeling subunits INI1 and Brm has also been observed [122, 123]. T cell activation by mitogenic stimuli induces recruitment of SWI/SNF complex subunits Jun-3, BRG-1, and ATF-3 [124]. In particular, BRG-1 has been shown to be recruited to the Ap1-III site located at the 3′ boundary of nuc-1 [124]. T cell activation also enhances the endogenous pools of inositol phosphate, increasing the activity of SWI/SNF by a yet-unexplained mechanism [125–127].

Tat has also been shown to facilitate enhanced transcriptional initiation through protein-protein interactions with Sp1 [128] (Figure 3). Tat residues 30–55 contact Sp1 and impact DNA-PK-mediated phosphorylation of Sp1, which increases gene expression driven by the HIV-1 LTR (Figure 3) [129]. Amino acids 56–101 of Tat contain the DNA-PK-binding domain. TFIIH has been shown to be able to bind the transactivation domain of Tat and phosphorylate the CTD of RNA pol II although a number of investigative groups have shown that different subunits of TFIIH may mediate Tat binding [130]. Interestingly, the ability of TFIIH to phosphorylate RNA pol II has been shown to be significantly increased after it was bound to Tat; in turn, the transactivation ability of Tat was enhanced in the presence of TFIIH [84]. Tat has been shown to be specifically associated with Tat-associated kinase, which corresponds to the *Drosophila* P-TEFb, composed of cyclin T1 and CDK9. Cyclin T1 interacts directly with the activation domain of Tat and has been shown to mediate high affinity and specific binding of Tat to TAR. After Tat binding to cyclin T1, CDK9 is recruited, and then CDK9 phosphorylates CTD of RNA pol II and promotes transcription elongation. Tat is able to effectively antagonize HIV-1 latency and promote active transcription by liberating P-TEFb from an inactive 7SK RNP complex. Moreover, Tat is proposed to engage in formation of a "super elongation complex" with elongation factors like ELL2, ENL, AFF4, PAF1, and others as previously reviewed [131] (Figure 3). Meanwhile, efficient transcription elongation of the HIV-1 genome in response to Tat has been

shown to lead to more Tat synthesis and generate a Tat-dependent positive feedback loop. However, mouse cyclin T1 has a cysteine-to-tyrosine substitution at position 261 that may lead to weak binding of mouse cyclin T1 to Tat, resulting in Tat losing the ability to interact with TAR, which results in low transactivation levels. However, changing the tyrosine residue back to cysteine at position 261 renders mouse cyclin T1 fully functional in Tat transactivation, demonstrating the importance of this residue in the transactivation process [132].

It has been reported that Tat is also able to interact physically with C/EBP$\beta$ *in vitro* and *in vivo* [133]. Tat amino acid residues from 47 to 67 are critical for interaction with C/EBP$\beta$, and specifically Tat increases the distribution of nuclear levels of C/EBP$\beta$. Moreover, Tat can activate C/EBP$\beta$ in human U-373MG astroglial cells in a dose-dependent manner [134]. Recently, coexpression of Tat and C/EBP$\beta$ has been shown to enhance C/EBP$\beta$ binding to the HIV-1 LTR [135]. The N terminus of HIV-1 Tat (residues 1–26) has also been shown to bind to the transactivation domain (amino acids 1–96) within NFAT1 [136]. HIV-1 Tat enhances NFAT1-driven transcription in Jurkat T cells through a direct protein-protein interaction between the two proteins.

Tat is a robust transactivating protein that induces a number of effects by modulating the expression of many cellular and viral genes. Tat was recently shown to be associated with the promoters of PTEN and PP2A subunits, and these interactions result in activation of apoptotic pathways in HIV-1-infected $CD4^+$ T cells [137]. These studies reinforce the fact that Tat, besides regulating the HIV-1 promoter, also affects cellular promoters. In addition to all of these interactions, Tat also interacts with a wide array of proteins, which have been summarized in Table 1. As shown here, there are a number of proteins with which Tat has been shown to interact. However, very little is known concerning a majority of these interactions and how they may or may not contribute to HIV-1 pathogenesis. This may be one reason why Tat inhibitors, discussed below, have universally failed to date to be effective in the therapeutic arena. In fact, given the large numbers of proteins already identified, future studies will undoubtedly need to take into account the interactome that Tat has with respect to the various proteins involved to truly begin to design inhibitors to attack these interactions. However, modeling this concept will be a future area that will need addressing.

## 6. Molecular Diversity in Tat

The high level of HIV sequence diversity generated during the course of HIV disease is, for the most part, due to the error-prone nature and low fidelity of reverse transcriptase, poor proofreading by the polymerase, and selective pressures exerted by the host immune response, combination antiretroviral chemotherapy, and perhaps other physiological pressures [3, 102, 138]. The HIV-1 genotypic variants and resultant phenotypes occur as important variables of viral replication during the course of the disease [139]. It has been reported that Tat can tolerate 38% sequence variation without any change in its transactivation potential [140].

Both blood-derived and brain-derived HIV-1 viruses show immense molecular heterogeneity between patients and HIV-1 subtypes [53]. The LTR and several HIV-1 genes including [tat], [env] (gp120 and gp41), [nef], and [vpr] have been linked to the pathogenesis of HIV-related neurologic disease [141]. The molecular diversity of HIV-1 Tat protein isolated from brains of patients infected with different HIV-1 subtypes has been examined. Recent studies examining Tat proteins representative of HIV-1 subtype B, C, and BF recombinants have demonstrated important structural and functional differences [142, 143]. BF recombinant HIV-1 isolates from Argentina appear to have a replicative advantage over subtype B isolates, possibly due to the differential ability of Tat to interact with the LTR. Subtype C Tat has been shown to be more highly ordered than subtype B Tat. In addition, subtype C Tat protein has been demonstrated to be consistently inferior to subtype B Tat in biological assays with respect to its ability to promote viral proliferation, induce TNF-$\alpha$ and IL-6 expression, and upregulate chemokine coreceptor expression [142]. However, studies have also shown that HIV-1 subtype C Tat exhibits greater transcriptional activity in the Jurkat $CD4^+$ T cell line compared with subtypes B and E and that this higher level of transactivation is not LTR sequence dependent but rather because of variations in the C-Tat sequence at amino acid residues 57 (Arg in B and E, and Ser in C) and 63 (Glu in B, E, and C), which are within and close to the basic domain, respectively [144]. In addition, in HIV-1 subtype C Tat, a serine residue replaces a cysteine at position 31. This variation affects the biological function of Tat, resulting in a deficient chemoattractant activity, low ability to bind to chemokine receptor 2, and reduced ability to stimulate TNF-$\alpha$ production [142, 145, 146] without affecting Tat transactivation. More recently, signature pattern analysis identified five amino acid positions in Tat (21A, 24N, 29K, 40K, and 60Q) that contained signature residues unique for Indian HIV-1C [147]. Interestingly in the eight patients analyzed to date in the DrexelMed HIV/AIDS Genetic Analysis Cohort [148], which contains mostly subtype B-infected patients, all of the Tat sequences analyzed contain these important cysteines (data not shown). Some length variation in exon 2 and the absence of a critical cysteine in the cysteine-rich domain have also been found in subtype C Tat [106]. When one compares the structural and functional differences between subtypes B and C Tat proteins [142], subtype C Tat may have a relatively higher ordered structure and be less flexible than subtype B Tat. Analysis of subtype D Tat sequences revealed an in-frame stop codon in exon 2, which results in removal of the 13–16 amino acids from the C terminus of Tat [103, 149, 150]. Thus, emerging data encompassing the structure and function of the Tat protein across different subtypes have enabled us to better understand Tat-mediated effects. However, additional studies need to be performed to truly delineate the complexity of Tat. To accomplish this more sequencing data for all subtypes will have to be obtained to allow an even deeper understanding of how and why genetic variation evolves and what are the driving forces in this evolution especially on genes like *tat* that are not directly affected by antiretroviral therapy

Table 1: List of all known Tat protein interactions and the nature of the functional activity compiled from PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and HIV-1, Human Protein Interaction Database (http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/).

| Human protein | Type of activity with Tat |
|---|---|
| 2′-5′-Oligoadenylate synthetase 2 isoform 1 | Interacts |
| 2′-5′-Oligoadenylate synthetase 3 | Interacts |
| 2′,3′-Cyclic nucleotide 3′ phosphodiesterase | Activates |
| 2′,5′-Oligoadenylate synthetase 1 isoform 2 | Interacts |
| 8-Oxoguanine DNA glycosylase isoform 1a | Upregulates |
| Actin, gamma 1 propeptide | Induces rearrangement of |
| Activated RNA polymerase II transcription cofactor 4 | Binds |
| Adaptor-related protein complex 2, alpha 1, 2 beta-1, mu-1, and sigma-1 | Interacts |
| Adenosine A2a receptor | Inhibited by |
| Adenylate cyclase 2–9 | Inhibits |
| AFF4 | Involved in transcription |
| Albumin precursor | Induces release |
| Alpha 1 actin precursor | Induces rearrangement |
| Alpha 1 type-I collagen preproprotein | Inhibits |
| Alpha 2 actin | Induces rearrangement |
| Alpha 2 type-I collagen | Inhibits |
| Alpha isoform of regulatory subunit A, B55, protein phosphatase 2 | Modulated by |
| Alpha-2-macroglobulin precursor | Inhibits |
| AMP-activated protein kinase gamma 2 subunit isoform a | Activates |
| AMP-activated protein kinase, noncatalytic gamma-1 subunit isoform 1 | Activates |
| Amyloid beta A4 protein precursor, isoform a | Inhibits |
| Annexin A2 isoform 2 | Downregulates |
| Apolipoprotein E precursor | Inhibited by |
| ATP-binding cassette subfamily B, C member 1 | Upregulates |
| ATP-dependent DNA helicase II | Interacts |
| ATP-dependent DNA helicase II, 70 kDa subunit | Interacts |
| Autoantigen La | Interacts |
| B-cell CLL/lymphoma 11B isoform 1 | Binds |
| B-cell lymphoma 6 protein | Upregulates |
| B-cell lymphoma protein 2 alpha isoform | Interacts |
| Baculoviral IAP repeat-containing protein 3 | Upregulates |
| BCL2-antagonist of cell death protein | Induces phosphorylation |
| BCL2-associated athanogene isoform 1L | Upregulates |
| BCL2-associated X protein isoform beta | Interacts |
| BCL2-like 1 isoform 1 | Upregulates |
| BCL2-like 11 isoform 6 | Interacts |
| Beta actin | Induces rearrangement |
| Beta isoform of regulatory subunit A, B55 and B56, protein phosphatase 2 isoform a | Modulated by |
| Beta tubulin 1, class VI | Binds |
| Beta-2-microglobulin precursor | Downregulates |
| Bone-morphogenetic protein 1, 2 isoform 1, precursor | Upregulates |
| Bone-morphogenetic protein receptor type-II precursor | Downregulates |
| Brain adenylate cyclase 1 | Inhibits |
| Breast cancer antiestrogen resistance 1 | Induces phosphorylation |
| BTAF1 RNA polymerase II, B-TFIID transcription factor-associated, 170 kDa | Interacts with |
| c-Src tyrosine kinase | Activates |
| Calcium/calmodulin-dependent protein kinase I | Downregulates |
| Calcium/calmodulin-dependent protein kinase IIA, IIB isoform 1 | Inhibits |
| cAMP-responsive element-binding protein 1 isoform A | Activates |
| cAMP-responsive element modulator isoform v | Activates |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| cAMP-dependent protein kinase catalytic subunit beta isoform 1 | Activates |
| cAMP-dependent protein kinase, regulatory subunit alpha 1 and beta 2 | Activates |
| cAMP-specific phosphodiesterase 4D | Activates |
| Cardiac muscle alpha actin 1 proprotein | Induces rearrangement |
| Caspase 3 preproprotein | Activates |
| Caspase 8 isoform A precursor | Upregulates |
| CC chemokine receptor 3 | Binds |
| CCAAT/enhancer binding protein | Binds |
| CCAAT/enhancer binding protein beta | Binds |
| CD180 antigen | Downregulates |
| CD28 antigen | Interacts |
| CD3D antigen, delta polypeptide isoform A precursor | Interacts |
| CD3E antigen, epsilon polypeptide (TiT3 complex) | Interacts |
| CD3G gamma precursor | Interacts |
| CD4 antigen precursor | Upregulates |
| CD40 antigen isoform 1 precursor | Upregulates |
| Cell division cycle 2 protein isoform 1 | Interacts |
| Cell division cycle 20 and 25C protein | Downregulates |
| Cell division cycle 37 protein | Regulated by |
| Cell division cycle 6 protein | Interacts |
| Chaperonin containing TCP1, subunit 4 (delta) | Interacts |
| Chemokine (C motif) ligand 1, 3, 7 | Upregulates |
| Chemokine (C-C motif) receptor 1 | Modulates |
| Chemokine (C-C motif) receptor 2 isoform B | Binds |
| Chemokine (C-C motif) receptor 4 | Upregulated by |
| Chemokine (C-C motif) receptor 5 | Upregulates |
| Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) isoform beta | Interacts |
| Chemokine (C-X-C motif) receptor 3 | Inhibits |
| Chemokine (C-X-C motif) receptor 4 isoform b | Binds |
| Chemokine (C-X3-C motif) ligand 1 | Inhibited by |
| Chemokine C-C motif ligand 4 isoform 1 precursor | Upregulates |
| Chromobox homolog 5 (HP1 alpha homolog, Drosophila) | Inactivates |
| Class II transactivator | Inhibits |
| Claudin 1 | Downregulates |
| Claudin 5 | Downregulates |
| Cleavage and polyadenylation specific factor 3, 73 kDa | Upregulates |
| Cofactor of BRCA1 | Associates with |
| Cofilin 1 (nonmuscle) | Downregulates |
| Cofilin 2 (muscle) | Downregulates |
| Collagen, type-III, alpha 1 preproprotein | Upregulates |
| Colony-stimulating factor 2 precursor | Upregulates |
| Complement component 1 inhibitor precursor | Upregulates |
| Complement component 1, q subcomponent-binding protein precursor | Binds |
| Core histone macro-H2A2.2 | Binds |
| CREB-binding protein isoform a | Binds |
| CREB3: cAMP-responsive element binding protein 3 (luman) | Inhibited by |
| CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1 isoform FCP1a | Binds |
| CXCL16 | Interacts |
| Cyclin A, A1, E1 | Interacts |
| Cyclin B1 | Upregulates |
| Cyclin C isoform a | Interacts |
| Cyclin D1 | Downregulates |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| Cyclin D3 | Interacts |
| Cyclin H | Binds |
| Cyclin T1 | Binds |
| Cyclin T2 isoform a | Binds |
| Cyclin-dependent kinase 2 isoform 1 | Enhances |
| Cyclin-dependent kinase 4 | Interacts |
| Cyclin-dependent kinase 5 | Downregulates |
| Cyclin-dependent kinase 7 | Binds |
| Cyclin-dependent kinase 8 | Interacts |
| Cyclin-dependent kinase 9 | Binds |
| Cyclin-dependent kinase inhibitor 1A | Activates |
| Cyclin-dependent kinase inhibitor 1B | Interacts |
| Cytochrome c | Induces release of |
| Cytoplasmic nuclear factor of activated T cells 3 isoform 1 | Activates |
| Cytoplasmic nuclear factor of activated T cells 4 | Activates |
| Delta isoform of regulatory subunit B56, protein phosphatase 2A isoform 1 | Modulated by |
| Dicer1 | Interacts |
| Dipeptidyl peptidase IV | Inhibits |
| Diubiquitin | Ubiquitinated by |
| DNA-dependent protein kinase | Modulates HIV gene expression |
| DNA-directed RNA polymerase II polypeptides | Interacts |
| DNA polymerase epsilon catalytic subunit | Interacts |
| DNA-damage-inducible transcript 3 | Enhanced by |
| DNA-directed RNA polymerase III 39 kDa polypeptide F | Activates |
| Dopamine transporter | Inhibits |
| Downregulator of transcription 1 | Inhibits |
| E1A-binding protein p300 | Complexes with |
| E2F transcription factor 1 | Inhibited by |
| E2F transcription factor 4 | Recruits |
| Early growth response 1, 2, 3 | Binds |
| Egr-1 in astrocytes | Tat-mediated toxicity in astrocytes |
| Elongation factor RNA polymerase II | Interacts |
| Elongin B isoform a | Interacts |
| Endonuclease G precursor | Activates |
| Endothelial differentiation-related factor 1 isoform alpha | Downregulates |
| Endothelin 1 | Upregulates |
| Enolase 1 | Inhibits |
| EP300 | Binds |
| Epidermal growth factor (beta urogastrone) | Interacts |
| Epsilon isoform of regulatory subunit B56, protein phosphatase 2A | Modulated by |
| Eukaryotic translation elongation factor 1 alpha 1, 2 | Interacts |
| Eukaryotic translation initiation factor 2-alpha kinase 2 | Interacts |
| Excision repair cross-complementing rodent repair deficiency, complementation group 2 protein | Binds |
| Excision repair cross-complementing rodent repair deficiency, complementation group 3 | Binds |
| Fas ligand | Interacts |
| Fc fragment of IgG, low affinity IIIa, receptor for (CD16) | Inhibits |
| Fibroblast growth factor 1 (acidic) isoform 1 precursor | Upregulates |
| Fibronectin 1 isoform 3 preproprotein | Competes with |
| Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) | Binds |
| Furin preproprotein | Cleaved by |
| Galectin 3 | Upregulates |
| Gamma isoform of regulatory subunit B55, protein phosphatase 2 isoform a | Regulated by |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| Gastrointestinal glutathione peroxidase 2 | Modulates |
| GATA-binding protein 2 | Synergizes with |
| GCN5 general control of amino-acid synthesis 5-like 2 | Binds |
| Gelsolin isoform a precursor | Downregulates |
| General transcription factor iib, e, f, and h | Interacts |
| General transcription factor IIIC, polypeptide 1, alpha 220 kDa | Activates |
| GLI-Kruppel family member GLI2 | Synergizes with |
| Glial fibrillary acidic protein | Upregulates |
| Glucocerebrosidase precursor | Interacts |
| Glucose-6-phosphate dehydrogenase isoform a | Activates |
| Glutamate receptor, ionotropic, N-methyl-D-aspartate 3A, 3B | Activates |
| Glutamate receptor, metabotropic 1 | Activates |
| Glutathione peroxidase 1 isoform 1, 4, 5, 6, 7 | Downregulates |
| Glutathione synthetase | Modulates |
| Glycogen synthase kinase 3 beta | Activates |
| Glycophorin A precursor | Downregulates |
| Granulin precursor | Binds |
| Grb2 | Binds through SH3 domains |
| Growth factor receptor-bound protein 2 isoform 1 | Recruits |
| Growth hormone 1 isoform 1 | Inhibited by |
| H2A histone family members | Binds |
| H2B histone family members | Binds |
| H3 histone family members | Binds |
| H4 histone family members | Binds |
| Heat shock 70 kDa proteins | Regulated by |
| Heparan sulfate proteoglycan 2 | Interacts |
| Heparanase | Interacts |
| Hexamethylene bis-acetamide inducible 1, 2 | Inhibited by |
| Histone 1, h2ad | Binds |
| Histone 2, H4 | Binds |
| Histone deacetylase 1 | Inhibited by |
| Histone h2a | Binds |
| Histone H2A | Binds |
| Histone H2B | Binds |
| Histone H4 | Binds |
| HIV TAT specific factor 1 | Stimulated by |
| HIV-1 Tat interactive protein 2, 30 kDa | Stimulates |
| HIV-1 Tat interactive protein, 60 kDa isoform 1 | Binds |
| HMT1 hnRNP methyltransferase-like 6 | Methylated by |
| Human immunodeficiency virus type-I enhancer binding protein 1 | Enhanced by |
| Inhibitor of DNA-binding 1 isoform a | Upregulates |
| Ini1/hSNF5 | Interacts |
| Inositol 1,4,5-triphosphate receptor, type 1, 2, 3 | Interacts |
| Insulin-like growth factor 1 (somatomedin C) | Inhibited by |
| Insulin-like growth factor-binding protein 4 precursor | Binds |
| Integrin alpha 3 isoform a precursor | Activates |
| Integrin alpha 5 precursor | Binds |
| Integrin alpha L precursor | Inhibits |
| Integrin beta 1 isoform 1A precursor | Interacts |
| Integrin beta 4 isoform 1 precursor | Interacts |
| Integrin beta chain, beta 3 precursor | Interacts |
| Integrin, beta 2 precursor | Inhibits |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| Integrin, beta 5 | Interacts |
| Intercellular adhesion molecule 1 precursor | Interacts |
| Interferon regulatory factor 1 | Binds |
| Interferon regulatory factor 7 isoform a | Upregulates |
| Interferon-stimulated gene 20 kDa | Upregulates |
| Interferon-induced protein 35 | Upregulates |
| Interferon-induced protein with tetratricopeptide repeats 3 | Upregulates |
| Interferon-induced, hepatitis C-associated microtubular aggregate protein | Upregulates |
| Interferon, alpha 1 | Interacts |
| Interferon, alpha-inducible protein (clone IFI-15 K) | Upregulates |
| Interferon, alpha-inducible protein 27 | Upregulates |
| Interferon, beta 1, fibroblast | Interacts |
| Interferon, gamma | Inhibited by |
| Interferon, gamma-inducible protein 16 | Upregulates |
| Interleukin 1 receptor antagonist isoform 2 | Upregulates |
| Interleukin 1 receptor, type I precursor | Upregulates |
| Interleukin 1, alpha proprotein | Downregulates |
| Interleukin 1, beta proprotein | Downregulates |
| Interleukin 10 precursor | Inhibited by |
| Interleukin 12A precursor | Downregulates |
| Interleukin 12B precursor | Downregulates |
| Interleukin 13 precursor | Induces release of |
| Interleukin 16 isoform 1 precursor | Inhibited by |
| Interleukin 19 isoform 1 precursor | Upregulates |
| Interleukin 2 precursor | Downregulates |
| Interleukin 2 receptor beta precursor | Downregulates |
| Interleukin 2 receptor, alpha chain precursor | Upregulates |
| Interleukin 20 precursor | Upregulates |
| Interleukin 3 precursor | Upregulates |
| Interleukin 4 isoform 1 precursor | Interacts |
| Interleukin 6 (interferon, beta 2) | Activated by |
| Interleukin 6 receptor isoform 1 precursor | Upregulates |
| Interleukin 7 precursor | Inhibits |
| Interleukin 7 receptor precursor | Inhibits |
| Interleukin 8 precursor | Downregulates |
| Jun oncogene | Binds |
| Karyopherin beta 1 | Binds |
| Kinase insert domain receptor (a type-III receptor tyrosine kinase) | Interacts with |
| Kinesin family member 2C | Downregulates |
| Kruppel-like factor 9 | Enhanced by |
| Lactate dehydrogenase A | Downregulates |
| Lactate dehydrogenase B | Downregulates |
| Lamin A/C isoform 1 precursor | Binds |
| Lamin B1 | Binds |
| Lamin B2 | Binds |
| Laminin alpha 5 | Upregulates |
| Laminin subunit beta 3 precursor | Upregulates |
| Laminin, alpha 1 precursor | Upregulates |
| Laminin, alpha 4 precursor | Upregulates |
| Laminin, beta 1 precursor | Upregulates |
| Laminin, beta 2 precursor | Upregulates |
| Laminin, beta 4 | Upregulates |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| Laminin, gamma 1 precursor | Upregulates |
| Laminin, gamma 2 isoform a precursor | Upregulates |
| Laminin, gamma 3 precursor | Upregulates |
| Low affinity immunoglobulin gamma Fc region receptor III-B precursor | Inhibits |
| Low-density lipoprotein-related protein 1 | Binds |
| LSD1/KDM1 | Modulates transcription |
| Lymphocyte-specific protein tyrosine kinase precursor | Activates |
| Lymphotoxin alpha precursor | Upregulates |
| MAD, mothers against decapentaplegic homolog 6 | Downregulates |
| Major histocompatibility complex, class I | Downregulates |
| Major histocompatibility complex, class II | Downregulates |
| Manganese superoxide dismutase isoform A precursor | Downregulates |
| Mannose receptor C type-1 precursor | Downregulates |
| Mannose receptor C type 2 | Downregulates |
| Matrix metalloproteinase | Activates |
| Menage a trois 1 (CAK assembly factor) | Binds |
| Metallothioneins | Upregulates |
| Methyltransferase-like protein 1 isoform a | Downregulates |
| Microtubule-associated deacetylase HDAC6 | Tat acetylation and transactivation |
| Mitogen-activated protein kinases | Activates |
| Mothers against decapentaplegic homolog 3 | Inhibited by |
| Mothers against decapentaplegic homolog 4 | Inhibited by |
| Mouse double minute 2 homolog isoform MDM2 | Regulated by |
| Myc protooncogene protein | Upregulates |
| MyoD family inhibitor domain containing isoform p40 | Binds |
| Myxovirus resistance protein 1 | Upregulates |
| Nasal embryonic LHRH factor | Associates with |
| Nerve growth factor, beta polypeptide precursor | Inhibits |
| Neutrophil cytosolic factor 1 | Interacts |
| Nitric oxide synthase 2A isoform 1 | Inhibits |
| Nitric oxide synthase 3 (endothelial cell) | Upregulates |
| NMDA receptor 1 isoforms | Activates |
| Notch 2 preproprotein | Interacts |
| Nrf2 transcription factor | Fusion to Tat |
| Nuclear factor I/C isoform 1 | Synergizes with |
| Nuclear factor kappa-B, subunit 1 | Interacts |
| Nuclear factor of activated T cells, cytosolic component 1 isoform A | Activates |
| Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2 isoform B | Binds |
| Nuclear factor of kappa light polypeptide gene enhancer in B cells | Activates |
| Nuclear receptor coactivator 1 isoform 1 | Stimulates |
| Nuclear receptor coactivator 2 | Stimulates |
| Nuclear receptor coactivator 3 isoform a | Enhanced by |
| Nuclear receptor subfamily 2, group F, member 1 | Interacts |
| Nuclease-sensitive element binding protein 1 | Binds |
| Nucleophosmin 1 isoform 1 | Binds |
| Nucleosome assembly protein 1 | Interacts |
| P-TEFb | Binds |
| p21-activated kinase 1 | Interacts |
| p300/CBP-associated factor | Interacts |
| p65 | Binds |
| Paired mesoderm homeobox 1 isoform pmx-1a | Downregulates |
| Pancreas-enriched phospholipase C | Activates |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| Paxillin | Induces phosphorylation of |
| Phorbolin 1 | Upregulates |
| Phosphatase and tensin homolog | Downregulates |
| Phosphodiesterases | Activates |
| Phosphoinositide-3-kinases | Interacts with |
| Phosphoinositide-specific phospholipase C beta 1 isoform a | Activates |
| Phospholipase C isoforms | Activates |
| Plasma glutathione peroxidase 3 precursor | Downregulates |
| Plasminogen activator inhibitor-1 | Modulates |
| Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit (45kDa ) | Interacts |
| Poly(ADP-ribose) polymerase family, member 1 | Regulates |
| Poly(A) polymerase alpha | Regulates |
| Poly(A) polymerase beta (testis specific) | Regulates |
| Poly(A) polymerase gamma | Regulates |
| Polymerase (DNA directed), beta | Upregulates |
| Polymerase (RNA) III (DNA directed) polypeptides | Activates |
| Polypyrimidine tract-binding protein 2 | Interacts |
| Polypyrimidine tract-binding protein 1 isoform a | Interacts |
| POU domain, class 2, transcription factor 1 | Binds |
| PRF1: perforin 1 (pore forming protein) | Downregulates |
| Prion protein preproprotein | Upregulates |
| PRKC, apoptosis, WT1, regulator | Upregulates |
| Programmed cell death 11 | Modulated by |
| Proliferating cell nuclear antigen | Interacts |
| Promyelocytic leukemia protein isoform 1 | Regulated by |
| Prostaglandin-endoperoxide synthase 2 precursor | Upregulates |
| Proteasome (prosome, macropain) 26S subunit, non-ATPase, 6 | Interacts |
| Proteasome (prosome, macropain) activator subunit 4 | Interacts |
| Proteasome 26S ATPase subunits | Interacts |
| Proteasome 26S non-ATPase subunits | Interacts |
| Proteasome activator subunits | Interacts |
| Proteasome alpha subunits | Interacts |
| Proteasome beta subunits | Interacts |
| Proteasome inhibitor subunit 1 isoform 1 | Interacts |
| Protein kinase C | Activates |
| Protein kinase D | Regulated by |
| Protein kinase, camp dependent | Activates |
| Protein kinase, DNA-activated, catalytic polypeptide | Binds |
| Protein phosphatase 1 | Through Cdk9 phosphorylation |
| Protein phosphatase 1 regulatory inhibitor subunit 8 isoform alpha | Binds |
| Protein phosphatase 1, catalytic subunits | Binds |
| Protein phosphatase 2, catalytic subunits | Modulated by |
| Protein phosphatase 3, catalytic subunits | Activates |
| Protein tyrosine phosphatase, nonreceptor type 23 | Upregulates |
| Protooncogene tyrosine-protein kinase SRC | Activates |
| PTK2 protein tyrosine kinase 2 isoform a | Activates |
| PTK2B protein tyrosine kinase 2 beta isoform a | Induces phosphorylation |
| Purine-rich element-binding protein A | Binds |
| RAD51 homolog protein isoform 1 | Interacts |
| RAN-binding protein 5 | Binds |
| Ras homolog gene family, member A | Activates |
| Ras-related C3 botulinum toxin substrate 1 isoform Rac1 | Activates |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| Ras-related C3 botulinum toxin substrate 2 | Activates |
| RD RNA-binding protein | Associates |
| RelB | Through inhibition of tnf$\alpha$ |
| Replication factor C2 (40 kDa) isoform 1 | Interacts |
| Replication factor C3 isoform 1 | Interacts |
| Replication factor C4 | Interacts |
| Replication factor C5 isoform 1 | Interacts |
| Replication factor C large subunit | Interacts |
| Replication protein A1, 70 kDa | Interacts |
| Reticuloendotheliosis viral oncogene homolog B | Interacts |
| Retinoblastoma 1 | Inhibited by |
| Retinoblastoma-like 2 (p130) | Binds |
| Rho GDP dissociation inhibitor (GDI) alpha | Downregulates |
| Ribosomal protein L3 isoform a | Interacts |
| RNA guanylyltransferase and 5′-phosphatase | Binds |
| RNA polymerase II, polypeptide H | Activates |
| S-phase kinase-associated protein 2 isoform 1 | Enhanced by |
| Secretoglobin, family 2A, member 2 | Upregulates |
| Selectin E precursor | Upregulates |
| Semaphorin 4D | Upregulates |
| SHC (Src homology 2 domain containing) transforming protein 1 isoform p52Shc | Induces phosphorylation of |
| Signal transducer and activator of transcription 1 isoform alpha | Upregulates |
| Signal transducer and activator of transcription 3 isoform 2 | Activates |
| Signal transducer and activator of transcription 6 | Interacts |
| Single-stranded DNA-binding protein 1 | Upregulates |
| Sirtuin 1 | Regulated by |
| SKI-interacting protein | Associates |
| SKIP | Interacts through c-Myc and Menin |
| Small GTPase protein E-Ras | Activates |
| Small inducible cytokine precursors | Inhibits |
| Small inducible cytokine subfamily E, member 1 | Upregulates |
| Small nuclear ribonucleoprotein polypeptide | Stimulated by |
| Solute carrier family 22, member 1 isoform a | Regulated by |
| Solute carrier family 5 (sodium/glucose cotransporter), member 1 | Inhibits |
| Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3 | Downregulates |
| Sp1 transcription factor | Interacts |
| SP110 nuclear body protein isoform a | Upregulates |
| Sp3 transcription factor isoform 1 | Interacts |
| Sp4 transcription factor | Interacts |
| Spermidine/spermine N1 acetyltransferase | Upregulates |
| Splicing factor, arginine/serine-rich 1 isoform 1 | Inhibits |
| Splicing factor, arginine/serine-rich 7 | Inhibits |
| Squamous cell carcinoma antigen recognized by T cells 3 | Regulated by |
| SRB7 suppressor of RNA polymerase B homolog | Interacts |
| Src homology 2 domain containing transforming protein C3 | Induces phosphorylation |
| Succinate dehydrogenase complex, subunit B, iron sulfur (Ip) | Binds |
| Superoxide dismutase 1, soluble | Downregulates |
| Superoxide dismutase 3, extracellular precursor | Downregulates |
| Suppressor of Ty 4 homolog 1 | Activated by |
| Suppressor of Ty 5 homolog | Stimulated by |
| SWI/SNF-related, matrix-associated, and actin-dependent regulator of chromatin | Binds |
| Syndecan 1–4 precursors | Binds |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| T-box 21 | Upregulates |
| T-cell receptor zeta chain isoform 1 precursor | Interacts |
| TAF12 RNA polymerase II, TATA box-binding protein (TBP)-associated factor, 20 kDa | Interacts |
| TAF9 RNA polymerase II isoform a | Interacts |
| TAR DNA-binding protein | Inhibited by |
| TAR RNA-binding protein 1 | Regulates |
| TAR RNA-binding protein 2 isoform a | Synergizes |
| TAT-interactive protein, 72 kDa | Binds |
| TATA box-binding protein | Interacts |
| TATA box-binding protein-associated factor 2F | Interacts |
| TBP-1 interacting protein isoform 2 | Inhibited by |
| TBP-associated factors | Interacts |
| Telomerase reverse transcriptase isoform 1 | Downregulates |
| Testis-specific histone H2B | Binds |
| TFIIA alpha, p55 isoform 1 | Stabilizes |
| TH1-like protein | Associates |
| Thrombospondin 1 precursor | Binds |
| Thyroid hormone receptor, alpha isoform 2 | Binds |
| Tight junction protein 2 (zona occludens 2) isoform 1 | Downregulates |
| Tissue inhibitor of metalloproteinase 1 precursor | Interacts |
| Tissue inhibitor of metalloproteinase 2 precursor | Interacts |
| TNF receptor-associated factor 4 | Inhibited by |
| Transcription elongation factor A (SII), 3 | Interacts |
| Transcription elongation factor A1 isoform 1 | Interacts |
| Transcription elongation factor A protein 2 isoform a | Interacts |
| Transcription elongation regulator 1 isoform 1 | Associates with |
| Transcription factor 3 | Regulated by |
| Transcription factor 4 isoform b | Inhibits |
| Transcription factor 7-like 2 (T-cell specific, HMG-box) | Regulated by |
| Transcription factor AP-4 (activating enhancer binding protein 4) | Regulated by |
| Transcription factor CP2 | Inhibited by |
| Transforming growth factor, alpha | Upregulates |
| Transforming growth factor, beta 1 | Inhibited by |
| Transforming growth factor, beta 2 | Upregulates |
| Transporter 1, ATP-binding cassette, subfamily B | Upregulates |
| Transportin 1 | Binds |
| Tripartite motif-containing 22 | Inhibited by |
| TUBB4Q: tubulin, beta polypeptide 4, member Q | Downregulates |
| Tubulin alpha 6 | Binds |
| Tubulin, alpha 1 | Binds |
| Tubulin, alpha 1a | Binds |
| Tubulin, alpha 2 isoform 1 | Binds |
| Tubulin, alpha 4 | Binds |
| Tubulin, alpha 8 | Binds |
| Tubulin, alpha, ubiquitous | Binds |
| Tubulin, beta | Downregulates |
| Tubulin, beta 2 | Binds |
| Tubulin, beta 2B | Downregulates |
| Tubulin, beta 4 | Binds |
| Tubulin, beta 6 | Binds |
| Tubulin, beta 8 | Binds |

Table 1: Continued.

| Human protein | Type of activity with Tat |
|---|---|
| Tubulin, beta, 2 | Binds |
| Tubulin, beta, 4 | Binds |
| Tumor necrosis factor (ligand) superfamily, member 10 | Inhibits |
| Tumor necrosis factor alpha | Interacts |
| Tumor necrosis factor receptors | Upregulates |
| Tumor protein p53 | Binds |
| Tumor protein p73 | Binds |
| Tyrosine hydroxylase isoform b | Downregulates |
| Ubiquitin B precursor | Ubiquitinated by |
| Ubiquitin C | Ubiquitinated by |
| Ubiquitin-activating enzyme E1 | Ubiquitinated by |
| Ubiquitin-conjugating enzyme E2D 1 | Ubiquitinated by |
| Upstream-binding protein 1 (LBP-1a) | Inhibits |
| Urokinase plasminogen activator preproprotein | Upregulates |
| V-akt murine thymoma viral oncogene homolog 2 | Activates |
| V-akt murine thymoma viral oncogene homolog 3 isoform 1 | Activates |
| V-fos FBJ murine osteosarcoma viral oncogene homolog | Interacts |
| V-Ha-ras Harvey rat sarcoma viral oncogene homolog isofrom 1 | Activates |
| V-rel reticuloendotheliosis viral oncogene homolog | Interacts |
| Vascular cell adhesion molecule 1 isoform a precursor | Upregulates |
| Vascular endothelial growth factor A isoform b precursor | Cooperates with |
| Vitronectin precursor | Competes with |
| Wolf-Hirschhorn syndrome candidate 2 protein | Downregulates |
| YY1 transcription factor | Inhibited by |
| Zinc finger and BTB domain containing 7A | Binds |

and therefore have direct selective pressures. The power of having large data sets can be demonstrated in a recent study which has identified two residues in Tat, positions 35 and 39, which appear to be coevolved. These residues, however, have two distinct functions with respect to the transactivation of the HIV-1 LTR-binding P-TEFb and promoting P-TEFb phosphorylation of the C-terminal domain in RNAPII, respectively [151]. In addition, understanding the genetic diversity of Tat in multiple subtypes has become and will continue to be increasingly important as vaccines in development will need to account for immunity to all of these variations.

## 7. Tat Genetic Variation and HIV-1-Associated Neurological Disorders

In addition to transactivation of the viral LTR, Tat exhibits a range of biological properties relative to HIV-1 pathogenesis [152], including the intracellular regulation of host gene expression to facilitate viral production as well as the extracellular detrimental effects on the cells of the immune and nervous systems. HIV-1 induces pathological consequences in a number of end organs including the brain [7, 153]. More than 30% of AIDS patients suffer from some form of HIV-1-induced neurological impairment including HIV-1-associated dementia (HAD) as well as other more subtle minor neurocognitive disorders [154, 155]. Despite the widespread use of highly active antiretroviral therapy and the resultant decrease in the incidence of HAD, the prevalence of HAD and other milder forms of HIV-related neurological disease has become increasingly common problems with respect to the clinical management of HIV/AIDS [156]. In particular, Tat has been implicated in the pathogenesis of HIV-associated neurological disease including HAD via a variety of mechanisms [157]. The neurotoxicity of Tat is further supported by the observation that the mRNA levels for Tat are elevated in brain extracts of patients with HAD [158]. Tat has been shown to act as an intracellular and extracellular mediator of neurotoxicity and to play a critical role in contributing to neurological injury in HAD [159]. Tat protein is secreted by HIV-1-infected cells and acts by diffusing through the cell membrane. It appears to act as a secreted, soluble neurotoxin and induces HIV-1-infected macrophages and microglia to release neurotoxic substances [160–162]. Some Tat variants have been reported to be dysfunctional with respect to LTR transactivation and may contribute to viral latency under certain conditions while still being able to stimulate the transcription of a number of cytokine genes [95]. Tat can also cooperate with cellular factors to enhance the neurotoxic effects on host cells [163]. Tat and cytokines IFN-$\gamma$ and TNF-$\alpha$ have also been demonstrated to synergistically increase expression of CXCL10 in human astrocytes, which provide an important

reservoir for the generation of inflammatory mediators, for instance, CXCL10 as a neurotoxin and a chemoattractant [164].

Phylogenetic analyses of Tat sequences from patients with and without HAD have shown clustering of sequences with respect to clinical diagnosis of neurological impairment as well as tissue of origin [165, 166]. Comparisons of matched brain and spleen-derived Tat sequences have suggested that greater sequence homology exists among brain-derived Tat clones than that observed between brain and spleen-derived clones [166]. Another study also showed sequence variations within patients segregated as CNS and non-CNS *tat* genes [167]. Additionally, significant sequence heterogeneity exists within brain-derived Tat in domains associated with viral replication and intracellular transport [166]. Nonsynonymous versus synonymous mutation rates among brain-derived Tat sequences isolated from patients with neurocognitive impairment were shown to be significantly greater than those isolated from patients without clinical evidence of neurological disease [165]. Importantly, most of the mutations present in the HAD-associated Tat sequences were located in the augmenting region (residues 57–78 amino acids), which affects viral replication. Interestingly, in these studies, variations at position 74 and 100 were correlated to Tat sequences isolated from brain-derived sequences. Collectively, results from these studies suggest that differing selective pressures act on individual HIV-1 genes within the CNS and that this differential selective pressure may influence both the development and subsequent severity of neurocognitive impairment.

Participants in a current longitudinal study of patients in the DrexelMed HIV/AIDS Genetic Analysis Cohort have had their LTR and their *env* and *tat* genes and proteins, where appropriate, analyzed from peripehral blood. The LTR was analyzed as a result of previous studies that identified the potential for single nucleotide polymorphisms in the LTR to be predictive of neurocognitive impairment [168–170]. Patients within the DrexelMed HIV/AIDS Genetic Analysis Cohort are followed longitudinally, with scheduled visits every 6 months. At each visit, patients are interviewed for clinical and social history, a blood sample is collected, and neurocognitive status is evaluated with a minineurological exam [171]. Recently, eight patients were analyzed with respect to Tat genetic variation. Longitudinal analyses of these 8 patients showed 7 of the patients had exhibited NI at some point in their clinical history; of these 7 patients, 3 patients had current NI and all 3 had Tat containing a change at amino acid position 100 (amino acid 100). Of the remaining 4 patients, 2 had the change at amino acid 100 with their following visit showing NI; the final 2 did not have a change at amino acid 100; however, these patients were lost to follow up for 23–36 months, at which time they presented with NI. The one patient without NI did not have a change at amino acid 100. Given the previous studies, which have identified this amino acid position as indicative of brain-derived sequences, this observation may point to the fact that a change at this amino acid position may occur in the periphery in patients before the onset of NI (perhaps in the HIV-1-infected monocyte-macrophage compartment).

The genetic variation observed within Tat has also been shown to alter the function of this protein and relate to pathogenesis. Importantly, HIV-1 Tat derived from HAD patients has been associated with greater neuronal death both *in vitro* and *in vivo* compared with Tat from non-HAD patients. This characteristic has been attributed, in part, to enhanced MMP-2 expression induced by brain-derived HIV-1 Tat variants [172]. Interestingly, however, these same brain-derived Tat isolates also appear to be limited in their ability to enhance viral gene expression despite the increased activation of host transcriptional machinery [173]. This observation differs from other reported studies, which showed that a subset of patients demonstrated reduced transactivation capacity of CNS-derived Tat proteins compared to those from matched lymphoid tissues; however, overall Tat proteins from the CNS, when compared to lymphoid compartments, maintained similar levels of transactivation function [167]. However, one must remember that these viral gene activation studies were all performed with a viral regulatory region derived from a non-CNS tissue source and may therefore not be naturally compatible with respect to optimal LTR activation by a Tat protein selected for CNS replication. This observation is of particular importance because previous studies [168, 174, 175] have demonstrated that LTRs derived from the CNS are likely to be structurally and functionally different from LTRs derived from other tissue sources. In fact, a current study has shown that Tat transactivates the corresponding HIV-1-infected patient-derived colinear LTR better than a non-co-linear Tat protein. These Tat clones were shown to have nonconsensus variations compared with IIIB or the consensus B sequence of Tat that might contribute to the alteration in their function [171]. Taken together, these reports point to the notion that genetic diversity of HIV-1 Tat likely contributes to the establishment and severity of HIV-1-associated neurological disease. However, additional studies of this nature are warranted. For instance, are there certain Tat residues that induce increased Tat secretion from cells both in the periphery as well as in the CNS? Are there variations that induce more neurotoxic effects? Are the Tat variants isolated from vRNA versus integrated DNA similar or different?

## 8. HIV-1 Tat as a Therapeutic Target

The HIV-1 Tat protein has long remained an attractive target for therapeutic intervention owing to its essential role in viral gene expression and activation of the HIV-1 LTR. As discussed before, Tat and the P-TEFb complex bind to TAR to promote efficient transcription of the full-length HIV genome. The expanding knowledge of Tat functional properties and its interactions with other cellular and viral partners has led to the identification of a varied range of compounds that can inhibit different Tat functions. The Tat and HIV-1 transactivation inhibitors fall broadly into the following categories: (1) inhibitors targeting TAR RNA (2), inhibitors targeting Tat protein, and (3) Tat-P-TEFb interaction inhibitors. In this section, we review the current status of the development of therapeutic strategies that target

Table 2: Tat-based therapeutics.

| Compound/class | Mechanism of action | Reference(s) |
|---|---|---|
| DRB | Purine nucleoside analog; inhibits cyclin-dependent kinases | [23] |
| Flavopiridol (flavonoids) | Inhibits cyclin-dependent kinases | [24] |
| Seliciclib | Inhibits cyclin-dependent kinases | [25] |
| 2′-O-Methyl/LNA oligoribonucleotides | Binds TAR | [26] |
| Phosphodiester/phophothiote oligonucleotides | Binds TAR | [27] |
| PNA-(TAR-16) | Polyamide nucleotide analog; binds TAR | [28] |
| Acetylpromazine | Binds 5′ bulge of TAR | [29] |
| O,O′-Bismyristoyl thiamine disulfide | Inhibits nuclear translocation of Tan and NF-$\kappa$B, via interaction with cysteine region | [30] |
| Cyclic peptides | Mimics basic region and binds TAR | [31] |
| Tat 9-K-biotin peptide | Binds TAR | [32] |
| CGP64222 | Peptoid/peptide similar to Tat-basic domain; binds TAR | [33] |
| CGP40336A (polyamine-acridine based) | Binds TAR | [34] |
| Aminoglycoside-arginine conjugates | Binds TAR in the major groove of the bulge and upper portion of the stem | [35] |
| Transdominant Tat mutants | Binds TAR | [36] |
| Biscationic diphenylfuran derivatives | Binds TAR | [35] |
| Neomycin (aminoglycoside) | Binds TAR, CXCR4, and other Tat targets | [37] |
| D-penicillamine | Binds Tat stably through cysteine residues | [38] |
| Stilbene (CGA137053) | Binds Tat directly | [39] |
| Suramin and derivatives | Competes with heparin/heparin sulfate for binding to the basic region of Tat; inhibits extracellular functions of Tat | [40] |
| Benzodiazepine derivatives | General inhibition of HIV-1 transcription and Tat transactivation | [41, 42] |
| Benzothiophene derivatives | — | [43] |
| Temacrazine (bistriazolonoacridones) | — | [44] |
| Fluoroquinolone derivatives | — | [45, 46] |

Tat and its functional interactions in the process of HIV-1 transcription (Table 2).

Compounds against the TAR RNA are the most numerous because they would block the primary functional interaction of Tat in the process of HIV-1 transcription. In this category, the compounds can be broadly divided into three classes: (1) peptide based, (2) oligonucleotide based, and (3) small-molecule based (for more detailed information refer to a previously published review [176]). It is well established that the arginine-rich motif of the Tat protein is required for it to bind to the TAR RNA trinucleotide bulge region [15, 90, 177]. Peptides corresponding to this region were found to compete for Tat binding and were shown to inhibit HIV-1 replication [32, 178]. Compounds directly binding to the three-base bulge of TAR RNA include 6-aminoquinolone [179, 180], quinoxaline-2, 3-diones [181], pyridine oxide derivatives such as JPL-32 [182, 183], and acridine derivatives such as CGP64222 and CGP40336A [33, 34]. All of these compounds exhibit strong inhibition of the Tat-TAR interaction by binding to the three-base bulge of TAR RNA. The peptide CGP64222 was later shown also to interact with CXCR4 [184], the chemokine receptor that acts as a coreceptor for the X4 or dual tropic HIV-1 strains. Other drugs that specifically interact with the bulge in TAR RNA include biscationic diphenylfuran derivatives and a new class of polyamine-acridine-based compounds [34, 185]. Aminoglycoside antibiotics such as neomycin and streptomycin and neamine and its derivatives have been shown to specifically bind RNA molecules and to block the conformation of the Tat-TAR complex by targeting the structure of TAR RNA [181, 186, 187]. Besides the aforementioned Tat-TAR inhibition strategies, the developments in the field of RNA interference have yet to be applied in full force for achieving a more targeted inhibition [188, 189].

Compounds binding directly to the Tat protein could inhibit HIV-1 replication. To achieve this goal, targeting the basic domain of Tat would be relevant because this very domain is required for nuclear localization, transactivation through TAR binding, and also for extracellular release and cellular uptake [190, 191]. It has been shown that, in the extracellular compartment, the basic domain of Tat can be targeted by several polyanions [192] such as heparin and heparan sulfates [40], thrombospondin [193], polysulfonated distamycin A derivatives [194], and sulfated polysaccharides [195], thereby blocking its internalization and also its extracellular activities. Another compound targeting Tat is the negatively charged polyacrylic acid, which could inhibit the Tat-TAR interaction with high affinity to Tat peptide, thereby blocking HIV-1 replication [196]. Negatively charged small molecules, such as the stilbene derivative, CGA-137053, was shown to inhibit HIV-1 replication by directly binding to Tat and inhibiting the formation of the Tat-TAR complex at low nanomolar concentrations [39]. One important aspect, which must be considered, is

that most of these interactions are largely due to electrostatic interactions with limited sequence specificity. Therefore, it would be highly relevant to evaluate their specificity in a relevant model system before they can be exploited in any therapeutic intervention strategy. Some transdominant-negative Tat mutants have also been shown to be potential antiviral therapeutics because they could inhibit the transactivation function of Tat, thereby inducing latency during viral infection [197–200]. Moreover, various biopolymeric drugs and anti-Tat antibodies have been demonstrated to be effective in inhibiting the extracellular activity and cellular uptake of Tat protein [176, 201]. Thus, targeting specific, conserved conformational epitopes on Tat might prove to be more beneficial. This approach can be aided tremendously by the emerging structural data on Tat. Some insights have already been provided by *ab initio* molecular dynamic studies on the Tat NMR structure and structural conformations of TAR [202, 203]. It would also be relevant to evaluate combination drug formulations to achieve an inhibition of the functional interactions of Tat at several levels [204]. This combination approach may also facilitate strategies that use relatively lower concentrations of these compounds that might improve overall toxicity levels. It might also be useful in targeting the small pool of latently infected cells that may hinder clearing the virus from the system.

The third approach is to use the understanding of the Tat-P-TEFb interaction to develop interventions to disrupt this interaction or to reduce the stability of this complex. P-TEFb (CDK9/cyclin T1) is an essential cofactor for Tat-mediated transactivation, and selective inhibition blocks HIV-1 replication without affecting cellular transcription, thus making it a potential target for anti-HIV-1 therapy. To this end, P-TEFb inhibitors such as 5,6-dichlororibofuranosylbenzimidazole, a purine nucleoside analogue; flavopiridol, a small molecular cyclin-dependent kinase inhibitor [23, 24]; Seliciclib, an inhibitior of CDK2/cyclin E and/or P-TEFb [25] have been evaluated in various *in vitro* studies and have been shown to effectively reduce HIV-1 replication. However, long-term HIV-1 replication studies showed that these inhibitors were more cytotoxic and less efficacious against HIV-1 in the primary cell cultures [205]. The failure of these known kinase inhibitors in providing anti-HIV efficacy has prompted studies to revisit the Tat-P-TEFb complex for small molecule inhibitors. In this study, molecular dynamics simulations are being utilized to understand the nature of interactions of Tat with CDK9 and Cyclin-T1 in a dynamic mode. These interactions are further mapped on to a pharmacophore-based screening paradigm to design small molecule inhibitors that show potent HIV-1 efficacy and low toxicity (Kortagere and Wigdahl, unpublished results).

In alternative approaches, promising results were demonstrated using anti-CycT1 human single-chain antibodies that targeted the cyclin domain and the TAR recognition motif, using transiently transfected cell lines [132] and stably transfected cells [206]. Protein chimeras like fusion of a truncated human CycT1 and a mutant CDK9 protein that lacked autophosphorylation activity have demonstrated the inhibition of Tat-mediated transactivation and HIV-1 gene expression [207]. Intracellular proteins that inhibit P-TEFb-like HEXIM1 have been evaluated to suppress HIV-1 replication [208, 209]. Moreover, the inhibition of a transcriptional coactivator like PCAF has been evaluated using antibodies against the bromodomain of PCAF [210]. These studies establish a therapeutic rationale, but more specificity is desired because targeting proteins involved in cellular homeostasis and activation pathways may have detrimental effects on the cells. The same complexity applies to studies that propose targeting specific posttranslation modifications on Tat such as phosphorylation [211] and acetylation [107]. Moreover, most of these studies are done in irrelevant cell types that dilute their therapeutic promise. Another aspect that gets overlooked is that it is extremely difficult to evaluate this effect since any such intervention will have an impact on normal cellular pathways. This underscores the value of in-depth analysis of the functional interactions in the HIV-1 replication paradigm because it would yield more specific targets with minimal host toxicity. Another aspect that has not been investigated in detail is the genetic variations observed within the Tat-coding sequences and how they might impact the structure and function of this vital transactivator. Efforts in our laboratory and others have indicated variation in the transactivation potential of different Tat sequences from HIV-1-infected patients (unpublished observations). Moreover, it has been established that sequence variation within specific domains of Tat was associated with increased viral replication and TNF-$\alpha$ production [141, 166, 212, 213]. These observations along with results that have shown that some Tat sequences exhibit minimal transactivation potential but have an ability to activate host gene expression [173] provide new directions where this aspect of Tat sequence variability can be included in strategies directed against Tat. The ideal outcome would be to achieve a competitive inhibition by using defective Tat mutants to inhibit Tat function, but a thorough understanding selected aspects of Tat function is a prerequisite to this line of investigation. These efforts again will be aided immensely by incorporating testing that includes Tat sequence and functional information across subtypes.

## 9. Conclusions

More than two decades of investigations have established the central role of Tat in the activation of HIV-1 LTR. Genetic- and structure/function-based studies have enabled us to understand the functional intricacies of Tat-dependent functions. All of these studies have motivated a number of researchers to use Tat as an important target in combination antiretroviral therapies, but to date none of these have materialized into clinically effective antiviral agents. One crippling factor has been the inability to assess the functions of Tat in relevant systems at a concentration that would be closer to that encountered *in vivo*. Concentrating efforts in a direction to elucidate the protein-protein interactome established by Tat will go a long way toward targeting specific breakpoints in HIV-1 pathogenesis. Isolation of mutant sequences of Tat from sites like the brain can also be used to identify tissue-specific functions of Tat that may have great bearing

on long-term use of Tat inhibitors. Moreover, it would be an important effort to consolidate studies concerning the structural information and functional interactions of Tat across different HIV subtypes and use this information to increase the spectrum of subtypes susceptible to Tat-based therapeutic inhibitors. Eradicating latent reservoirs by the elimination of integrated HIV-1 provirus or irreversibly blocking LTR activation or Tat transactivation activity will provide the next major step forward in controlling the HIV-1 pandemic.

## Authors' Contribution

Luna Li and Satinder Dahiya contributed equally in the preparation of this paper.

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*Review Article*

# Hepatitis C Variability, Patterns of Resistance, and Impact on Therapy

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Hepatitis C (HCV), a leading cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma, is the most common indication for liver transplantation in the United States. Although annual incidence of infection has declined since the 1980s, aging of the currently infected population is expected to result in an increase in HCV burden. HCV is prone to develop resistance to antiviral drugs, and despite considerable efforts to understand the virus for effective treatments, our knowledge remains incomplete. This paper reviews HCV resistance mechanisms, the traditional treatment with and the new standard of care for hepatitis C treatment. Although these new treatments remain PEG-IFN-$\alpha$- and ribavirin-based, they add one of the newly FDA approved direct antiviral agents, telaprevir or boceprevir. This new "triple therapy" has resulted in greater viral cure rates, although treatment failure remains a possibility. The future may belong to nucleoside/nucleotide analogues, non-nucleoside RNA-dependent RNA polymerase inhibitors, or cyclophilin inhibitors, and the treatment of HCV may ultimately parallel that of HIV. However, research should focus not only on effective treatments, but also on the development of a HCV vaccine, as this may prove to be the most cost-effective method of eradicating this disease.

## 1. Introduction: Burden of Hepatitis C

Hepatitis C (HCV) is a leading cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma, as well as the most common indication for liver transplantation in the United States. The annual incidence of infection in the USA has declined from about 230,000 cases per year in the 1980s to an estimated 17,000 cases in 2007 [1, 2]. This decline has been largely attributed to changes in injection practices motivated by a concern for human immunodeficiency virus (HIV) risk [3]. Approximately 3.2 million persons have chronic HCV infection in the United States; however, the reservoir of chronically infected persons is still estimated at approximately 2.35%, representing approximately 160 million worldwide infected individuals [4]. Aging of the currently infected population is expected to result in an increase in the burden of hepatitis C in the next decade [5]. During that period, the number of HCV-related cirrhosis cases is estimated to increase by 31% and that of hepatocellular carcinoma (HCC) cases is estimated to increase by approximately 50% [5]. Estimates of hepatitis C prevalence range from <0.5% in very low endemic countries (e.g., northern European countries) to staggering rates of approximately 20% in highly endemic areas, including urban centers and the Nile Delta in Egypt [6].

## 2. HCV Virus

HCV, like hepatitis B virus (HBV) and HIV, is prone to develop resistance to antiviral drugs. Viral dynamics include daily virion production of $10^{12}$ with a half-life of 2-3 hours for free virions and less for intracellular virions. It has a very rapid mutation rate, with 2 error-prone viral polymerases that lack proofreading, and no overlapping reading frames, which make it prone to developing resistance. However, given the moderate infected cell turnover and the absence of a viral reservoir, or in other words, the lack of host genome integration or episomal persistence in infected cells [7], HCV has the full potential for eradication.

Hepatitis C is a flavivirus (of which yellow fever is the prototype) from the genus *Hepacivirus*. An HCV particle consists of a core of genetic material (RNA), surrounded by an icosahedral protective shell of protein, and is further encased in a lipid envelope. Two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope [8]. The virus particle diameter is approximately 30–60 nm. The genome of 9,600 bases codes for ten proteins, though HCV isolates from different parts of the world differ in their length [9]. The 5′ and 3′ ends of the RNA are not translated into proteins (UTR) but are important to translation and replication of the viral RNA. The 5′ UTR has a ribosome binding site [10] (IRES—Internal ribosome entry site) that starts the translation of a very long protein containing about 3,000 amino acids (the polyprotein). The large polyprotein is later cut by cellular and viral proteases into the 10 smaller proteins that allow viral replication within the host cell or assembly into the mature viral particles [11].

Structural proteins made by the hepatitis C virus include the core protein E1 and E2, while nonstructural proteins include NS2, NS4, NS4A, NS4B, NS5A, and NS5B.

The core protein has 191 amino acids and can be divided into three domains based on hydrophobicity: domain 1 contains mainly basic residues with two short hydrophobic regions; domain 2 is less basic and more hydrophobic and its C-terminus is at the end of p21; domain 3 is highly hydrophobic and acts as a signal sequence for E1 envelope protein. Both envelope proteins (E1 and E2) are highly glycosylated and important in cell entry. E1 serves as the fusing subunit and E2 acts as the receptor binding protein.

NS2 protein is a 21–23 kilo Dalton (kDa) transmembrane protein with protease activity.

NS3 is 67 kDa protein with serine protease activity at the N-terminal end and NTPase/helicase activity at the C-terminal end. It is located within the endoplasmic reticulum and forms a heterodimeric complex with NS4A, a 54 amino acid membrane protein that acts as a cofactor of the proteinase.

NS4B is a small (27 kDa) hydrophobic integral membrane protein with 4 transmembrane domains. Like NS3, it is located within the endoplasmic reticulum and plays an important role in recruitment of other viral proteins. It induces morphological changes in the endoplasmic reticulum forming a structure called the "membranous web." NS5A is a hydrophilic phosphoprotein, which plays an important role in viral replication, modulation of cell signaling pathways, and the interferon response. The NS5B protein (65 kDa) is the viral RNA-dependent RNA polymerase.

Despite considerable efforts made to understand the basic structure and function of the virus, and the importance of this understanding for the development of antiviral treatment, our knowledge is far from complete. The exact mechanisms of HCV entry into hepatocytes have not yet been fully understood. Potential entry pathways into host cells may occur through complex interactions between virions and cell-surface molecules, such as CD81, LDL receptor, SR-BI, DC-SIGN, Claudin-1, and Occludin [12, 13], and ultimately via receptor-mediated endocytosis. Fusion of the virion envelope with cellular membranes delivers the nucleocapsid to the cytoplasm. After decapsidation, translation of the viral genome occurs in the cytoplasm, leading to the production of a precursor polyprotein, which is then cleaved by both cellular and viral proteases into three structural (virion-associated) and seven nonstructural (NS) proteins as discussed above. Via NS proteins, the viral genome attaches to the RNA replication complex, which is associated with rearranged cytoplasmic membranes. RNA replication takes place via the viral RNA-dependent RNA polymerase (RdRp) NS5B, which produces a negative strand RNA intermediate. The negative strand RNA then serves as a template for the production of new positive strand viral genomes. New genomes can then be translated, further replicated or packaged within new virus particles. New virus particles are thought to bud into the secretory pathway and are released at the cell surface. Release from the hepatocyte may involve the very low density lipoprotein secretory pathway [14].

## 3. HCV Resistance and Mechanisms of Action of IFN-$\alpha$ and Ribavirin

HCV exists as a mixture of populations of genetically distinct but closely related virions in every patient, including potentially drug-resistant variants that are present when antiviral therapy is initiated, thus conferring a quasispecies distribution. However, given its intracytoplasmatic replication and lack of intranuclear replication, there is no known potential for intracellular persistence [15, 16]. Drug-resistant variants often show reduced "replication fitness," are undetectable with current technology, and have not gained much attention prior to development of the new direct acting antivirals (DAAs) [17, 18]. More sensitive techniques, such as ultradeep pyrosequencing have been used to identify resistant variants prior to treatment, but these are not routinely used in current clinical practice [19–21]. Potent antiviral therapy eliminates sensitive strains, while resistant variants are uncovered and can expand. For many years, the recommended standard of care therapy for chronic HCV remained a combination of pegylated alpha-interferon (IFN-$\alpha$) and ribavirin; however, neither drug exerts viral pressure. In other words, treatment failure is not due to selection of IFN-$\alpha$ or ribavirin-resistant variants but is more likely to occur due to inherent host factors (such as the presence of certain single nucleotide polymorphisms (SNPs) upstream of the IL-28B locus that correlate with the rate of SVR), inappropriate drug regimens and viral factors. Interferons are cellular proteins able to induce an antiviral state in their target cells, as well as cytokine secretion, recruitment of immune cells, and cell differentiation. Their metabolism and mechanisms were recently reviewed [22].

Immediately after injection, IFN-$\alpha$ binds to receptors present on various cells including hepatocytes, triggering a sequence of intracellular reactions that activate IFN-inducible genes (ISGs). The products of these genes are responsible for the IFN-$\alpha$-mediated antiviral effects, achieved via two mechanisms. One is the induction of a nonvirus-specific replication inhibition in infected cells. IFN-$\alpha$ was found to directly inhibit HCV replication in vitro in the subgenomic replicon (a synthetic replication system

using HCV nonstructural proteins in various cell lines) [23]. The second mechanism involves immunomodulatory effects that enhance the host's specific antiviral immune response, thus clearing the infected cells [22]. The induction of the antiviral state could potentially extend to noninfected cells, thus reducing the chance that they will become infected. Upon the interaction of IFN-$\alpha$ with its receptor, many complex effects are generated, including the induction of major histocompatibility complex class I (MHC I) antigen expression, activation of macrophages, natural killer cells and T lymphocytes, production of primarily T-helper 1 (Th1) cells, and decreased production of T helper-2 (Th2) cells. PEG-IFN-$\alpha$ also interacts with cytokines such as CCL chemokines and tumor necrosis factor (TNF)-$\alpha$. Soluble TNF-$\alpha$ receptors (sTNF-R), which are released by activated neutrophils, mononuclear blood cells, and fibroblasts [24, 25] in response to mediators, such as interferon and TNF-$\alpha$ itself [25–28], retain their ability to bind circulating TNF-$\alpha$ and are important in regulating its activity. These sTNF-R may contribute to the anti-inflammatory action of IFN-$\alpha$. All of these effects suggest that IFN-$\alpha$ simply accelerates the host immune response, though no studies have clearly proven it.

HCV genotypes 1 and 4 are intrinsically more resistant to IFN-$\alpha$ than genotypes 2 and 3. More importantly, the sensitivity to IFN-$\alpha$ varies within each genotype. Consequently, the clearance of infected cells in patient who are interferon responders occurs much slower in genotypes 1 and 4, as compared to 2 and 3 [29, 30]. The mechanisms underlying these differences are yet to be defined. The combination of IFN-induced proteins and pathways responsible for inducing an antiviral state has not been completely mapped out, though various effectors have been proposed including the 2′–5′ oligoadenylate synthetase (2′–5′ OAS) system, Mx proteins, double-strand-RNA-dependent protein kinase (PKR), as well as other, less well-characterized/unknown IFN-induced intracellular pathways [22].

Ribavirin is a synthetic guanosine analog that undergoes intracellular phosphorylation, with the ultimate product, ribavirin triphosphate, being responsible for ribavirin's effects. *In vivo*, ribavirin has a moderate (<0.5 log HCV RNA reduction) and transient inhibitory effect of only 2-3 days duration on HCV replication, and this is only seen in about 50% of patients [31]. These effects are consistent with those seen in *in vitro* studies, and the modesty of the effect could be related to the drug's weak inhibitory action on the RNA-dependent RNA polymerase (RdRp) [32]. Fortunately, this effect is too weak and transient to account for selecting viral resistance to ribavirin. Despite its apparently weak antiviral effects, ribavirin remains essential to HCV treatment, as it appears to accelerate the clearance of infected cells through unknown mechanisms and to prevent viral breakthrough during treatment, and relapses following treatment, in patients on IFN-$\alpha$ [33]. Studies have suggested that ribavirin is an RNA mutagen, causing loss of viral "fitness" via lethal nucleoside accumulation during replication [34], though no excessive mutagenesis was noted during ribavirin therapy in HCV infection [35, 36]. Ribavirin, much like IFN-$\alpha$, has immunomodulatory effects, including preferentially driving the immune system to produce more Th1 cells relative to Th2 [37]. Some reports have suggested that ribavirin can also amplify the intracellular IFN-$\alpha$ responses, via unknown mechanisms [38].

## 4. HCV Clearance

According to Tang et al., IFN-$\alpha$ attachment to interferon alpha receptors (IFNAR) [39] and the very rapid activation of Interferon Stimulated Genes (ISG) after interferon alpha administration [40] could explain the effect of IFN-$\alpha$ as a potent immunostimulant of an innate response in the first few hours and an adaptive response after the first four weeks. [41]. The degree of the ISG-induced innate response may result in a rapid decline of HCV replication (as measured by a decrease of HCV-RNA levels), which, if significant enough, causes very distinct CD4 and CD8 responses (shifting the immune response from innate to adaptive). Clinically, this phenomenon defines a group of patients known as rapid responders (HCV RNA serologic negativity achieved by week 4 of treatment) and differentiates them from patients with a less vigorous early reduction in viral load, known as slow responders.

Sustained virologic response (SVR) is achieved when no virus is detected in the blood for six months after finishing treatment and prognosticates a 99% chance of indefinitely eradicating HCV, which for many experts is equivalent to curing HCV. Several events need to work in concert in order to achieve SVR, including (1) successfully achieve a rapid phase 1 effect by turning off viral replication, (2) effectively suppress the viral load throughout treatment, and (3) induce a solid and persistent Phase 2 effect [42].

It has been hypothesized that phase 2 is driven by the host's adaptive immune response in the context of sustained inhibition of virus production, while restoration of the innate immune response by viral inhibition leads to clearance of residual HCV-infected cells [43].

Both IFN-$\alpha$ and direct antiviral agents (DAAs) have very potent antiviral abilities and induce a very strong phase 1 response. Ribavirin is needed to prevent viral breakthrough during treatment and relapses after treatment, in patients who respond to the antiviral effect of IFN-$\alpha$ [33], and it appears to enhance the clearance of any residual infected cells, through unknown mechanisms, in patients treated with IFN-$\alpha$ combined with ribavirin or when the two are combined with DAAs [44]. Therefore, in addition to achieving a vigorous Phase 1 effect via the administration of IFN-$\alpha$, use of ribavirin and DAAs may produce a similar vigorous Phase 1 as well as a gradual yet vigorous second phase, which combined with long-term treatment could ensure cure. Without a sufficient duration of treatment to promote the Phase 2 effect until all infected cells have been cleared, HCV replication will resume shortly after treatment completion and patients will experience a relapse.

## 5. HCV Resistance to Specific Viral Inhibitors (Direct Antiviral Agents [DAAs])

HCV is prone to the development of resistance to specific antiviral inhibitors due to the quasispecies nature of the

virus, its rapid dynamic, and the double error-prone RdRp [15], all leading to the development and/or persistence of drug-resistant mutants. It has been proposed that all single nucleotide-mutant drug-resistant viruses and all combinations of double-nucleotide mutant viruses preexist before treatment in most patients [45].

Based on experience with HIV and combined antiretroviral therapy (cART), it appears that the long-standing administration of specific viral inhibitors leads to formation of viral variants carrying amino acid substitutions that alter the original drug target, therefore, diminishing the therapeutic effects of the given drug. Similar substitutions present in a preexistent viral population may also allow those viruses to continue to replicate at a significant level during drug treatment. This phenomena may allow further mutations to accumulate, thus imparting a *partial resistance pattern*. Additionally, *compensatory mutations* may restore the viral capability to replicate at near the pretreatment stage [46]. *Resistance* is associated with a pattern where amino acid substitution confers a high level of drug resistance without diminishing the viral fitness in the presence of the drug. Cross-resistance between medications targeting the same site/function occurs both *in vitro* and *in vivo* and appears to be mediated by amino acid substitutions that confer reduced susceptibility to both drugs. The ability of viruses to replicate irrespective of the presence or absence of a drug has huge therapeutic implications, as it will lead to further evolution of the viral population. In theory, further evolution can result in a more fit, drug-resistant viral population that may remain in a patient, even in the absence of drug pressure. This further evolution should be preventable by quickly discontinuing the antiviral drug when a patient has a confirmed increase in HCV RNA levels while adhering to therapy, implying development of resistance. It is extremely important that clinicians adhere to the "futility rules," which are defined below, to limit the development of viral resistance.

Resistance can be measured *in vitro* and *in vivo. In vitro,* resistance is measured on cell-free enzyme assays as the drug concentration needed to inhibit viral replication. Effective concentration (EC) defines the drug concentration required to inhibit the viral replication by 50% or 90% (E50 or E90). Less susceptible (hence more resistant) viruses will require more drug to be inhibited, thus are associated with an increase in E50 or E90. There is no consensus on what level of increase is needed to conclude that a given amino acid substitution confers resistance. In addition, the results *in vitro* must be cautiously interpreted in the clinical context because some viral variants with low-level resistance in vitro may be more aggressive in vivo than variants with higher-level resistance [47]. *In vivo,* resistance is influenced by genetic factors, the fitness of the virus, and drug exposure and is measured by viral load while on therapy. The best determinant of replication *in vivo* appears to be the fitness of the resistant variant [48] as a poorly fit virus is less clinically significant despite its being highly resistant, unlike a less resistant but fitter virus. Viral fitness implies the relative capacity of a viral variant to replicate in a given environment. Resistance mutations frequently compromise viral function and thus reduce viral fitness compared to wild-type virus in a drug-free environment.

HCV has a relatively low genetic barrier to development of resistance to NS3/4A protease inhibitors. The genetic barrier refers to the number and type of nucleotide changes required for a virus to acquire clinical resistance to an antiviral agent [45]. Low genetic barrier to resistance implies that a single substitution can confer high resistance, while high genetic barrier to resistance implies that at least 3 substitutions are required to confer resistance. The protease inhibitors are able to select resistant variants *in vitro* based upon presence or development of amino acid substitutions. These substitutions appear to be located near the NS3 protease catalytic triad. These mutant variants render protease inhibitors unable to prevent viral polyprotein processing and, therefore, allow continued generation of mature viral proteins even in the presence of this class of drug [49–57].

Boceprevir and telaprevir are two recently developed NS3/4A protease inhibitor drugs.

Boceprevir- and telaprevir-based triple therapies are approved for the treatment of chronic hepatitis C, in combination with interferon-alpha and ribavirin, in the United States and Europe, where they are marketed under the brand names Victrelis and Incivek (Incivo in Europe), respectively. These drugs have opened up a new era in HCV therapy. They also illustrate quite well the significance of resistance. They share significant cross-resistance *in vitro,* with any given substitution producing different levels of resistance to the two drugs [52]. Most available data is derived from studies performed on patients treated with telaprevir (a reversible, selective, orally bioavailable inhibitor of the HCV NS3/4A serine protease) used in combination with IFN and ribavirin. Based on the level of drug present in serum, telaprevir is able to select resistant viral populations within days or weeks. The most resistant but least fit variants (with substitution at the 156 position) are selected early during therapy, while later they are quickly replaced by fitter variants carrying substitutions at various positions including 155, 36 and 155, 36, and 156 at the time of breakthrough [58, 59].

*5.1. Triple Therapy with Telaprevir.* At baseline, the main variants conferring resistance to telaprevir in patients with HCV genotype 1a (R155K, V36M) were detectable in 0.6–1.2% by population-based sequencing. In genotype 1b, variants conferring resistance to telaprevir (T54A, V36A, and A156T) were either undetectable, or detectable in a very small percentage of patients (0.07% for A156S, V36M and 2.1% for T54S) [60].

On-treatment virologic failure rates, defined as futility rules having been met ("futility rules" are defined below in the paper), were found to differ for HCV subtype 1a versus 1b and were also dependent on the prior treatment status of the patients. In treatment-naive patients, virologic failure was observed in 10% of patients with subtype 1a HCV and 3% of patients with subtype 1b HCV. Among previous treatment nonresponders, the on-treatment virologic failure rate was 38% and was again found to be higher in patients with subtype 1a than with subtype 1b. Finally, the on-treatment

Table 1: Mutations conferring resistance to telaprevir.

| HCV resistant variant | Patients without resistant variants at followup |
|---|---|
| T54A | 94% |
| A156S | 88% |
| V55A | 86% |
| V36M | 75% |
| R155K | 68% |
| T54S | 68% |

virologic failure rate for prior treatment relapsers was low at 10% [61, 62].

Population and clonal amino acid analysis of HCV species from patients who develop protease inhibitor resistance indicate that drug-resistant viral populations disappear in time, as illustrated by the follow-up studies for telaprevir. For genotype 1a HCV, the median time to lose resistant variants V36M and R155K was approximately 6 and 10 months, respectively, whereas loss of both mutations required approximately 13 months. For patients with subtype 1b HCV, the median half-life of resistant variants was shorter at 3 months for the common variants T54A, V36A, and A156T and 9 months for the more rarely occurring A156S variant [63]. Additional long-term followup, with a median duration of 29 months (range, 7 to 49 months) after treatment failure, showed that resistant variants were no longer detected in 85% of patients, and the rate of disappearance of resistant variants again depended on the specific mutation(s) (Table 1) [64].

*5.2. Triple Therapy with Boceprevir.* At baseline, the main observed variants conferring resistance to boceprevir in patients with HCV genotype 1a include V36M, T54S, R155K, which varies from the typical genotype 1b resistance profile, T54A, V55A, A156S, I/V170A, both of which occur with an overall low frequency [65]. As with telaprevir, viral drug resistance is more common at baseline and develops more frequently in subtype 1a than 1b, as subtype 1a HCV virus has a lower genetic barrier to resistance. Thus, for the most prevalent resistance variant, R155K, only 1 nucleotide exchange is required for subtype 1a HCV to develop drug resistance, whereas 2 exchanges are needed to generate the same resistance for subtype 1b HCV. Conversely, phenotypic resistance analysis in the HCV replicon [50, 52, 53, 66] showed that resistance-associated variants found in patients with subtype 1b HCV awarded a greater degree of resistance, with a 5- to 16-fold loss of susceptibility to boceprevir, than that found in patients with subtype 1a, with only 2- to 4-fold loss of susceptibility.

Current data confirm that the overall sustained virologic response (SVR) rate was lower in patients infected with HCV subtype 1a (53% to 64%) than in those infected with HCV subtype 1b (66% to 73%), again likely due to lower genetic barrier to resistance for subtype 1a. The likelihood of achieving SVR for genotype 1 prior treatment nonresponders was around 60% with boceprevir (combined with pegylated interferon/ribavirin) with an on-treatment failure rate of 40%, which is comparable to teleprevir. For treatment-naive patients, SVR rates approached 70–75% with boceprevir combination therapy, with a virologic failure rate of 20%–25%. For relapsers, the SVR reaches 75%, thus conferring an on-treatment virologic failure rate of 25% [67–70].

Long-term follow-up data on resistance-associated variants selected in non-SVR patients while on treatment showed that only approximately 20% of patients still had resistant variants detectable by population-based sequencing at 6–14 month followup [71]. The disappearance rate for the different NS3 protease resistance mutations was variable and is shown in Table 1.

## 6. Treatment Failure in Triple Therapy and Clinical Implications

Resistance is not an "all or none phenomenon." Clinically significant resistance is usually associated with an "escape" pattern [72], where viral replication recovers quickly to pretreatment levels while amino acid substitution confers a high level of drug resistance without impairing fitness in the presence of the drug. If the virus is not very fit, the viral replication process will resume more gradually [46, 73–75]. Clinical resistance occurs if drugs levels are not sufficient to inhibit viral replication, and highly resistant viruses may need very high drug levels to inhibit their replication, which may not be achievable within acceptable safety parameters. In addition, sufficient drug trough levels must be maintained over time to achieve long-term viral suppression. Antiviral efficacy *in vivo* may remain stable if resistant variants replicate at low level and/or if the drug retains partial efficacy. Various patterns of HCV treatment failure have been reported, including viral nonresponse (persistent HCV RNA positivity on treatment), viral breakthrough on treatment, and viral relapse after treatment completion. In compliant patients, failure to respond to triple therapy derives mostly from lack of response to IFN-$\alpha$ and ribavirin, and consequent selection of preexistent viral species inherently resistant to DAAs. Conversely, treatment failure in noncompliant patients frequently results from de novo generation of viral mutants resistant to DAAs. This latter phenomenon is thought to account for most cases of viral breakthrough and relapse.

As noted above, the failure rates in treatment-naïve patients are 20%–30% on triple therapy. In previously treated patients, the failure rates range as high as 50%–60%. Development of persistence of viral resistance depends in part on several host and treatment-related variables [61, 62, 69, 70]. Failure rates are higher in populations with patients with less favorable genetic background (IL-28 phenotypes CT or TT), including the African-American population, prior treatment nonresponders, HIV- or HBV-coinfected patients, post-liver-transplant patients, noncompliant patients, and patients with advanced fibrosis/cirrhosis. Multiple studies, including SPRINT 1- and -2, RESPOND-2, PROVE, REALIZE, and ADVANCE [67, 68, 76–79] have shown that the final treatment outcome with inclusion of

Table 2: Role of IL-28B in HCV treatment.

| IL-28B SNP | PR (IDEAL) | | Telaprevir (advance) | | Boceprevir (SPRINT-2) | |
|---|---|---|---|---|---|---|
| | ITT population | Adherent population | TVR/PR | PR control | BOC/PR* | PR control |
| CC | 69% | 79% | 90% | 64% | 80–82% | 78% |
| CT | 33% | 38% | 71% | 25% | 65–71% | 28% |
| TT | 27% | 26% | 73% | 23% | 55–59% | 27% |

* Includes BOC/RGT and BOC/PR48 arms [61, 69, 80, 81].

DAAs was very much dependent on the degree of viral responsiveness seen during the first course of therapy with IFN-$\alpha$ and Ribavirin, a variable that likely reflects genetic host factors such as IL-28B genotype.

Certain single nucleotide polymorphisms (SNPs) upstream of the IL-28B locus correlate with the rate of SVR in patients treated with PEG-IFN-$\alpha$ and ribavirin, and recent data reveals a similar, though much less discrepant correlation in patients treated with triple therapy (Table 2).

Given the concerning data regarding development and persistence of drug-resistant mutants and on-treatment clinical resistance (data reviewed earlier in this paper), strategies should be employed to predict triple therapy failure early by monitoring viral kinetics. On-treatment "futility rules" exist and should be followed strictly. These rules define conditions that, if met, mandate immediate treatment discontinuation and are based upon viral kinetics. Failure to follow these rules could contribute to the development and persistence of more fit drug-resistant viruses, the presence of which, in turn, could jeopardize the patient's future chances to respond to newer antiviral treatments. Telaprevir is taken, with PEG-IFN-$\alpha$ and ribavirin, at a dose of 750 mg every eight hours for the first 12 weeks of the treatment. If HCV RNA is equal to or greater than 1000 IU/mL at weeks 4 or 12, virologic failure has occurred and triple therapy should be discontinued immediately. If the virus is detected to any degree at week 24, dual therapy should also be discontinued at that point, as the likelihood of achieving SVR is very low. When using boceprevir, the treatment algorithm is different and involves a 4-week period of PEG-INF-$\alpha$ and ribavirin dual therapy, also known as the "lead in" period, followed by triple therapy with addition of 800 mg of boceprevir every eight hours for a variable period of time; PEG-IFN-$\alpha$ and ribavirin will continue until treatment completion. A viral load equal to or greater than 100 IU/mL at week 12 is equivalent to virologic failure, and all three drugs should be stopped to avoid resistance. Alternatively, any detectable viral load at week 24 again implies again lack of response, and treatment should be terminated.

Failing triple therapy raises concerns regarding possible adverse consequences for the liver disease itself and also for the potential to respond to future DAA-based therapy. The rate of HCV replication ultimately returns to the pre-treatment stage following treatment failure. Consequently, the disease appears to resume its progression to cirrhosis at the same pretreatment rate. As HCV is not a cytopathic virus, most of the hepatocellular damage is caused by the host immune system targeting the persistently infected hepatocytes. In terms of impact for future therapy, one can hypothesize that if protease inhibitor-resistant viruses, which have acquired greater fitness during therapy with DAA's, remain the dominant viral population within a host then, at the time of retreatment, the use of medications with cross-resistance with telaprevir and boceprevir would not be expected to work. Patients failing triple therapy should be advised that many new HCV drugs are under development and may become available outside study protocols within several years, but it is yet to be defined whether these patients will be appropriate candidates for retreatment with these drugs. These patients must continue to be clinically monitored for signs of disease progression.

## 7. Conclusions

A new standard of care for treating genotype 1 hepatitis C infected patients is now available, for both treatment-naïve patients and treatment-experienced patients. This treatment remains PEG-IFN-$\alpha$ and ribavirin-based but adds either telaprevir or boceprevir, protease inhibitors that became the first DAA drugs specifically targeting the HCV NS3/4A protease, and that were approved by the FDA in May, 2011. This new "triple therapy" has allowed patients to achieve dramatically greater rates of viral cure than previously, but treatment failure remains a possibility. Failure to achieve a viral cure with this regimen most likely results from the low viral genetic barrier to resistance to the protease inhibitors, allowing *de novo* formation of drug-resistant viral mutants, superimposed on a weak host response to pegylated IFN-$\alpha$ allowing persistence of drug-resistant viruses that were present at baseline. Treatment failure would allow continued progression of the liver disease and may affect candidacy for and/or responsiveness to the next line of DAAs in development. The future may belong to agents such as nucleoside/nucleotide analogues, nonnucleoside RNA-dependent RNA polymerase inhibitors, or cyclophilin inhibitors, and the treatment of hepatitis C may ultimately parallel that of HIV in the near future, with use of various combinations of "drug cocktails."

The need for a vigorous host response to IFN in order to achieve a viral cure with current therapies, and patients' failure to achieve such a response, or intolerant of IFN-related side effects, has led many investigators to develop drugs that could provide an IFN-free curative regimen. IFN-free regimens are no longer a dream, but a reality that may be available in the clinic in the next 5 years. It is possible that some of these regimens will also be RBV-free. PSI-7977, now GS-7977, a potent uridine nucleotide analog in Phase 3 development, demonstrated >90% SVR rates

in the PROTON trial, where it was used in combination with PEG/RBV to treat patients infected with HCV GT1, and 100% SVR rates were achieved in the ELECTRON trial when used to treat patients infected with HCV GT2/3 with an interferon-free regimen using only PSI-7977/RBV. These high SVRs are attributable to PSI-7977's antiviral potency, a lack of detectable preexisting resistant HCV variants, and excellent safety and tolerability profile [82]. Another 12-week, interferon-free combination of two experimental, once-daily drugs (ABT-450, a hepatitis C protease inhibitor that requires blood-level boosting with the HIV protease inhibitor Norvir, and ABT-072, an HCV nonnucleoside polymerase inhibitor) plus ribavirin achieved SVR at 12 weeks in 91% of noncirrhotic first-time treatment takers with HCV genotype 1 and an IL-28B CC genotype, according to data from the PILOT trial, presented on April 19, at the 47th Annual Meeting of the European Association for the Study of the Liver (EASL) in Barcelona [83]. Researchers at EASL also presented data from the COPILOT trial, an interferon-free clinical trial combining ribavirin, Norvir-boosted ABT-450 and ABT-333 (a twice-daily HCV nonnucleoside polymerase inhibitor) in first time-treatment takers and null responders with HCV genotype 1. ABT-450 and ABT-333 maintained hepatitis C virus levels undetectable for 12 weeks after completing treatment in over 90% of first-time treatment takers and 47% of treatment experienced people [84]. Having more, and more effective, treatments should not, however, diminish our efforts to develop a hepatitis C vaccine, as the cost of treatment continues to increase, the currently available agents continue to carry the potential for severe side effects, and cure rates still fall short of the ideal goal of 100%.

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*Research Article*

# *In Silico* and *In Vitro* Comparison of HIV-1 Subtypes B and CRF02_AG Integrases Susceptibility to Integrase Strand Transfer Inhibitors

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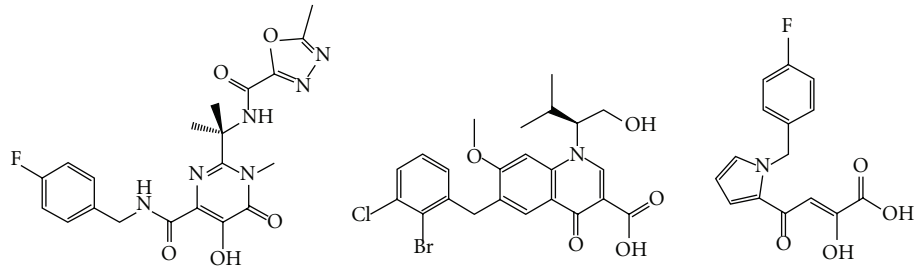
Most antiretroviral medical treatments were developed and tested principally on HIV-1 B nonrecombinant strain, which represents less than 10% of the worldwide HIV-1-infected population. HIV-1 circulating recombinant form CRF02_AG is prevalent in West Africa and is becoming more frequent in other countries. Previous studies suggested that the HIV-1 polymorphisms might be associated to variable susceptibility to antiretrovirals. This study is pointed to compare the susceptibility to integrase (IN) inhibitors of HIV-1 subtype CRF02_AG IN respectively to HIV-1 B. Structural models of B and CRF02_AG HIV-1 INs as unbound enzymes and in complex with the DNA substrate were built by homology modeling. IN inhibitors—raltegravir (RAL), elvitegravir (ELV) and L731,988—were docked onto the models, and their binding affinity for both HIV-1 B and CRF02_AG INs was compared. CRF02_AG INs were cloned and expressed from plasma of integrase strand transfer inhibitor (INSTI)-naïve infected patients. Our *in silico* and *in vitro* studies showed that the sequence variations between the INs of CRF02_AG and B strains did not lead to any notable difference in the structural features of the enzyme and did not impact the susceptibility to the IN inhibitors. The binding modes and affinities of INSTI inhibitors to B and CRF02_AG INs were found to be similar. Although previous studies suggested that several naturally occurring variations of CRF02_AG IN might alter either IN/vDNA interactions or INSTIs binding, our study demonstrate that these variations do affect neither IN activity nor its susceptibility to INSTIs.

## 1. Introduction

The *pol*-encoded HIV-1 integrase (IN) is a key enzyme in the replication mechanism of retroviruses. It catalyses the covalent insertion of the viral cDNA into the chromosomes of the infected cells [1]. Two reactions are required for covalent integration of viral DNA. First, IN binds to a short sequence located at either end of the long terminal repeat (LTR) of the vDNA and catalyzes an endonucleotide cleavage, 3′-processing reaction, resulting in the removal of two nucleotides from each of the 3′-ends of LTR and the delivery of hydroxy groups for nucleophilic attacks. The trimmed DNA is then used as a substrate for strand transfer (ST) reaction, leading to the covalent insertion of the DNA into the host genome [1]. Inhibitors of the strand transfer reaction—INSTIs—constitute a novel family of antiretroviral (ARV) drugs, with raltegravir (RAL) at the cape, which is a first INSTI approved for AIDS treatment. Other inhibitors in advanced phase of development are elvitegravir (ELV) and GSK572.

Human immunodeficiency virus type one (HIV-1) exhibits an exceptional level of genetic variability, which may influence the viral properties such as infectivity, transmissibility, or response to antiviral treatment [2]. The most prevalent HIV-1 group M genetic forms are subtypes A, B, C and circulating recombinant form CRF02_AG.

Analysis of the global distribution of HIV-1 subtypes and recombinants in the two followed three-year periods,



RAL ELV L731,988

Scheme 1

2000–2003 and 2004–2007, indicated a broadly stable distribution of HIV-1 subtypes worldwide with a notable increase in the proportion of circulating recombinant forms (CRFs), a decrease in unique recombinant forms (URFs), and an overall increase in recombinants [3]. Particularly, in 2004–2007, CRF02_AG accounted for 8% of all global infections, following subtypes C (48%), A (12%), and B (11%). CRF02_AG is the predominant HIV strain circulating in West and West Central Africa [4–6]. Recently the recombinant CRF02_AG form was identified in the Amazon region of Brazil [7] and in China [8].

In France the frequency of antiretroviral-naive chronically HIV-infected patients infected with a non-B subtype reached 42% in 2006/2007, having increased significantly since 1998 (10%) and 2001 (33%). This evolution in subtype distribution was mainly due to a higher proportion of patients originating from sub-Saharan countries. Among these non-B subtypes, the most prevalent was CRF02_AG with a stable proportion around 20% between 2001 and 2006/2007 [9].

Enzymatic and virological data support the concept that naturally occurring polymorphisms in different non-B subtypes can affect the susceptibility of HIV-1 to different antiretroviral drugs, the magnitude of resistance conferred by major mutations, and the propensity to acquire some resistance mutations [10]. The genetic variation between viral isolates retroviral enzymes is estimated up to 25–35%; particularly the *pol* gene exhibits high variation, about 10–15% for reverse transcriptase (RT) and 8–12% for integrase (IN) [11]. Integrase inhibitors are active *in vivo* against B and non-B subtypes. Furthermore, *in vitro* studies suggested that subtype C integrase is equally susceptible to INSTIs [12]. Similarly, the analysis of *pol* gene in infected patients showed that highly prevalent polymorphisms have little effect on INSTIs susceptibility [13]. Nevertheless, the comparison of IN sequences of B and CRF02_AG strains showed that CRF02_AG sequence differs from the B sequence by 13 residues (K/R14, V/I31, L/I101, T/V112, T/A124, T/A125, G/N134, I/V135, K/T136, V/I201, T/S206, L/I234 and S/G283) [14]. Based on a model of the B HIV-1 integrase/DNA complex [15], it was suggested that several of these variations K/R14, T/V112, T/A125, G/N134, K/T136, and T/S206 may impact IN interaction with DNA or IN susceptibility to INSTIs. Later we compared the genetic barriers between B and CRF02_AG strains; we found that the variability between subtypes impacted the genetic barrier for G140C/S and V151I with a higher genetic barrier being calculated for subtype CRF02_AG suggesting a great difficulty in selecting these mutations for CR02_AG compared to subtype B [16].

Integrase is a 288 amino acids enzyme, which consists in three structurally distinct functional domains [17]. Structures reporting HIV-1 IN single- or two-domain data allow the generation of biologically relevant models, representing either unbound dimeric enzyme or IN complexes with viral (vDNA) and/or host DNA (hDNA) [18]. The X-ray structures of full-length prototype foamy virus (PFV) IN complex with its cognate DNA and integrase strand transfer inhibitors (INSTIs; RAL, ELV, and others first- and second-generation INSTIs) were recently solved [19, 20]. The reported structures were used for homology modeling of the unbound IN and IN bound to vDNA from CRF02_AG and B strains. Further, the constructed models were used to estimate the susceptibility of both INs to strand transfer inhibitors, RAL, ELV and L731,988 (Scheme 1). Results from molecular modeling were compared to experimental data obtained with B and CRF02_AG INs which were isolated from plasma samples of HIV-1-infected patients and then cloned and expressed *in vitro*.

## 2. Results and Discussion

*2.1. Analysis of CRF02_AG IN Sequences from INSTI-Naive Infected Patients.* The complete sequence of the IN coding region of the *pol* gene was amplified and cloned from the plasma samples of CRF02_AG HIV-1-infected patients. Four IN sequences, $N^1$ to $N^4$, harbored several variations among the thirteen residues that were shown to be subjected to polymorphic substitutions between CRF02_AG and B HIV-1 sequences (K/R14, V/I31, L/I101, T/V112, T/A124, T/A125, G/N134, I/V135, K/T136, V/I201, T/S206, L/I234 and S/G283; Table 1) [14]. Sequence $N_1$ (CRF02_AG 33CR) displayed the seven variations K14R, T112V, T125A, G134N, K136T, T206S, S283G; $N_2$CRF02_AG 49CR the five variations T112V, T125A, G134N, K136T, S283G; $N_3$ (CRF02_AG 68CR) the five variations K14R, T112V, T125A, K136T, T206S; $N_4$ (CRF02_AG 52CR Q148K) the two variations T125A and T206S, along with the INSTI resistance mutation Q148K. Although Q148K is involved in INSTIs resistance, the patient from whom the IN coding DNA was derived was

Table 1: Amino acid variations at the positions putatively affecting the susceptibility to INSTI in 4 isolated HIV-1 subtype CRF02_AG IN coding sequences.

| Position | B consensus | Subtype CRF02_AG | | | |
|---|---|---|---|---|---|
| | | $N_1$ (33CR) | $N_2$ (49CR) | $N_4$ (52CR Q148K) | $N_3$ (68CR) |
| 14 | K | R | K | K | R |
| 112 | T | V | V | R | V |
| 125 | T | A | A | A | A |
| 134 | G | N | N | G | G |
| 136 | K | T | T | K | T |
| 206 | T | S | T | S | S |
| 283 | S | G | G | S | S |

Compared with HIV-1 subtype B IN, seven variations present at positions 14, 112, 125, 134, 136, 206, and 283 of CRF02_AG 33CR IN; five variations at positions 112, 125, 134, 136, and 283 of CRF02_AG 49CR IN; five variations at positions 14, 112, 125, 136, and 206 of CRF02_AG 68CR IN; CRF02_AG 52CR Q148K has two variations at positions 125 and 206, and an INSTI-resistant mutation Q148K, the R112 was not considered.

not exposed to the INSTI-containing treatment. Thereby we presume that Q148K may be a naturally occurring amino acid substitution.

*2.2. Structural Comparison of HIV-1 B and CRF02_AG Integrases.* In order to determine the potential impact of the natural variations on the protein activity and susceptibility to INSTIs, we built models of the IN structures corresponding to the consensus B sequence and the CRF02_AG variant differing from B subtype by twelve residues. The 18-aas C-terminal end containing the S283G was omitted since the structure of this domain was not resolved by X-ray analysis and the folding of this part of protein is extremely difficult to predict in the apo state, due to its essential length and its highly solvent-exposed position.

Comparative structural analysis were performed considering 6 IN models generated by homology modeling (Figure 1). Models 1(B) and 2 (CRF02_AG) (Figure 1(a)) represent the unbound homodimer of integrase ($IN^{1-270}$), which depicts the conformational state of the enzyme just before the 3′-processing of vDNA (apo state); models 3′(B) and 4 (CRF02_AG) (Figure 1(b)) represent the IN dimer in complex with vDNA (holo state), which depicts the active unit of the IN·vDNA strand transfer intasome; models 5 (B) and 6 (CRF02_AG) (not shown) were derived from models 3 and 4 by removing vDNA.

Models 1 and 2 were constructed from the crystallographic structures of HIV-1 IN-isolated domains or pairs of domains. Overall, the analysis of the models representing the HIV-1 IN conformational state before 3′-processing (apo state) did not show any significant structural change between the two subtypes (Figures 1(a) and 1(c)).

Models 3 and 4 were constructed from the crystallographic structure of the IN·vDNA complex of the PFV intasome [19, 20]. Although the sequence identity between HIV-1 and PFV INs is low (22%), the structure-based alignment of the two proteins demonstrates high conservation of key secondary structural elements and the three PFV IN domains shared with HIV-1 IN have essentially the same structure as the isolated HIV-1 domains. Moreover, the structure of the PFV intasome displays a distance between the reactive 3′ ends of vDNA that corresponds to the expected distance between the integration sites of HIV-1 IN target DNA (4 base pairs). Consequently, we are confident that the PFV IN X-ray structure represents a good template for the HIV-1 IN model generation [21]. To obtain a robust alignment, we adjusted the targets (HIV-1 INs from B and CRF02_AG subtypes) and template (PFV IN) sequences manually, considering each structural domain separately, in order to take into account the conservation of the secondary structure (see Section 4).

Again, models 3 and 4, representing the IN·vDNA intasomes of both strains, superimposed perfectly and no structural dissimilarity was observed (Figures 1(b) and 1(d)). Most of the variations are located far from the active sites, and the nearest two mutated residues to the active site, at positions 134 and 136, are exposed to the solvent and apparently did not affect significantly the structure. Similarly for 3′-processing, strand transfer activities of B and CRF02_AG recombinant proteins were assayed and compared. In agreement with the modeling results, activities of both INs were comparable (Figure 2(c)).

It is worth noting that large structural and conformational changes are observed between the apo (models 1 and 2) and holo (3 and 4) states regarding the relative positions of the IN domains (RMSD, root mean square deviation, of 31 Å, based on $C_\alpha$) (Figure 1(e)). These structural modifications result in different contacts between IN domains, N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CDD). As such, in models 1 and 2 (apo state) no interaction was detected between CTD and CCD, whereas the two domains interact tightly in models 3 and 4 (holo state). The NTD-CCD interface also exhibits substantial changes: in the apo form the NTD-CCD interface belongs to the same monomer subunit whereas in the holo form the interface is from two different subunits. Moreover, IN undergoes important structural transformation leading to structural reorganization of the catalytic site loop upon vDNA binding; the coiled portion of the loop reduces from 10 residues (140–149 aas) in the apo form to 5 residues (140–144 aas) in the holo form (Figure 1(f)). This partial folding of the catalytic loop is probably stabilized through intra-IN domain-domain interactions and interactions with vDNA which contribute in the helix $\alpha 4$ elongation.

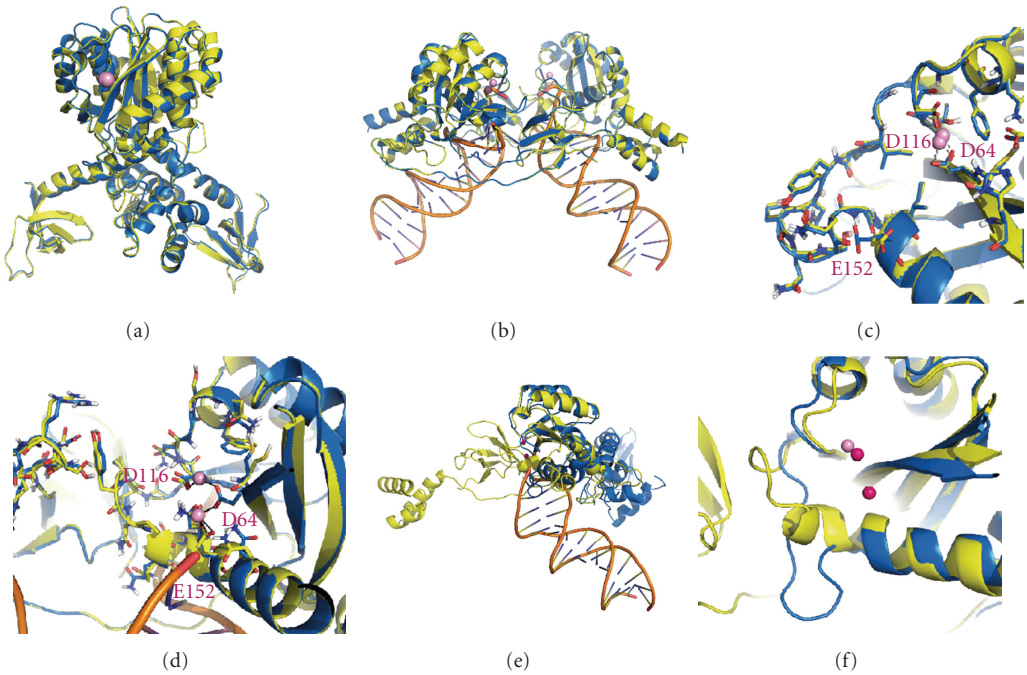



Figure 1: Structural models of the HIV-1 INs from B and CRF02_AG strains. (a) Superimposition of models 1 and 2, representing the enzyme before the 3′ processing from B (in blue) and CRF02_AG (in yellow) strains; (b) Superimposition of models 3 and 4, representing the IN·DNA pre-integration complex from B (in blue) and CRF02_AG (in yellow) strains; (c) and (d) Comparison of the catalytic site and loop 140–149 structure in models 1/3 (in blue) and 2/4 (in yellow) respectively. The proteins are shown as cartoons, $Mg^{+2}$ ions as spheres (in pink). (e and f) superimposition of the structural subunits from models 1 (in blue) and 3 (in yellow) and the structural details of the active site and loop 140–149.

*2.3. In Vitro Enzymatic Comparison of Recombinant HIV-1 B IN and CRF02_AG IN.* To confirm experimentally the absence of divergence between INs from both strains CRF02_AG and B, $N_1$ to $N_4$ sequences were expressed and purified (Figure 2(a)) and their enzymatic activities were compared to the one of HxB2 B IN. First, the DNA binding activities of recombinant INs were compared using a steady-state fluorescence anisotropy assay (Figure 2(b)) [22]. In this assay, the binding of IN to a fluorophore-labeled dsODN substrate mimicking one end of the viral DNA is monitored by the increase of the steady-state anisotropy value, resulting from the restriction of the substrate movements. As shown in Figure 2(b), no significant difference in DNA binding activity of recombinant subtype B IN and the CRF02_AG INs was observed within a range of IN concentrations of 100 to 250 nM, thereby indicating that the variations in IN sequence did not affect the binding affinity of the enzyme. Then, 3′- processing of HIV-1 B IN and CRF02_AG INs was compared *in vitro*. No significant difference of 3′-processing activity of recombinant HIV-1 B IN and CRF02_AG INs was found within a range of IN concentrations of 50 to 400 nM (Figure 2(c)). Impaired 3′-processing and strand transfer activity, but conserved DNA binding ability of CRF02_AG 52CR Q148K were observed, in agreement with previous study [23]. Finally we decided to analyze 3′-processing kinetics of recombinant HIV-1 B IN and CRF02_AG 33CR IN in the presence of increasing concentrations of IN 50 nM to 200 nM recombinant IN proteins with an increasing incubation time, using both *in vitro* 3′-processing activity assay and steady-state fluorescence anisotropy-based assay (Figure 3). Again, no difference could be detected. This result was further confirmed by steady-state fluorescence anisotropy assay (data not shown).

In agreement of the modeling result, *in vitro* study confirmed that the enzymatic activities of both INs were comparable.

*2.4. Docking of INSTIs.* Although B and CRF02_AG INs are structurally similar, residue variations may impact the interaction and subsequent activity of the inhibitors. To address this hypothesis, the three inhibitors RAL, ELV, and L731,988 (Scheme 1) were docked onto INs by using two different docking algorithms, Glide and AutoDock. RAL and ELV coordinates were taken from the crystallographic structures of PFV intasome cocomplexes [19, 20], L731,988 was built from scratch (see Section 4). The three compounds were considered in their deprotonated form, as it has been clearly established that diketo acids (DKAs) mainly exist in this form in solution [24]. The binding energies obtained by Glide and Autodock scoring functions are reported in Table 2.

The inhibitors were first docked onto the unbound IN, models 1 and 2 (apo state), with a single $Mg^{2+}$ ion within the catalytic site. All three inhibitors are positioned at the catalytic site far from the catalytic site flexible loop. For subtype B, values of binding energies obtained with Glide range in a relatively narrow interval from −8.49 to

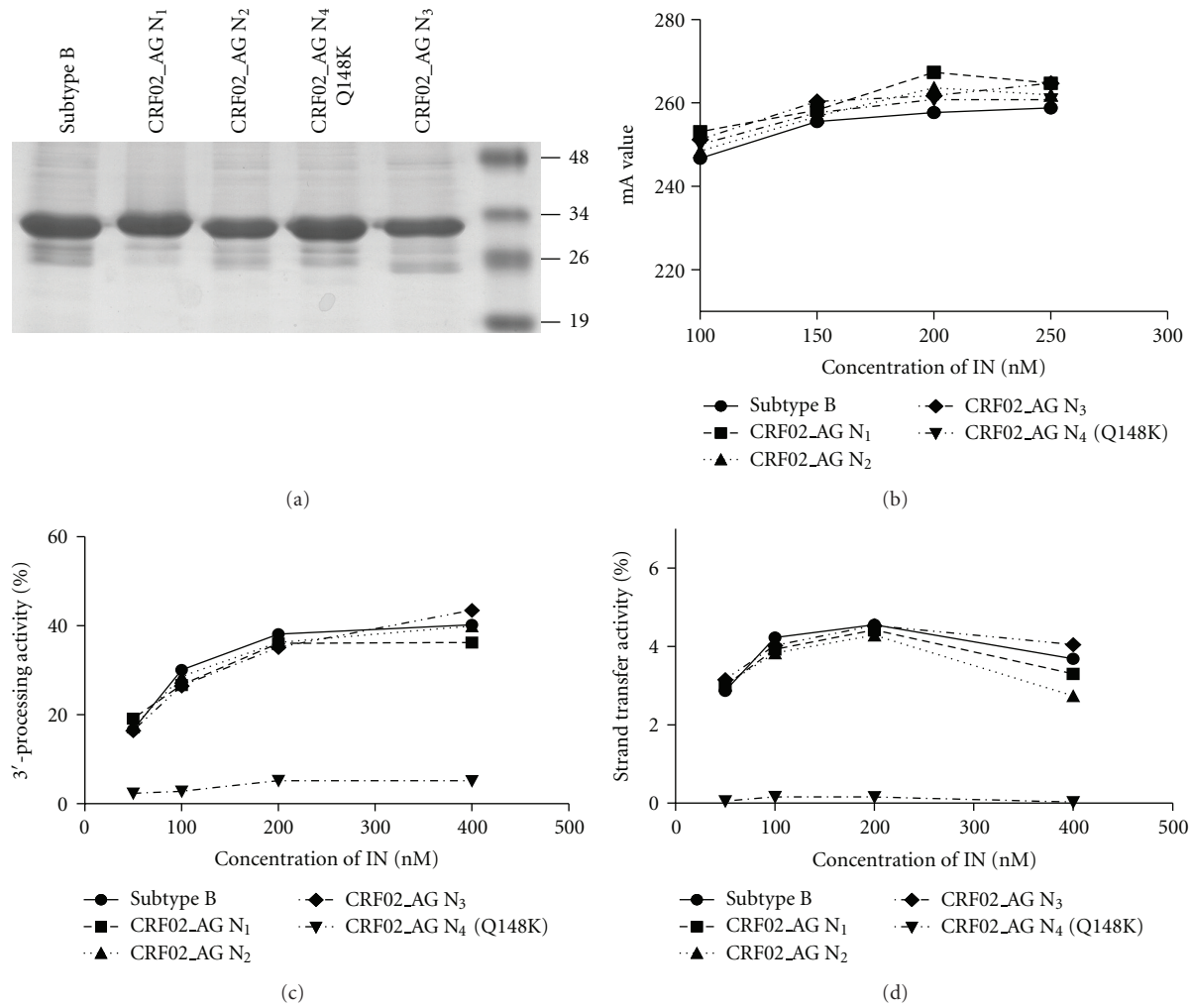



Figure 2: Purification of recombinant HIV-1 INs from B and CRF02_AG subtypes and comparison of their activities. (a) Purification products $N_1$, $N_2$, $N_3$ and $N_4$ of recombinant HIV-1 INs from B and CRF02_AG subtypes. (b)–(d) Comparison of DNA binding, 3′-processing and strand transfer activities, respectively, of the HIV-1 IN from B and CRF02_AG as a function of IN concentration.

−7.42 kcal/mol while those obtained with AutoDock range from −8.72 to −6.65 kcal/mol. Scores obtained for a given inhibitor display some variations from one strain to another and between the two docking programs. ELV best pose in model 1 (B subtype) predicted by Glide is very close to that in model 2 (CRF02_AG subtype). Small differences relate to an improved affinity of ELV to model 2 evidenced by a better score (−8.20 kcal/mol) and by the formation of an additional H-bond between the hydroxy group of ELV and E152 side chain (Figures 4(a) and 4(b)). RAL poses in models 1 and 2 differ strongly. In both cases RAL coordinates similarly the $Mg^{2+}$ cations by its ketoenolate functionality, but the inhibitor adopts opposite positions, more specifically in model 1 its fluorobenzyl ring is oriented towards Y143, while in 2 towards Q148. L731,988 poses are also different in models 1 and 2, characterized by distinct pyrrole ring positions, close to E152 in 1 and to Y143 in 2. Such presence of alternative poses is likely due to a large pocket formed by the accessible active site and the open conformation of the folded loop which allow a large number of conformations and orientations with equivalent binding affinity for the flexible RAL and L731,988 molecules. Consequently no significant difference can be assessed between the binding of the three studied inhibitors to the unbound IN from strains B and CRF02_AG.

Further the inhibitors were docked onto models 3 and 4 representing preintegration complexes, $IN{\cdot}2Mg^{2+}{\cdot}DNA$, from B and CRF02_AG subtypes, respectively. Docking resulted in a binding for the three inhibitors with significantly higher scores than those found for the apo IN. This finding agrees well with the previously published experimental data that showed a high affinity of L-731,988 only to the IN conformations adopted after assembly with the viral DNA [25]. Glide scores ranked in a range from −10.22 to −8.73 kcal/mol, while AutoDock scores range from −13.45 to −11.11 kcal/mol. Comparisons of the poses produced by the two docking software were found similar, and consequently we focus here on the analysis of Glide results.

The three compounds are positioned in the catalytic site and chelate the $Mg^{2+}$ cations in agreement with the mechanism of action of these molecules, which are strand transfer

Table 2: Docking binding energies of RAL, ELV and L731,988 on the HIV-1 IN from B and CRF02_AG strains predicted by Autodock and Glide. The targets are the IN model with one $Mg^{2+}$ cation in the active site (apo state, models 1 and 2) and IN·DNA model with two $Mg^{2+}$ cations (holo state, models 3 and 4).

| Target | | The free binding energies (kcal/mol) | |
|---|---|---|---|
| | Inhibitor | Autodock | Glide |
| IN B (apo) | RAL | −6.83 | −8.05 |
| | ELV | −8.22 | −7.42 |
| | L731,988 | −7.81 | −8.49 |
| IN CRF02_AG (apo) | RAL | −6.65 | −7.68 |
| | ELV | −8.72 | −8.20 |
| | L731,988 | −8.31 | −7.85 |
| IN·DNA_B (holo) | RAL | −11.43 | −10.22 |
| | ELV | −12.45 | −9.17 |
| | L731,988 | −11.50 | −8.73 |
| IN·DNA CRF02_AG (holo) | RAL | −11.11 | −9.98 |
| | ELV | −13.45 | −9.16 |
| | L731,988 | −11.93 | −8.82 |
| IN* B (holo) | RAL | −8.29 | −8.36 |
| | ELV | −11.62 | −8.92 |
| | L731,988 | −12.19 | −8.96 |
| IN* CRF02_AG (holo) | RAL | −7.98 | −8.46 |
| | ELV | −11.80 | −8.93 |
| | L731,988 | −11.58 | −8.82 |

inhibitors [26]. RAL binding mode is characterized by higher scores in both models 3 (B subtype) and 4 (CRF02_AG subtype), respectively, to the other two inhibitors. RAL predicted poses are identical in models 3 and 4 (Figures 4(a), 4(b), 4(c) and 4(d)). It binds bidentaetly both metal cofactors of the active site acting as a 1–5, and 1–4-type ligand, with the enolic oxygen atom as an oxo-bridge between two $Mg^{2+}$ cations. Additional stabilization of inhibitor RAL is achieved by $\pi$-staking of fluorobenzyl ring upon Cyt16 of DNA substrate. Similar to RAL, ELV coordinates the $Mg^{2+}$ cofactors bidentantly through the 1–5 type $\beta$-ketoenolate moiety and 1–3 geminal carboxylic oxygen atoms, with a carboxylic oxygen atom as an oxo-bridge at the bicationic cluster. A few differences of ELV binding in models 3 and 4 refer to slightly different conformation of the chlorofluorobenzyl moiety. L731,988 molecule shows different binding poses in models 3 and 4. In model 3 (B subtype) L731,988 coordinates bidentately one $Mg^{2+}$ cation by the oxygen atoms from keto functionality of ketoenolate and carboxylate groups, acting as a ligand of 1-6 type. The second $Mg^{2+}$ cation is coordinated only by the carboxylate oxygen atom. In model 4 (CRF02_AG) L731,988 inhibitor shows exclusively one coordination to the one $Mg^{2+}$ cation (Figures 4(e) and 4(f)).

The predicted binding poses of RAL correlate well with those observed in the X-ray structure of the PFV intasome complex [19, 20]. Undoubtedly, the presence of the second catalytic $Mg^{2+}$ cation, the partial loop folding, and the DNA substrate bearing are presumably the driving determinants for the tight binding of ST inhibitors in the catalytic site. It was perfectly evidenced by Cherepanov that a series of INSTIs fixed similarly to the PFV intasome [19]. Apparently the crystallographic data or static models derived from these data are not suitable means to explain the specificity of inhibitor recognition by a target. Consequently, considering the similar scoring values for a given inhibitor and closed poses, no significant dissimilarity can be assessed between the binding of studied inhibitors to the IN·$2Mg^{2+}$·DNA complex from strains B and CRF02_AG.

To validate the *in silico* predictions regarding the susceptibility of subtypes B and CRF02_AG INs, the efficiency of INSTIs (RAL, ELV, and L731,988) on recombinant INs proteins was determined by *in vitro* strand transfer assay in the presence of increasing concentration of INSTI (see Section 4). As to all of the three studied INSTIs, no significant difference in $IC_{50}$ values against recombinant HIV-1 INs from B and CRF02_AG strains was observed (Table 3). $IC_{50}$ of RAL, ELV, and L731,988 against HIV-1 INs from B and CRF02_AG strains are 41.8, 93.4, 855 nM and 13.7–25.9, 48.9–66.8, 193–291 nM, respectively. The experimental ranking of the three compounds was predicted correctly by Glide scoring function.

The docking calculations evidenced that (i) the IN·DNA complex represents the best target for the studied inhibitors and (ii) the co-complexed vDNA partially shapes the inhibitors binding site. To further explore the role of vDNA, substrate was removed from the IN·vDNA complex and inhibitors were docked again on unbound IN with a fold corresponding to the holo state, models 5 and 6. The binding energies of RAL are depreciated upon vDNA removal in B and CR02_AG subtypes while ELV and L731,988 binding scores are less affected.

Docking scores are nearly similar between the two strains while poses display some variations, as already observed on the apo form. Surprisingly, the AutoDock results show

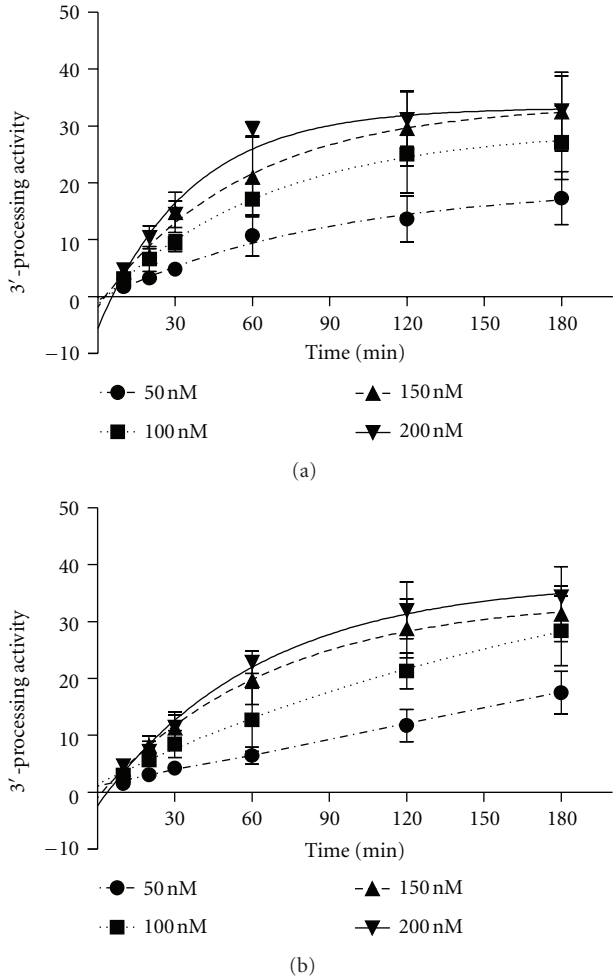



Figure 3: Kinetic comparison of HIV-1 B and CRF02_AG 33CR IN. (A) The kinetic features of recombinant HIV-1 B IN and (B) CRF02_AG IN ($N_1$) were determined *in vitro* using 3′-processing activity assay, in the presence of 50, 100, 150, and 200 nM recombinant IN proteins with an incubation time of 10, 20, 30, 60, 90, 120, and 180 min, respectively.

the lower score for RAL binding to both models 5 and 6, while the binding of the two other inhibitors are characterized by better scores, closer to those obtained with models 3 and 4. In contrast the scores produced by Glide are identical between the inhibitors and the subtypes. Chelation of the $Mg^{2+}$ ions by the inhibitors is still maintained but the interaction patterns differ from those predicted in models 3 and 4. Indeed, in model 5 (B subtype) RAL chelates the first $Mg^{2+}$ cation through the nitrogen atom of the oxadiazole ring, and the oxygen atom of the carboxamide moiety; the second $Mg^{2+}$ is coordinated by 1–4 oxygen atoms of pyrimidinone fragment. In model 6 RAL mode of coordination resembles that observed in model 4; however, stabilizing $\pi$-stacking interactions were vanished. Again, the large volume of the binding pocket and the lack of stabilizing protein-ligand and DNA–ligand interactions can explain such variety. Consequently, unbound IN in the holo conformation, as unbound IN in the apo conformation, does not appear as a suitable target for the inhibitors RAL and

Table 3: $IC_{50}$ of 3 INSTIs against recombinant HIV-1 B IN and CRF02_AG IN.

| | $IC_{50}$ (M) | | |
|---|---|---|---|
| | RAL | ELV | L731,988 |
| Subtype B | $4.185e-008$ | $9.340e-008$ | $8.554e-007$ |
| CRF02_AG $N_1$ | $1.373e-008$ | $5.562e-008$ | $2.115e-007$ |

ELV. L731,988 appears as a weaker binder, as confirmed by the experimental $IC_{50}$ values.

Molecular modeling approaches were used to investigate the effect of the natural variations showed by CRF02_AG strain on the *in vitro* activities of the enzyme and its susceptibility to INSTIs as compared to the ones of the consensus B integrase. We found that the structural models of unbound (apo state) and viral DNA-bound (holo state) integrase showed very similar folding and tertiary structure for the two studied strains. The structural models of the IN·vDNA complex superimposed perfectly. This similarity was confirmed by comparable strand transfer activity for IN variants in 14, 112, 125, 134, 136, 206, and 283 positions. Consequently, the naturally occurring variations in the HIV-1 IN subtype CRF02_AG – K14R, V31I, L101I, T112V, T124A, T125A, G134N, I135V, K136T, V201I, T206S, V234I, and S283G, which were suggested to modify IN structure, do not affect significantly *in vitro* DNA binding activity, either 3′-processing or strand transfer reaction. Furthermore, docking results revealed that the modes of binding and docking conformations of three studied inhibitors are comparable for B and CRF02_AG strains and these INSTIs possessed similar IN inhibitory activity against B and CRF02_AG HIV-1 strains. Altogether these results demonstrate the absence of difference in susceptibility and confirm previously reported observations for subtype B and C HIV-1 INs [12]. Thus, in contrast to the lower baseline susceptibilities of recombinant A/G subtype virus to protease inhibitors (PIs) and reduced susceptibility of some A/G isolates to abacavir, INSTIs potentially provide an excellent therapeutic options for the treatment of HIV-1 subtype CRF02_AG-infected patients [10].

In the targets all three molecules are positioned similarly with keto-enol moiety in an orientation encouraging coordination of the two metal cofactors in the active site. Furthermore, independently of the method, the three INSTIs displayed a more favorable binding onto the IN·vDNA complex (holo state) than on the unbound enzyme (apo state), in good agreement with their mechanism of action [26]. Same difference in theoretically predicted modes of RAL binding was reported early by Loizidou [27]. The observed conformational and structural transformation of IN upon DNA binding led to an important change in the folding and conformation of the catalytic site loop which in turn favors a formation of the binding pocket accommodating the INSTIs. The binding modes of ELV and L731,988 were practically not altered by the removal of the viral DNA. Conversely removing vDNA had a significant effect on the docking results RAL, thereby highlighting the role of vDNA for RAL recognition most likely due to the halogenated benzyl moiety

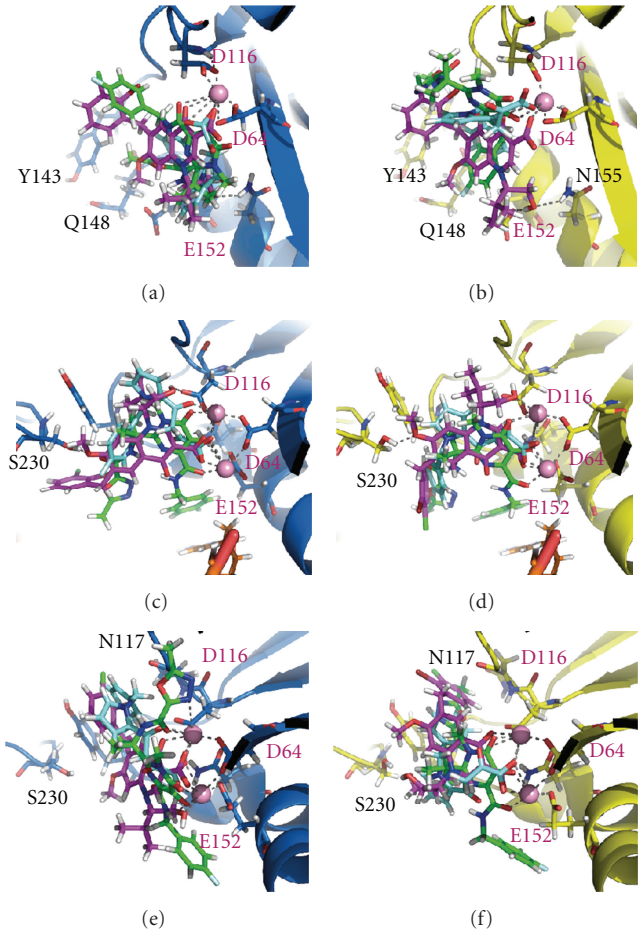



Figure 4: RAL (green), ELV (magenta), and L731,988 (cyan) best poses predicted by Glide. The inhibitors were docked into the active site of unbound IN (top) and IN·DNA complex (middle) and IN in holo conformation without DNA (bottom) from of the B (in blue) and CRF02_AG (in yellow). Proteins and DNA are shown as cartoons, inhibitors as sticks, and $Mg^{+2}$ cations as balls.

that displaces the unpaired 5′-adenine and stacking with the Cyt16 through $\pi$-$\pi$ interactions. Although such interaction is thought to be involved in all the IN strand transfer inhibitors examined [19], our results suggest that ELV and L731,988 binding determinants differed in part from the ones of RAL.

It should be noted that slight differences were observed between the results obtained with Glide and AutoDock scores, which can be ascribed to the impact of electrostatic interactions in the studied molecular systems. Indeed Glide uses higher negative charge localized on the two oxygen atoms of the hydroxypyrimidinone of RAL than AutoDock ($-1.22$ and $-0.5e$ versus $-0.183$ and $-0.265e$). Also, within the AutoDock scoring function, the carboxylate charges used for ELV ($2 \times -0.64e$) and L731,988 ($2 \times -0.62e$) are more than two oxygen atoms attached to the pyrimidine cycle of RAL. To verify this hypothesis, we repeated the docking calculations of ELV and L731,988 using the charges of two oxygen atoms attached to the pyrimidine ring of RAL instead of those assigned by Gasteiger charges. The new binding energies of both inhibitors increased from $-12.45$ and $-11.50$ to $-7.95$ and $-7.80$ kcal/mol for ELV and L731,988, respectively. Since these atomic charges contribute highly in the binding energy as the atoms coordinate $Mg^{2+}$ ions, they are likely responsible for the discrepancies found between the theoretical binding energies and the experimental $IC_{50}$ values. The experimental ranking of the three inhibitors based on $IC_{50}$ is RAL > ELV > L731,988, as predicted by Glide while the ranking predicted by the AutoDock is ELV > L731,988 ≥ RAL. The high negative charges of the carboxylate oxygen atoms of ELV and L731,988 may be the obstacle to have inhibitory actions on integrase, as efficient as RAL, since these charges increase the desolvation free energy and so increase the binding penalty for these inhibitors.

Studies investigating the presence and frequency of polymorphisms in the HIV-1 gene of treatment-native patients are extremely important for tracing the virus evolution and the epidemiology of HIV infections worldwide. Associated crucial questions concern the effect of polymorphisms on viral enzymatic activities, susceptibility towards inhibitors, and inhibitor resistance pathways. The absence of accurate experimental data characterising the IN and/or IN·vDNA complex structures essentially perplexes an exploration of these essential topics. Since the beginning of clinical AIDS treatment with RAL in 2007, only a few attempts to probe RAL binding to integrase from different retroviral strains have been reported. Particularly, molecular docking of RAL into the IN catalytic core domain structure with the inhibitor 5CITEP as a viral DNA mimic has depicted different

binding modes and affinities of RAL to IN from B and C subtypes [27]. Differences between the binding modes of several compounds to IN from B and C subtypes were also communicated [28].

In this context, our combined theoretical (structural modeling) and experimental (biochemical) evaluation of subtype CRF02_AG variation impact/effect on IN interaction with DNA or IN susceptibility to INSTIs contribute to the understanding of polymorphism effects at the molecular and structural level. Our experiments have revealed that IN from subtype CRF02_AG has similar enzymatic activity to IN from subtype B, and the susceptibility of the two INs to strand transfer inhibitors is comparable. Results from molecular modeling and inhibitor docking were found in agreement with *in vitro* observations.

Biochemical studies have revealed the impact of HIV-1 natural polymorphism on the susceptibility of protease (PR)—the other retroviral enzyme—to inhibitors [29]. Recent structural and biophysical studies have also shown that sequence polymorphisms of B and CRF01_AE strains can alter protease activity and PR inhibitors binding [30]. In this protein, the variations between the two strains directly impact the conformation of the flap hinge region and the protease core region that play crucial roles for the enzyme functions.

By contrast, the residues showing natural variations in the HIV-1 integrases from B and CRF02_AG strains are located outside the catalytic region and outer to the binding site of the strand transfer inhibitors. Such type of polymorphism may allow the virus to preserve the integrase structural and functional properties as observed in this study.

The methods we applied could be used for the study of other retroviral substrains emerging at the moment or to appear in the future in order to evaluate and optimize the efficiency of novel specific antiretrovirals. Consequently, our study contributes particularly to this topic and closely relates to a clinically and therapeutically—significant question—does the HIV-1 integrase polymorphisms influence the susceptibility towards integrase inhibitors?

## 3. Conclusions

The naturally occurring variations in HIV-1 subtype CRF02_AG IN, such as K14R, V31I, L101I, T112V, T124A, T125A, G134N, I135V, K136T, V201I, T206S, V234I, and S283G, do not affect notably integrase structure, neither *in vitro* enzymatic activity, 3′-processing, nor strand transfer reaction. Docking results of all the considered inhibitors into the unbound IN model show the considerably low scores respectively, to docking into the pre integration IN·DNA complex. The docking scores and inhibitor poses confirm that the generated structure of the HIV IN·DNA complex is the appropriate biologically relevant model used to explain the inhibition mechanism of the strand transfer inhibitors. All the three studied molecules are polydentate ligands able to wrap around the metal cations in the active site. The results of the docking are in perfect agreement with the proposed mechanism of action for INSTIs. Docking results reveal that the modes of binding and docking conformations of three studied molecules are identical for the HIV-1 IN from B and CRF02_AG strains. The proposed mechanism of the integrase inhibition based on considering of different conformational states, unbound IN, and IN·vDNA complex holds for the two studied strains.

## 4. Methods

*4.1. Molecular Modeling.* All calculations were carried out on a Linux station (4×2 cores) running Centos 5.4. The IN models were constructed using Modeller package 9V8 [31]. The sequence alignment was performed using ClustalW server [32, 33] (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The docking of ST inhibitors, RAL, ELV and L731,988 (Scheme 1), onto the IN models 1–6 was performed using two algorithms, GLIDE [34] incorporated in the Schrödinger suite (Schrödinger Inc.) and Autodock 4.2 [35]. Figures were produced with PyMol [36].

*4.2. Models of the HIV-1 IN from B and CRF02_AG Strains.* 3D models of the full-length IN homodimer, $IN^{1-270}$ (unbound IN, or *apo* state, resp. to DNA) containing one $Mg^{2+}$ cation in each active site were generated by homology modeling from crystallographic structures of isolated pairs of IN domains. Two structures of the HIV-1 IN, one containing the N-terminal domain (NTD) and the catalytic core domain (CCD) ($IN^{1-210}$, PDB code: 1K6Y) [37] and the other containing the CCD and the C-terminal domain (CTD) ($IN^{56-270}$, PDB code: 1EX4) [38], were chosen as the initial templates. These structures represent multiple mutants of the HIV-1 subtype B IN, the mutations being W131D/F139D/F185K in 1K6Y and C56S/W131D/F139/F185K/C180S in 1EX4. Both structures were superimposed and CCD domain ($IN^{56-210}$) of 1EX4, determined at lower resolution (2.8 Å) than 1K6Y (2.4 Å), was deleted. The disordered residues 271–288 were also omitted. Sequences of the WT HIV-1 INs from B and CRF02_AG strains, which differ by 13 amino acids (K/R14, V/I31, L/I101, T/V112, T/A124, T/A125, G/N134, I/V135, K/T136, V/I201, T/S206, V/I234 and S/G283), were aligned to the templates sequences using ClustalW. The missing CCD-NTD linker (47–55 aas) was constructed by an *ab initio* approach with Modeller 9V8, based on, discrete optimized protein energy (DOPE) scoring function [39]. 100 models were generated for each IN, from B and CRF02_AG strains. The conformation of the folded loop $IN^{140-149}$ with a well-shaped hairpin structure [40] was reconstructed by a loop-generating algorithm based on database searches (Protein Loop Search). $Mg^{2+}$ cation was inserted into the active site (D64, D116, and E152) as reported in structure 1BI4 [41] and minimized by molecular mechanics (MM) under constrains using CHARMM [42]. We shall refer to these generated models as model 1 (B strain) and model 2 (CRF02_AG strain).

*4.3. Models of the HIV-1 IN from B and CRF0_AG Strains in Complex with vDNA.* 3D models of the IN·vDNA pre integration complex (*holo* state respectively to DNA) from B and CRF02_AG strains were generated by homology

modeling following a two-step procedure. The coordinates of the recently published crystal structure of the PFV IN·vDNA complex cocrystallized with RAL (PDB code: 3OYA, resolution of 2.65 Å) [19, 20] was used as template. The sequence alignment of the HIV-1 IN dimer (B strain) and the PFV IN was performed using ClustalW. The sequence identity between these two INs is 22%. Nevertheless, structure-based alignment of INs from the PFV and HIV-1 demonstrates high conservation of key structural elements and consequently, the PFV IN X-ray structure provides a good template for the HIV-1 IN model generation. In order to increase the quality of our model, the NED domain (residues 1 to 50), only present in PFV IN, was removed from the corresponding sequence. Then, the sequences of the structural domains of HIV-1 and PFV INs were aligned separately, taking into account the conservation of the secondary structure. The obtained sequence alignment was used for homology modeling of the HIV-1 intasome. The interdomains linker were constructed using the *ab initio* LOOP module in Modeller [43]. For both subtypes B and CRF02_AG models, distance restraints were applied to reproduce key interactions reported in earlier experimental studies [37, 44–46]. 100 models were generated for each IN, from B and CRF02_AG strains, and those with the lowest energy were retained. We shall refer to these models as model 3 (B strain) and model 4 (CRF02_AG strain). Two additional models 5 and 6 were generated by removing vDNA from models 3 and 4.

*4.4. Refinement of Models 1–6 and Quality Check out.* Hydrogen atoms were added by the HBUILD facility in CHARMM [42]. The resulting models were slightly minimized while constraining carbon-$\alpha$ to remove clashes. The stereochemical quality of the models was assessed with Portable ProCheck [47], which showed that more than 97% of the residues in all models had dihedral angles in the most favorable and allowed regions of the Ramachandran plot, indicating high model quality.

*4.5. Molecular Docking.* Initial molecular geometries of ELV and RAL were taken from the X-ray structures 30YA (RAL) and 3L2U (ELV) of PFV IN·vDNA complexes [19, 20]. The 3D structure of the compound L731,988 was generated by ChemBioOffice 2010 [48]. The models of all inhibitors (Scheme 1) in deprotonated form were minimized with density functional theory (DFT) B3LYP 6-31G* method implemented in *Gaussian03* program [49]. Inhibitors RAL, ELV, and L731,988 were docked onto models 1–6 using two algorithms, GLIDE [34] and AutoDock 4.2 [35]. The receptor is considered as a rigid body while the ligand is treated fully flexible.

In AutoDock 4.2, the graphical user interface (GUI) was used for the preparation of the inhibitor and receptor files. Grid maps of interaction energies for various atom types were constructed with a grid box of dimension $25\times25\times25$ Å$^3$ centered on the active site. Calculations were performed with a population size of 150, number of energy evaluations of $5\times10^6$, maximum number of generations of 27,000, mutation and crossover rate of 0.02 and 0.8 respectively. The number of runs was set to 100 to explore a large number of poses of the highest affinity and the Solis and Wets algorithm was used to relax the best 10% of the obtained conformations.

In the Schrödinger suite receptor grids were generated by Glide 4.5 within an enclosing box of size 20 Å centered on the active site. Inhibitors were docked flexibly to these pre-computing grids using standard precision (SP) scoring function. For each compounds, the best-scored pose was saved and analyzed.

*4.6. Cloning of IN Gene.* IN cDNA was derived from naïve HIV-1 subtype CRF02_AG infected patients. Plasmid pET15b- HIV-1subtype B IN (HBX2) was our lab's conservation [50]. Amplification of IN coding sequence was carried out with specific primers at 94°C for 10 min, then 28 repeat cycles (94°C for 30 s, 55°C for 45 s, and 72°C for 1 min) followed by incubation at 72°C for 10 min. PCR products corresponding to the entire IN sequences were purified and ligated into pGEM-T Easy vector (Promega) and sequenced (Eurofins MWG operon). Then IN gene was inserted into expression vector pET-15b (Novagen) after digested with Nde I and BamH I and verified by sequencing. Forward primer: 5′-CATATGTTTTTAGATGGCATAGATAAAGCC-3′; backward primer for CRF02_AG 33CR, 49CR: 5′ -GATCCTAATCCTCATCCTGTCTACCTGC-3′; backward primer for CRF02_AG 52CR Q148K: 5′-GATCCTAATCCTCATCCTGTCCACTTGC-3′; backward primer for CRF02_AG 68CR: 5′-GGATCCTAATCTTCATCCTGTCTACTTGC-3′.

*4.7. Expression and Purification of IN.* His-tagged INs were produced in *Escherichia coli* BL21-CodonPlus (DE3)-RIPL (Agilent) and purified under nondenaturing conditions as previously described [50, 51].

*4.8. Steady-State Fluorescence Anisotropy-Based Assay.* Steady-state fluorescence anisotropy values were recorded on a Beacon 2000 Instrument (Panvera, Madison, WI, USA), in a cell maintained at 25°C or 37°C under thermostatic control. The principle underlying the anisotropy-based assay was published elsewhere [52, 53]. DNA-binding assay was carried out at 25°C for 20 minutes in a buffer containing 10 mM HEPES pH 6.8, 1 mM dithiothreitol, and 7.5 mM magnesium chloride in the presence of 12.5 nM-double stranded DNA substrate (21-mer oligodeoxynucleotide mimicking the U5 viral DNA end, fluorescein-labeled at the 3′-terminal GT) and 100, 150, 200, and 250 nM recombinant IN, respectively. In kinetic study, steady-state fluorescence anisotropy-based 3′-processing activity assay was performed in the presence of 50, 100, 200, and 250 nM recombinant IN proteins and 12.5 nM double stranded fluorescein-labeled DNA substrate, at 37°C for 10, 20, 30, 60, 90, 120 and 180 min.

*4.9. IN 3′-Processing and Strand Transfer Activity Assay. In vitro* 3′-processing and strand transfer activities assays were carried out using the 21/21-mer or 21/19-mer double

stranded oligodeoxynucleotides marked with [$\gamma$-$^{32}$P] ATP-respectively, as previously described [51]. The duration of the assays was 3 hours, at temperature 37°C, in a buffer containing 10 mM HEPES pH 6.8, 1 mM dithiothreitol, and 7.5 mM magnesium chloride in the presence of 12.5 nM double stranded DNA substrate and 100 nM recombinant IN. The kinetic study was carried out by testing *in vitro* 3′-processing activity in the presence of 50, 100, 150, and 200 nM recombinant IN proteins, at 37°C for 10, 20, 30, 60, 90, 120 and 180 min, respectively.

*4.10. Susceptibility to INSTIs.* Susceptibility of INs to INSTI was determined by testing in vitro strand transfer activity in the presence of increasing concentration of INSTI in DMSO. Inhibition by the drug was expressed as a fractional product (percentage of the activity of the control without drug). The 50% inhibitory concentration ($IC_{50}$), defined as the concentration of drug that results in 50% inhibition, was calculated from inhibition curves fitted to experimental data with Prism software, version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

## Abbreviations

| | |
|---|---|
| HIV-1: | Human immunodeficiency virus |
| vDNA: | Viral DNA |
| hDNA: | Host DNA |
| IN: | Integrase |
| CRF02_AG: | Circulating recombinant form |
| LTR: | Long terminal repeat |
| PFV: | Prototype foamy virus |
| NTD: | N-terminal domain |
| CCD: | Catalytic core domain |
| CTD: | C-terminal domain |
| ST: | Strand transfer |
| INSTI: | Integrase strand transfer inhibitor |
| DKAs: | Diketo acids |
| RAL: | Raltegravir |
| ELV: | Elvitegravir |
| PI: | Protease inhibitors |
| RMSD: | Root mean square deviation. |

## Conflict of Interests

The authors have declared no competing interests.

## Authors' Contribution

X. Ni, S. Abdel-Azeim, and E. Laine contributed equally in this work. J.-F. Mouscadet and L.Tchertanov conceived and designed the experiments. X. Ni, S. Abdel-Azeim, E. Laine, R. Arora, and O. Osemwota performed the experiments. X. Ni, S. Abdel-Aziem, and E. Laine analysed the data. A.-G. Marcelin and V. Calvez contributed with reagents/materials/analysis tools. E. Laine, J.-F. Mouscadet, and L. Tchertanov wrote the paper.

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*Review Article*

# Glances in Immunology of HIV and HCV Infection

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Since the identification of HIV and HCV much progress has been made in the understanding of their life cycle and interaction with the host immune system. Despite these viruses markedly differ in their virological properties and in their pathogenesis, they share many common features in their immune escape and survival strategy. Both viruses have developed sophisticated ways to subvert and antagonize host innate and adaptive immune responses. In the last years, much effort has been done in the study of the AIDS pathogenesis and in the development of efficient treatment strategies, and a fatal infection has been transformed in a potentially chronic pathology. Much of this knowledge is now being transferred in the HCV research field, especially in the development of new drugs, although a big difference still remains between the outcome of the two infections, being HCV eradicable after treatment, whereas HIV eradication remains at present unachievable due to the establishment of reservoirs. In this review, we present current knowledge on innate and adaptive immune recognition and activation during HIV and HCV mono-infections and evasion strategies. We also discuss the genetic associations between components of the immune system, the course of infection, and the outcome of the therapies.

## 1. Introduction

*1.1. Human Immunodeficiency Virus Infection.* Human immunodeficiency virus (HIV) is a retrovirus of the Lentiviridae family. This positive strand RNA virus infects specific cell populations of the immune system through its receptor specificity. At present, more than 33 million people are infected with HIV. HIV infection is characterized by an acute and a chronic phase, possibly leading to AIDS. Immediately after infection the viral load increases with exponential growth kinetics and $CD4^+$ T cells rapidly decline [1, 2]. The peak of this growth curve coincides with the onset of a strong host immune response resulting in decreasing viral load and increasing number of circulating virus-specific $CD4^+$ T cells. Then, the acute phase of HIV infection is accompanied by a selective and dramatic depletion of $CD4^+CCR5^+$ memory T cells predominantly from mucosal surfaces. This loss is largely irreversible and ultimately leads to the failure of the host immune defenses to clear the infection [3, 4]. This allows HIV to establish life-long latency and chronic infection.

Over the chronic phase of infection, the viral load remains stable, whereas $CD4^+$ T cell levels gradually decline [5]. The chronic phase is clinically latent, but eventually without therapeutic intervention, the infection progresses to the symptomatic phase characterized by increased viral load and rapidly decreasing $CD4^+$ T cell and also $CD8^+$ T cell levels, making patients prone to opportunistic infections [6].

During HIV infection, both innate and adaptive immune responses are raised, but are insufficient or too late to eliminate the virus. Additionally, the very same cells and responses aimed at eliminating the virus seem to play deleterious roles by driving chronic immune activation that plays a central part in immunopathogenesis and progression to AIDS [7, 8].

*1.2. Hepatitis C Virus Infection.* Hepatitis C virus (HCV) is a positive-stranded RNA virus belonging to the Flaviviridae family. Six major HCV genotypes have been identified and more than 100 subtypes have been identified through the world on the basis of molecular relatedness of conserved and non-conserved regions. Furthermore, several distinct but

closely related HCV sequences coexist within each infected individual. These are referred to as quasi-species and reflect the high replication rate of the virus and the lack of a proof-reading capacity of the RNA-dependent RNA polymerase.

More than 170 million people worldwide are chronically infected with HCV, which is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Individuals infected with HCV have two possible outcomes of infection, clearance or persistent infection, determined by a complex set of virus-host interactions [9].

The majority of episodes of primary HCV infection are asymptomatic and infection is often detected incidentally at the time of routine health examinations or when donating blood. After initial exposure to HCV, 54–80% of infected person develop persistent viremia despite the generation of HCV-specific antibodies detected by ELISA and HCV-specific cellular immune responses [10–12]. This indicates that anti-viral immune response is functionally ineffective in the majority of exposed individuals. A correlation between syntomatic disease and viral clearance has been reported, possibly due to a more vigorous immune response, which also results in greater liver injury [11]. In chronic HCV infection, the liver is typically infiltrated by mononuclear cells including $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, as well as natural killer (NK) cells ($CD56^+CD3^-$) and NK T cells ($CD56^+CD3^+$).

Knowledge of the early immune response at the site of infection derives from recent studies on experimentally infected chimpanzees [13, 14], the only animal model used to study immune responses during the natural course of infection. Research into viral-host protein interactions has recently progressed rapidly with the development of an effective *in vitro* HCV culture system, which was previously unavailable [15–18].

*1.3. HIV/HCV Coinfection.* Infection with HCV is the most common co-infection in people with HIV, and HCV is categorized as an HIV-related opportunistic illness [19]. Complications related to HIV/HCV co-infection are becoming an increasingly important medical issue. Owing to shared modes of transmission, as many as 30 to 40% of people with HIV may also be co-infected with HCV. HCV is approximately 10 times more infectious than HIV through percutaneous blood exposures. Though HCV is less likely than HIV to be transmitted sexually or from mother to baby, some studies have shown that the risk of sexual or perinatal (mother to baby) HCV transmission is higher in $HIV^+$ individuals. Moreover, HIV/HCV co-infection is a significant problem among injection drug users (IDUs) as well as men who have sex with men (MSMs). The introduction in the mid-1990s of highly active antiretroviral therapy (HAART) for HIV has caused a sharp drop in the number of deaths from AIDS. This means that people with HIV are living longer. Therefore, if they are co-infected, the complications from HCV have more time to develop [20]. These complications (cirrhosis, liver cancer, end-stage liver disease) generally develop over 20–30 years. Liver disease from HCV is now the leading non-AIDS cause of death in the U.S. in co-infected individuals with HIV.

The ways in which co-infection with HIV and HCV affect the body are still poorly understood. Most studies indicate that HIV can worsen hepatitis C. HIV/HCV co-infection has been associated with a faster rate of hepatitis C disease progression, higher HCV viral loads, and a greater risk of developing severe liver damage. The impact of HCV on HIV disease is less clear, but a majority of studies suggest that hepatitis C does not accelerate HIV disease progression. Hepatitis C can affect HIV treatment by increasing the frequency of liver toxicity related to antiretroviral drugs. There is also the potential for interactions between drugs used to treat HIV and HCV infections. However, with careful medical monitoring, many co-infected people can be successfully treated for both HIV and HCV. Some experts believe it is better to begin HIV treatment first in order to control HIV replication and increase the CD4 count, since hepatitis C treatment works better in people with stronger immune systems [21]. Some research suggest that even after starting HIV treatment, CD4 counts do not increase as rapidly in co-infected people as in people with HIV alone. However, in people with early-stage HIV disease and advanced hepatitis C, it may be better to start HCV treatment first, so the liver can more easily handle HIV drugs since many HIV medications are metabolized by the liver and some can cause liver toxicity (hepatotoxicity).

## 2. Antiviral Immune Response

*2.1. Innate Immune Responses.* The innate immune system constitutes the first line of defense against invading pathogens and it functions during the early phase of infection, before the development of specific adaptive immunity. It is based on epithelial barriers, the complement system, and cells with phagocytotic and antigen presenting properties, such as granulocytes, macrophages, monocytes, Langerhans cells, dendritic cells (DCs), NK cells, and $\gamma\delta$ T cells [22, 23]. It plays a part in early restriction of the virus and in shaping the adaptive immune response, but at the same time participates in the establishment and spread of infection. DCs and NK cells are vital mediators of the innate immune system and promote the development of adaptive immune responses [24]. DCs are of pivotal importance because they are among the earliest targets of HIV and are crucial for activating and conditioning virus-specific T cells, a process that is largely influenced by the preceding innate immune response [25]. Myeloid DCs (mDC)s are professional antigen presenting cells present in blood, skin, and mucosal tissues, whereas plasmacytoid DCs (pDCs) are located in blood and secondary lymphoid organs and play important roles in innate immune responses to viruses through the production of type I Interferons (IFN) [26].

Unlike adaptive immunity, the innate immune cells do not use T cell receptors; they are not major histocompatibility complex restricted; and they apparently lack memory, which is essential in vaccination. An early non specific protective response may limit microbial replication and dissemination, and allow adaptive immunity sufficient time to mount an effective protective response.

Innate immune responses to viral infections generally feature the induction of both cellular responses via NK cells, and antiviral proteins, notably IFN-$\alpha$ and IFN-$\beta$, which generate an antiviral state [27]. The recent discovery of a fundamental pathogen recognition system which boosts innate immunity and drives the induction of type I IFN has dramatically advanced understanding of host responses to viral infection [28]. This system is based on pathogen-associated molecular patterns (PAMPs), which are recognized by specific PAMP receptors expressed in the host cell, initiating signals that ultimately induce the expression of antiviral effectors genes [29].

The receptors of the innate immune system that recognize PAMPs are called pattern-recognition receptors (PRRs). Among PRRs, the family of Toll-like receptors (TLRs) have been studied most extensively. TLRs are membrane bound receptors and 10 different TLRs have been identified in humans. Of those, viral recognition is primarily mediated by TLR9 recognizing DNA, as well as by TLRs 7/8 and TLR3 sensing single-stranded (ss) RNA and doublestranded (ds) RNA, respectively [30, 31]. In addition, C-type lectin receptors, such as DC-SIGN, Dectin-1, and mannose receptor, have emerged as cell surface PRRs that play important roles in induction of immune responses against various pathogens [32]. DC-SIGN, in particular, has been attributed essential roles as an adhesion receptor, in mediating interactions between DCs and T cells, and as a PRR inducing specific immune responses [33].

*2.1.1. HIV.* The innate immune cells involved in protection of HIV infection are Langerhans cells in vaginal and foreskin epithelia [34, 35]; $\gamma\delta^+$ T cells in rectal and vaginal epithelia; and macrophages, DCs, and NK cells in the subepithelial tissues.

Interestingly, opposing roles for mDCs and pDCs in HIV infection have been described [36]. Whereas mDCs enhance HIV infection through capture and subsequent transmission of the virus, pDCs inhibit HIV replication in T cells through the antiviral activities of IFN-$\alpha$ [37]. Since pDCs are the major producers of type I IFN, it has been suggested that abnormal migration and localization patterns of this important cell type may be a key defense strategy of HIV [38].

NK cells play a major role in preventing the early spread of viruses by producing cytokines and directly killing infected cells. NK cells may be crucial for early control of HIV infection and can have important roles in editing the function of DCs, thereby affecting their ability to prime antiviral effector T cells. They kill cells by releasing perforin and granzyme that cause the target cell to die by apoptosis. NK cells do not require activation in order to kill target cells, do not express T cell antigen receptors (TCR), whereas they express Fc receptor (FcR) to lyse cells through either to activate or to suppress their cytolytic activity. In general, NK receptors recognise missing self (MHC), induced self, or modified self (stress signals) proteins as their ligands [33].

As in NK cells, $\gamma\delta$ T cells express the inhibitory receptors KIR, which recognize major histocompatibility complex class I molecules and inhibit cytotoxic responses [38]. The NKG2D activating receptors are also found on $\gamma\delta$ T cells, and they recognize the human MHC class I chain-related proteins MICA and MICB.

The CC chemokines RANTES, MIP-1$\alpha$, and MIP-1$\beta$ are produced by activation of macrophages, DCs, T cells, NK cells, and $\gamma\delta$ T cells. These 3 CC chemokines can block the CCR5 coreceptors and prevent HIV infection *in vitro* [39]. There is *in vivo* evidence indicating that raised concentrations of CC chemokines downmodulate the cell surface expression of CCR5. An inverse correlation was established between the concentration of the 3 CC chemokines and the proportion of cells expressing CCR5 in macaques immunized with SIV gp120 and p27 [40].

$\gamma\delta$ T cells are involved in innate immunity and mucosal protection; they produce Th1 and Th2 types of cytokines [41], and they lyse HIV-infected target cells [39]. $\gamma\delta^+$ T cells also generate antiviral suppressor factors MIP-1$\alpha$ (CCL3), MIP-1$\beta$ (CCL-4), and RANTES (CCL-5), which can prevent SIV infection by binding to and down modulating the CCR5 coreceptors [42].

Defensins have emerged as further components of innate immunity and may contribute to mucosal protection against HIV-1 infection especially that of oral mucosa [43].

Finally, important intracellular innate antiviral factors are represented by APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic polypeptide-like-3G), TRIM-5 (Tripartite motif-containing protein 5), and tetherin.

APOBEC is packaged into retroviral virions, and it deaminates viral cytidine to uridine, rendering them nonfunctional and inhibiting viral replication. APOBEC3G has also been found to inhibit HIV by an additional mechanism, possibly at or prior to the reverse transcription stage of the viral RNA [44]. This innate mechanism of resistance to retroviral infection is counteracted by the HIV-1 viral infectivity factor (Vif), which protects the virus by preventing incorporation of APOBEC3G into virions and by rapidly inducing its ubiquitination and proteasomal degradation [44].

Tetherin is a human cellular protein, an IFN-inducible factor of the innate immune system, which exerts antiviral activity against HIV-1 and other enveloped viruses by tethering nascent viral particles to the cell surface, thereby inhibiting viral release. In HIV-1 infection, the viral protein U (Vpu) counteracts this antiviral action by downmodulating tetherin from the cell surface [45].

TRIM5 is a protein encoded in humans by the TRIM5 gene [46]. The alpha isoform of this protein, TRIM5$\alpha$, is a retrovirus restriction factor, which mediates species-specific early block to retrovirus infection. TRIM5$\alpha$ was isolated as a rhesus macaque protein responsible for blocking infection by HIV-1. The human version of TRIM5$\alpha$ does not target HIV-1, but can inhibit strains of the murine leukemia virus (MLV) as well as equine infectious anemia virus (EIAV) [47].

*2.1.2. HCV.* While the majority of HCV-infected patients progress to chronic hepatitis, a small fraction of individuals are able to clear the virus. Resolution of infection occurs

within the first few weeks of infection, suggesting that innate immune functions may be critical for early control. Although all nucleated mammalian cells are able to secrete type I IFN, the first response to HCV infection is thought to be IFN-$\beta$ production by infected hepatocytes. In hepatocyes, recognition of viral motifs at the cell surface is mediated via TLR3, while the retinoic acid-inducible gene (RIG)-I acts as a intracellular PAMP receptor for double-stranded (ds)RNA. These two independent signaling pathways are suggested to be involved in the activation of interferon regulatory factor (IRF)-3 and of nuclear factor kappa B (NF-$\kappa$B) leading to a transcriptional response that results in the secretion of IFN-$\alpha/\beta$ from the infected cell. Activation of NF-$\kappa$B also induces the expression of proinflammatory cytokines and chemokines that function to amplify the inflammatory response and facilitate leucocyte recruitment to act in concert with IFN-$\alpha/\beta$ in the host response to HCV [48].

Secreted IFN-$\alpha/\beta$ acts in both autocrine and paracrine pathways by binding IFN receptors to trigger activation of Jak-STAT pathway, leading to induction of IFN-stimulated genes (ISGs), which are the effectors of the host response to virus infection [49].

NK cells, which are present in greater numbers in the liver than in other organs, contribute to the pathogen-induced immune responses. Given the difficulties in generating efficient adaptive immune responses within the liver, the role of innate immune mechanisms in the induction of defensive responses is probably greater than in other tissues [50]. In the inflamed HCV-infected liver, activation of innate immune response induces NK cells to secrete IFN-$\gamma$ and to up regulate the expression of the chemokines CXCL9 and CXCL10. These chemokines bind CCR5 and CXCR3 on liver infiltrating lymphocytes and guide them into the space of Dissè and the parenchymal tissue. In addition, NK cells have a strong cytotoxic potential and are able to rapidly attack target cells without prior immunization.

*2.2. Adaptive Immune Responses.* Adaptive immunity refers to antigen-specific defense mechanisms, induced during the chronic phase of viral infection, designed to remove a specific antigen. There are two major branches of the adaptive immune responses: humoral immunity and cell-mediated immunity. The first involves the production of antibody molecules in response to an antigen and is mediated by B-lymphocytes; the second involves the production of cytotoxic T-lymphocytes (CTL), activated macrophages, activated NK cells, and cytokines in response to an antigen and is mediated by T-lymphocytes.

*2.2.1. HIV.* Soon after infection, p24 antigen is detectable in serum and disappears as the person seroconverts, developing an antibody response to envelope and core antigens. Most of the humoral antibody response is directed at the viral envelope and has little or no neutralizing effect. Neutralizing antibodies are directed at particular epitopes within the variable loop regions of gp120 and the pre-fusion complex of gp41 [51].

$CD8^+$ T cells mediated immunity is the most effective: upon recognition of viral antigen presented by the epitope-bearing MHC-I restricted molecule, $CD8^+$ T cells become activated CTLs, killing the presenting cell by induction of apoptosis through the release of cytotoxic molecules as perforin and granzyme A/B, or by activating the Fas-ligand pathway [52]. CTL responses are detectable throughout the course of infection, generally being lost only late in the disease. These responses maintain significant pressure on viral replication and are important for the initial control of HIV infection and for the determination of viral set point. In general, the response is narrowly focused in the first weeks to months of infection and then broadens during the asymptomatic phase, prior to decreasing both in breadth and magnitude late in the disease.

In HIV infection, as in other viral infections, recognition of viral antigenic peptides activates a $CD4^+$ T helper response, expressing a wide range of cytokines (including IL-2, IFN-$\gamma$ and tumor necrosis factor (TNF)-$\beta$) which co-ordinate a multi-cellular cell-mediated response against the invading virus. Activated proliferating HIV-specific $CD4^+$ T cells are detectable in early infection. The virus infects activated cells more easily because they express high levels of the co-receptor CCR5 and also replicates more efficiently in proliferating cells. HIV-specific $CD4^+$ T cells are preferentially infected early the disease process and subsequently become difficult to detect. Antigen-specific $CD4^+$ T cells are detected at only low levels at other stages of infection, except in sub-populations of individuals capable of controlling their infection naturally, referred to as long-term non-progressors (LTNP). Similarly, the majority of HIV-specific $CD4^+$ T cells detected are able to make IFN-$\gamma$ but not IL-2 [53]. This lack of appropriate CD4 help likely compromises $CD8^+$ T cell responses and neutralizing antibody responses, especially those to new variants of the virus as they arise. Later in the disease, also $CD4^+$ T cells recall responses to other pathogens and antigens are progressively lost, compromising immune responses to a range of pathogens.

Within the T cell compartment, much interest has been focused on an altered balance between proinflammatory Th17 cells and regulatory T cells (Tregs) in HIV infection. Th17 cells are $CD4^+$ T cells that produce IL-17 and play a central role in host defence against bacterial, fungal, and viral infections at mucosal surfaces [54]. It has been recently reported that there is a significant loss of Th17 cells in the gastrointestinal tract of HIV-1 infected individuals [55]. Because IL-17 serves to maintain the integrity of the mucosal barrier, loss of Th17 cells may permit the increase in microbial translocation across the gastrointestinal mucosa that is observed in pathogenic lentiviral diseases. Recent studies suggest that the replenishment of Th17 $CD4^+$ T cells in the gut mucosa during highly active antiretroviral therapy, or during nonpathogenic simian immunodeficiency virus infections in the nonhuman primate models, correlates with better restoration and function of the gut mucosal immune system [56].

Tregs represent a small subpopulation of T cells involved in preventing or inhibiting autoimmune and inflammatory disorders [57], but much controversy exists regarding the

role of Tregs in HIV pathogenesis. One study demonstrated expansion of Tregs during HIV infection positively correlating with $CD4^+$ T cell activation and rapid disease progression, indicating a detrimental role of Tregs in the immune control of HIV infection [58]. Tregs are major producers of transforming growth factor, (TGF-) $\alpha$, which promotes tissue fibrosis and limits immune reconstitution [59]. In direct contrast, however, other studies have reported decreased levels of Tregs in HIV-infected individuals [60], and in one study, depletion of Tregs in HIV infection was found to be associated with immune activation [61].

*2.2.2. HCV.* There is only limited evidence for a significant role for anti-HCV antibody responses in viral clearance, as recovery from primary infection does not correlate with HCV-specific antibody titers or levels of antibodies directed against the E1 and E2 glycoproteins [62, 63].

The humoral immune response in primary HCV infection is of low titer and, with exception of responses against the core protein, the generation of the response is delayed [64]. HCV-specific antibodies become detectable in the serum after the appearance of the cellular immune response and the subsequent increase in alanine transaminase (ALT). Another characteristic aspect of the humoral response against HCV infection is the restriction of antibodies to the IgG1 subclass, without the usual switching to IgG3 (or IgG4) subclasses that typically occurs with maturation of an antiviral humoral response [64]. Interestingly, there may be an association between the development of IgG2 antibodies and viral clearance. The IgG2 predominance has been linked to a Th1 bias in $CD4^+$ T cell responses [65].

Direct evidence for a protective role of HCV-specific antibodies, derives from limited *in vivo* studies in which chimpanzees were protected against infection with an HCV inoculum that was neutralized with HCV-specific antibodies *in vitro* [66]. Thus far, the hypervariable region-1 (HVR-1) and other regions of the HCV-envelope glycoproteins that are thought to bind to the putative HCV receptor complex have been proposed as targets for neutralizing antibodies [67]. Mutations in the HVR-1 have been associated with the emergence of the quasispecies leading to escape from neutralization [68, 69]. This area of the genome is a region that undergoes a high rates of nucleotide substitution in acute HCV infection [70] implying that neutralizing antibodies do provide significant selective pressure on the quasispecies and hence arguing for the importance of this aspect of the humoral response in clearance.

Despite this circumstantial evidence for a role of neutralizing antibodies, individuals with hypogammaglobulinaemia clear the virus at a similar rate to the general population [71].

The decrease of viral titer coincides with the appearance of HCV-specific T cells and IFN-$\gamma$ expression in the liver [72], which suggests that viral clearance is T cell mediated. These events coincide with the induction of genes that encode proteins of the adaptive immune responses, such as MHC class II proteins, immunoproteasome subunits, and chemokines.

HCV-specific $CD4^+$ T cells are essential in the generation of a successful HCV-specific immune response. At the time of clinical presentation and ALT elevation, vigorous proliferation of HCV-specific $CD4^+$ T cells with concomitant IL-2 and IFN-$\gamma$ production is readily detectable in the blood of patients who later recover and clear the infection [73–75]. In contrast, lack of an HCV-specific $CD4^+$ T cell response or failure to maintain it for a sufficient time, particularly in the face of viral mutations or quasi-species shifts, is associated with development of persistent infection and chronic hepatitis [73].

As with $CD4^+$ T cell responses, a strong multispecific $CD8^+$ T cell response produced early in infection is associated with viral clearance [76–80]. $CD8^+$ T cell responses directed against multiple epitopes spanning the HCV polyprotein were seen in chimpanzees that resolved infection, whereas animals that developed chronic infection generated narrow responses [80].

During acute HCV infection, $CD8^+$ T cells appear functionally impaired, with reduced proliferation, IFN-$\gamma$ production, and cytotoxicity [76, 81, 82] and increased levels of programmed death (PD)-1 [83]. However, the dysfunction of HCV-specific $CD8^+$ T cells resolves, and IL-7 receptor $\alpha$-positive (i.e., $CD127^+$) memory $CD8^+$ T cells become detectable, as soon as HCV-specific $CD4^+$ T cell responses develop and the HCV titer decreases [76, 82, 84].

## 3. Mechanisms of Immune Evasion

To replicate and spread successfully, viruses have evolved a number of strategies to evade host defenses. These include escape from T-cell recognition, resistance to immunological effectors functions, and active subversion of the immune response. Because of the high replication rate and the lack of proofreading capacity of both HIV and HCV DNA polymerases, the sequence and immunogenicity of the main viral populations can rapidly change and provide means to escape from emerging humoral and cellular immune responses.

*3.1. HIV.* The envelope of HIV is the primary target for humoral responses, and the virus has developed numerous mechanisms for avoiding the effect of neutralizing antibodies that are primarily directed towards envelope protein epitopes. Firstly, many of the neutralizing epitopes are cryptic, hidden within the protein structure of the molecule, and exposed only transiently. To be effective at these sites, antibodies require a high affinity to compete with natural ligands. Secondly, major neutralizing epitopes are protected by protein glycans, which form a shield to provide steric hindrance against anti-gp120 interaction [85]. Finally, the glycoproteins are highly mutable, conferring upon virus the ability to mutate rapidly away from effective neutralizing antibodies with minimal cost to viral fitness. Resistance to antibodies by point mutation on the V2/V3 loop and N-linked carbohydrate glycans on gp120 has been indicated as hindering antibody neutralization [86].

Similarly, the virus has developed multiple mechanisms to avoid recognition by $CD8^+$ CTL; these include both mutational and non-mutational mechanisms. Viral proteins, including Nef, Tat, and Vpu, interfere with antigen presentation by downregulating the expression of MHC-1 molecules on the surface of these cells [87]. Most of these act at a post-transcriptional site. Conversely, selective upregulation of HLA-C and E by Nef may protect infected cells from NK cell attack. Virus can mutate at targeted epitopes to avoid MHC restricted recognition, by decreasing binding affinity of the epitope to the presenting protein MHC-1 [88, 89]. Alternatively, mutations may alter the recognition of antigen peptide by a particular TCR, either resulting in no activation of the T cell or delivering an antagonistic signal to the T cell, preventing normal activation [90]. Mutations may also alter the chemistry of antigen processing through the proteasome and peptide-loading complex, so that peptide is no longer presented. Recent studies have indicated the expression of cryptic epitopes from proteins expressed through alternative reading frames.

Successful elimination of the virus requires a robust innate immune response and efficient priming of adaptive immunity. DC, are central in both these process. Evidences indicate that DC function is impaired during HIV-1 infection, contributing to a lack of effective antiviral adaptive immunity. This is either due to direct viral interactions with DCs or a result of indirect mechanisms, such as the production of IL-10 by monocytes during infection. pDCs activated by HIV-1 produce type I IFNs, which, in addition to inhibiting viral replication, may contribute to bystander $CD4^+$ T cell death. Furthermore, evidence shows that pDCs produce T cell-attracting chemokines, which may facilitate viral spread by providing a source of new T cells for HIV to infect. Finally, HIV-exposed pDCs prime regulatory T cells, which could impair mDC function and block effector T cell activity, further blunting adaptive immunity [91, 92].

*3.2. HCV.* HCV, after the initial interaction with the host immune system, uses several mechanisms to nullify the selective immunological pressure during the later phases of infection, including the alteration of its antigenic epitopes to escape immune surveillance. The human immune responses to HCV and the countermeasures of the virus are directly relevant to antiviral therapy given the main use of IFN-$\alpha$ in standard treatment. The best understood immune-evasive strategies include inhibition of protein kinase R (PKR) by the viral NS5A and E2 proteins [93], cleavage of cellular signal transduction molecules downstream of RIG-1 and TLR3 by the NS3/4A protein, and altering IL-12 production through secretion of the core protein.

Another evading strategy of HCV targets TLR7 expression, mRNA stability, and signalling. Recent studies have identified a significantly decreased TLR7 expression in the presence of HCV, both *in vitro* and *in vivo*. It was suggested that HCV may directly interfere with the transcriptional regulation of TLR7 mRNA. Despite decreased TLR7 expression in HCV-replicating cells, enhanced activation of IRF-7 was observed [94].

Many researchers have explored the hypothesis that the failure of HCV-infected individuals to mount an effective T cell response, thus developing chronic HCV infection, was due to the impairment of DC function [95, 96]. However, in spite of extensive studies, there is little overall consensus. The majority of studies observed decreased frequencies of both mDC and pDC during chronic infection [97–102]. It has been shown that the numbers of mDC and pDC in the liver of patients with chronic HCV infection were markedly increased, as compared to controls. Thus, it is difficult to determine if accumulation of DCs in the liver is casually related to the decrease of DC number in peripheral blood.

There is less agreement about the effect of chronic HCV infection on the function of circulating mDC and pDC. mDC from patients with chronic HCV infection have been reported to be inefficient at driving naïve T cell proliferation in allogeneic mixed-lymphocyte reaction (MLR) [97, 98, 101, 103]. However, in other studies, the ability to induce T cell proliferation in MLR or antigen-specific T cell responses was intact in DC obtained from patients with HCV [102, 104, 105]. A common finding in many studies is that IL-12 secretion by mDC is defective in cells obtained from patients with chronic HCV infection, while secretion of IL-10 increased as compared to healthy individuals [98, 99, 101, 106].

Many studies also described the impaired function of pDC in patients with HCV, resulting in abnormal IFN-$\alpha$ secretion in chronic HCV infection [106].

The impairment of DC function may not only influence T cell priming but may also preclude productive cross-talk between NK and DC. The abnormality of the NKG2A-expressing subpopulation also affects DC/NK cross-talk during chronic HCV infection [50].

Cross-sectional studies have indicated that NK cells are perturbed in chronic HCV infection. There may be a decrease in the number of circulating NK cells and skewing of NK cell subset distribution toward increased numbers of the cytokine-producing $CD56^{bright}$ population, relative to the cytotoxic $CD56^{dim}$ subpopulation. In addition, recent studies have shown that binding of recombinant HCV E2 protein and crosslinking of CD81 inhibit NK cell functions, such as cytotoxicity, proliferation, and IFN-$\gamma$ and TNF-$\alpha$ secretion *in vitro* [50, 107–109].

## 4. Polymorphisms in Host Response Genes

*4.1. HIV.* Epidemiological studies of genetic polymorphisms in human populations have enabled new ways to understand antiviral response and immune activation during HIV infection. The natural course of HIV infection is characterized by considerable variations among infected individuals, and is related to both viral and host factors, including genetic differences.

One of the first studies addressing this question was the description of almost complete protection from HIV infection conferred by homozygosity of a 32 base deletion in CCR5 (CCR5-l32) or a mutation in CCR2 (CCR2-64I) [110]. They provide strong protection and are found more frequently in LTNP than in rapid progressors.

Moreover, certain HLA alleles are associated with control of virus replication and slower progression to AIDS [111]. For virus replication control, a strong CTL response is crucial. CTLs are activated by binding to antigenic peptides presented by HLA, initiating the immune response. Many studies report the effect of HLA polymorphisms on the progression of AIDS [112, 113]. Many HLA alleles have been correlated with rapid disease progression, as HLA-A24, -A29, -B35, -C4, -DR1, and -DR3, whereas patients carrying HLA-B14, -B27, -B57, -C8, or -DR6 appear to develop AIDS more slowly [114, 115]. It is known that LTNPs remain asyntomatic and clinically healthy for more than 10 years without therapy [116, 117]. A number of studies [118–120] have shown a higher frequency of the HLA-B27 and HLA-B57 alleles in this group of patients, suggesting also a role of these alleles in the pathogenesis of HIV [111]. It has been shown that $CD8^+$ T cells of individuals expressing HLA-B27 or -B57, targeted a defined and highly conserved region within the HIV-1 p24 Gag (amino acids 240 to 272) early in infection, and responses against this region contributed over 35% to the total HIV-1-specific T cell responses in these individuals. In contrast, this region was rarely recognized in individuals expressing HLA alleles associated with rapid disease progression [121].

The HLA genotyping could help identifying those patients with either a mild or aggressive course of their natural HIV-1 infection. This might influence the timing to start HIV therapy and allow a risk stratification that could be relevant in countries with limited access to antiviral drugs. Moreover, under antiretroviral therapy, HLA type may influence the risk of developing drug-resistant viruses, and therefore address the choice of drug regimens in naïve patients. Finally, since HLA loci vary by ethnic groups and regions, it may affect the response to specific vaccines in different parts of the world.

Recently, the association between polymorphisms in the inflammasome component NLRP3 and susceptibility to HIV infection has been demonstrated and adds to other studies linking inflammasome activation and IL-1/IL-18 production with HIV pathogenesis [122].

For the association of TLRs polymorphisms and HIV-induced inflammation, somewhat more insight exists. One study reported an association between two single nucleotide polymorphisms (SNP)s in TLR9 and rapid HIV progression as measured by $CD4^+$ T cell decline [123], although the investigators did not evaluate the precise effect of these SNPs on TLR9 signalling. In contrast, a different TLR9 polymorphism has been linked to slow disease progression and found less frequently among individuals with high viral set point [124]. In addition, a frequent functional TLR7 polymorphism resulting in significantly less IFN-$\alpha$ production has been associated with accelerated disease progression and may also be associated with increased HIV susceptibility, since this mutation was present more frequently in patients than in controls [125]. The importance of TLR7 signalling was further supported by a recent article demonstrating sex differences in the TLR7-mediated response of pDCs to HIV: pDCs from women produce markedly more IFN-$\alpha$ in response to HIV-derived TLR7/8 ligands than pDCs from men, resulting in a higher degree of immune activation in women for a given viral load [126]. At the genetic level, this may be explained by the fact that TLR7 is X-linked and therefore women may have higher expression of this receptor due to unbalanced X-inactivation. Clinically, the more robust IFN-$\alpha$ response in women is translated into women exhibiting lower viral loads early in infection but progressing faster to AIDS for any given viral load [127]. Taken together, these studies support the idea of type I IFN having dual functions, including antiviral activities and immune activation.

*4.2. HCV.* Combination therapy with Peg-IFN plus ribavirin is the standard treatment for patients with chronic HCV infection [128]. However, it eradicates HCV in only about half of the patients infected with genotype 1. The outcome of IFN-based therapy for HCV is dependent on the genetic systems of both the human host and the virus. Host genetic factors that influence the outcome of therapy include gender, race, and variation in genes of the immune system.

The dominant role of the immune-modulator IFN-$\alpha$ in treating HCV infection has driven an extensive search for genetic associations between components of the immune system and outcome of therapy [129–131].

To date, the most prominent human genetic associations with outcome of therapy are SNPs within the IL28B gene. The strongest association was found for SNP rs12979860, located about 3 kb upstream of the IL28B coding region. Patients with a CC genotype at this SNP were more than twice as likely to achieve sustained virologic response (SVR) as patients with a CT or TT genotype [132]. Suppiah et al. [133] estimated that the cumulative effect of the favorable allele at the IL28B locus is to increase SVR by 32% relative to a population in which the allele is absent. The favorable association of the CC genotype was found in patients of both European and African-American descent, and differential prevalence of the CC genotype explained approximately half of the twofold poorer response rate to treatment found in African Americans. Several reports have associated failure of response also with the presence of SNP rs8099917 and rs12980275. All these associations were shown to be significant for genotypes 1 and 4 but not for genotypes 2 and 3 [134, 135].

The IL28B gene encodes the type 3 IFN, namely, IFN$\lambda$ that has been shown to inhibit HCV replication; so the variations in the IL28B gene mutations presumably affect the efficacy of the innate immune response against HCV during therapy [134].

Interestingly, the pretreatment expression levels of genes involved in IFN-stimulated genes (ISGs) have been found to be related to treatment outcome. The allele OASL rs12819210 constitutes an important independent factor that is significantly associated with SVR in peg-IFN-plus-ribavirin treatment, notably improving the predictive value of IL-28B rs12979860. In addition to OASL and IL-28B, the IFIT1 rs304478 A/A genotype favors a better therapy outcome, especially in patients with HCV-1 [136].

The most common associations with clearance after primary infection has been with the class II alleles, DQB1*0301, and DRB1*1101 which are in close linkage disequilibrium. It has been demonstrated that immunodominant HCV epitopes are presented by DRB1*1101 [137] and that T cell lines recognize HCV peptides presented by DQB1*0301 [138]. Similarly, another allele that has been associated with HCV clearance, DRB1*0101, was associated with a greater magnitude and broader range of epitope responses in an HCV-specific T cell proliferation assay [139].

By contrast, only very few of class I HLA allele associations have been described in association with clearance of HCV viremia. KIR2DL3 and its ligand, HLA-C1 has been associated with an increased likelihood of spontaneous and treatment-induced HCV clearance [140–142]. It has been postulated that this gene combination is protective because the KIR2DL3 binds HLA-C with a lower avidity than other inhibitory KIR and thus NK cells expressing this specific inhibitory receptor have a lower threshold for activation [143, 144].

NK cells are also inhibited by the heterodimeric receptor CD94 : NKG2A, which has the oligomorphic MHC class I molecule HLA-E as ligand. In a genetic study, homozygosity for the HLA-$E^R$ allele has been shown to be protective against chronic infection with HCV genotypes 2 and 3 [145]. This protective effect was thought to be due to an effect on HLA-E-restricted T cells, although the HLA-$E^R$ allele may have a lower affinity for peptides and hence be expressed at lower levels. This could, therefore, lead to less inhibition of NK cells via the CD94 : NKG2A receptor [146]. The presence of HLA-B27 allele has also been associated with viral clearance [147] and a functional mechanism for this effect has been reported with the recognition of an HLA-B27 restricted CD8 epitope [148].

Genetic associations with outcome of therapy have also been reported for the genes encoding KIR2DL5, IL-6, IL-12B, IL-18, CCL5, TNF-$\alpha$, IFN-$\gamma$, osteopontin, GNB3, and CTLA4. Several SNPs of TLR, relevant adaptor molecules and cytokines mediated by TLR signaling can affect various clinical outcomes in Caucasian patients with chronic HCV. However, the role of these polymorphisms in acute infection has not been elucidated [149]. However, other studies have failed to find associations with many of these genes. The inconsistencies in these data appear to be due to differences among the studies in racial make-up and HIV co-infection status of the cohorts, the definition of therapeutic response, the therapy employed (IFN-$\alpha$ monotherapy, IFN plus ribavirin, or pegylated IFN plus ribavirin), and the HCV genotype infecting the study participants.

Other immunogenetic polymorphism have recently been reported to influence outcome of HCV infection such as polymorphisms of the promoter region of the IL-10 gene with the IL-10-592 AA genotype associated with self limiting infection, and the IL-10-1082GG genotype associated with persistent infection [150, 151]. Another study demonstrated an association between the IL-10 ATA haplotype (IL-10-1082A, -819T, -592A) and spontaneous HCV clearance [151]. The authors postulated that the reduced production of IL-10 associated with this haplotype may result in a Th1-polarized $CD4^+$T cell response, which may be associated with enhanced viral elimination.

The presence of polymorphisms of the TGF-$\beta$1 gene promoter that reduce expression of TGF-$\beta$1 is also associated with increased rates of clearance of HCV infection [152].

Simultaneous genotyping for all these SNPs could be a useful tool for individualizing antiviral therapy for chronic hepatitis C.

## 5. Conclusions

HIV and HCV share many characteristics: both are RNA viruses and have similar blood-to-blood transmission routes. Despite these viruses markedly differ in their virological properties and in their pathogenesis, they share many common features in their immune escape and survival strategy.

In the last years, much effort has been done on the study of the AIDS pathogenesis and on the development of efficient treatment strategies and a fatal infection has been transformed in a potentially chronic pathology. Much of this knowledge is now being transferred in the HCV research field, especially in the development of new drugs, although a big difference still remains between the outcome of the two infections, being HCV eradicable after treatment, whereas HIV eradication remains at present unachievable due to the establishment of reservoirs.

In the era of effective HIV therapy, chronic HCV infection is a leading cause of liver disease and mortality in HIV-infected patients. Whereas treatment of HIV with ART appears to slow the progression of liver disease, co-infected patients remain at greater risk for HCV disease progression than patients with HCV mono-infection. Accordingly, effective HCV treatment is a priority in this population.

The knowledge of the immunogenetic background may enable to calculate a composite genetic risk, which will be of increasing importance in the tailored management of HIV and HCV mono-infected or co-infected patients. Obtaining a molecular understanding of the mechanisms by which the genetic associations modulate the outcome of therapy would substantially improve therapy. In addition, the immunologic characterization of the patients could be useful to speculate on the need for immunotherapy for these patients.

## Authors' Contribution

The first two authors contributed equally to this work.

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*Review Article*

# Interplay between HIV-1 and Host Genetic Variation: A Snapshot into Its Impact on AIDS and Therapy Response

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As of February 2012, 50 circulating recombinant forms (CRFs) have been reported for HIV-1 while one CRF for HIV-2. Also according to HIV sequence compendium 2011, the HIV sequence database is replete with 414,398 sequences. The fact that there are CRFs, which are an amalgamation of sequences derived from six or more subtypes (CRF27_cpx (cpx refers to complex) is a mosaic with sequences from 6 different subtypes besides an unclassified fragment), serves as a testimony to the continual divergent evolution of the virus with its approximate 1% per year rate of evolution, and this phenomena *per se* poses tremendous challenge for vaccine development against HIV/AIDS, a devastating disease that has killed 1.8 million patients in 2010. Here, we explore the interaction between HIV-1 and host genetic variation in the context of HIV/AIDS and antiretroviral therapy response.

## 1. Introduction

The evidence for HIV to be the causative agent of AIDS was documented way back in 1983, and, hitherto, the dreadful HIV remains unconquered [1]. As of 2010, 34 million people are living with HIV infections and 2.7 million people have been newly infected in that year alone [2]. This alarming statistics have accelerated much research into the biology of HIV, seeking clues on "Achilles heel" so as to curtail its spread and eventually to eradicate it.

## 2. HIV-1 Origin and Diversity

HIV-1 and HIV-2 cause AIDS, and HIV-1, with its tremendous diversity, outwits HIV-2 by its ability to inflict a more virulent form of the disease and has global distribution [3]. Both viruses originated in Africa, and viral zoonosis resulted in the rampant AIDS epidemic. Simian immunodeficiency virus (SIV) from chimpanzees ($SIV_{CPZ}$) is closely related to HIV-1, while SIV from sooty mangabeys ($SIV_{SM}$) forms the closest to HIV-2 [4, 5]. HIV-1 viruses fall under three main phylogenetic lineages, namely, M (Main), O (outlier), and N (non-M/non-O), all considered to have originated from chimpanzees dwelling in the eastern equatorial forests of Cameroon, West Central Africa, with O group viruses through a gorilla intermediate [6–8]. SIV infected *Pan troglodytes troglodytes* (Ptt) chimpanzees gave rise, through cross-species transmission, to HIV-1 groups M and N viruses while SIV-infected gorillas (*Gorilla gorilla*; SIVgor), which themselves contracted infection originally from chimpanzees, gave rise to formation of group O HIV-1 viruses. Recently, a variant of HIV-1 group O virus has been detected—P group—which resembles more closely to SIVgor than O group virus, in individuals of Cameroon origin [9, 10]. Studies have estimated the timing for origin of each lineage of HIV—HIV-1 group M, O, and N at 1931 (1915–1941), 1920 (1890–1940), and 1963 (1948–1977), respectively [11–13]. HIV-2 viruses are considered to have originated around 1930s [13].

Group M HIV-1 viruses are further subdivided into nine major subtypes, namely, A-D, F-H, J, and K. Sub-subtypes have been reported for clade A (A1 and A2) and F (F1 and F2) viruses. Group M also includes circulating recombinant forms (CRFs). Figures 1(a) and 1(b) illustrate global distribution of HIV subtypes and most common CRFs, respectively. Circulating recombinant forms arise as a result of

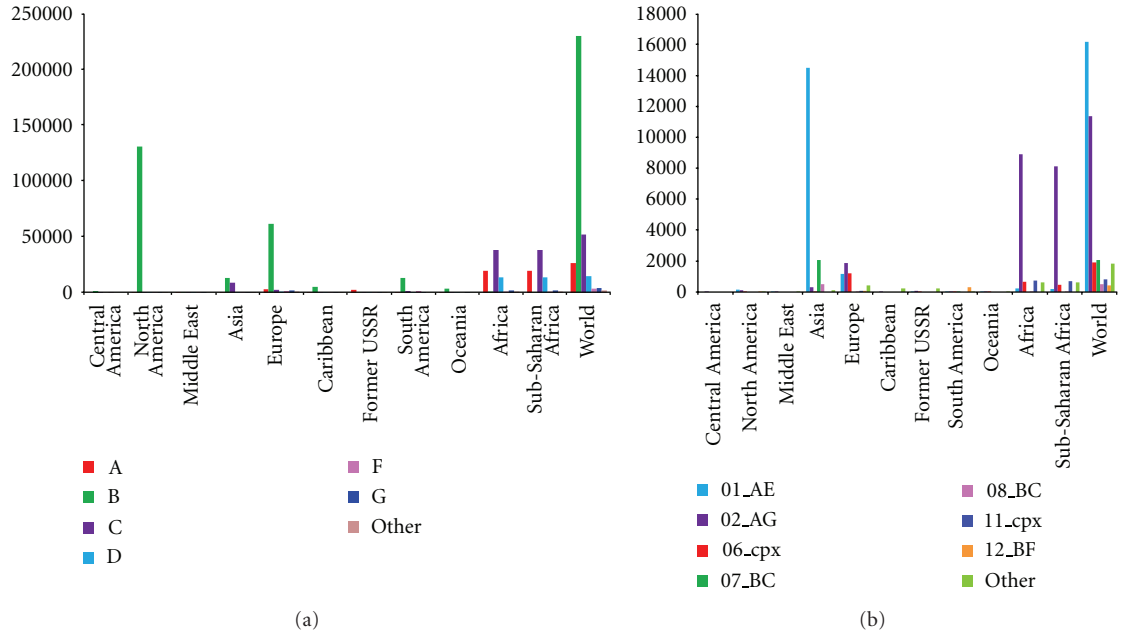
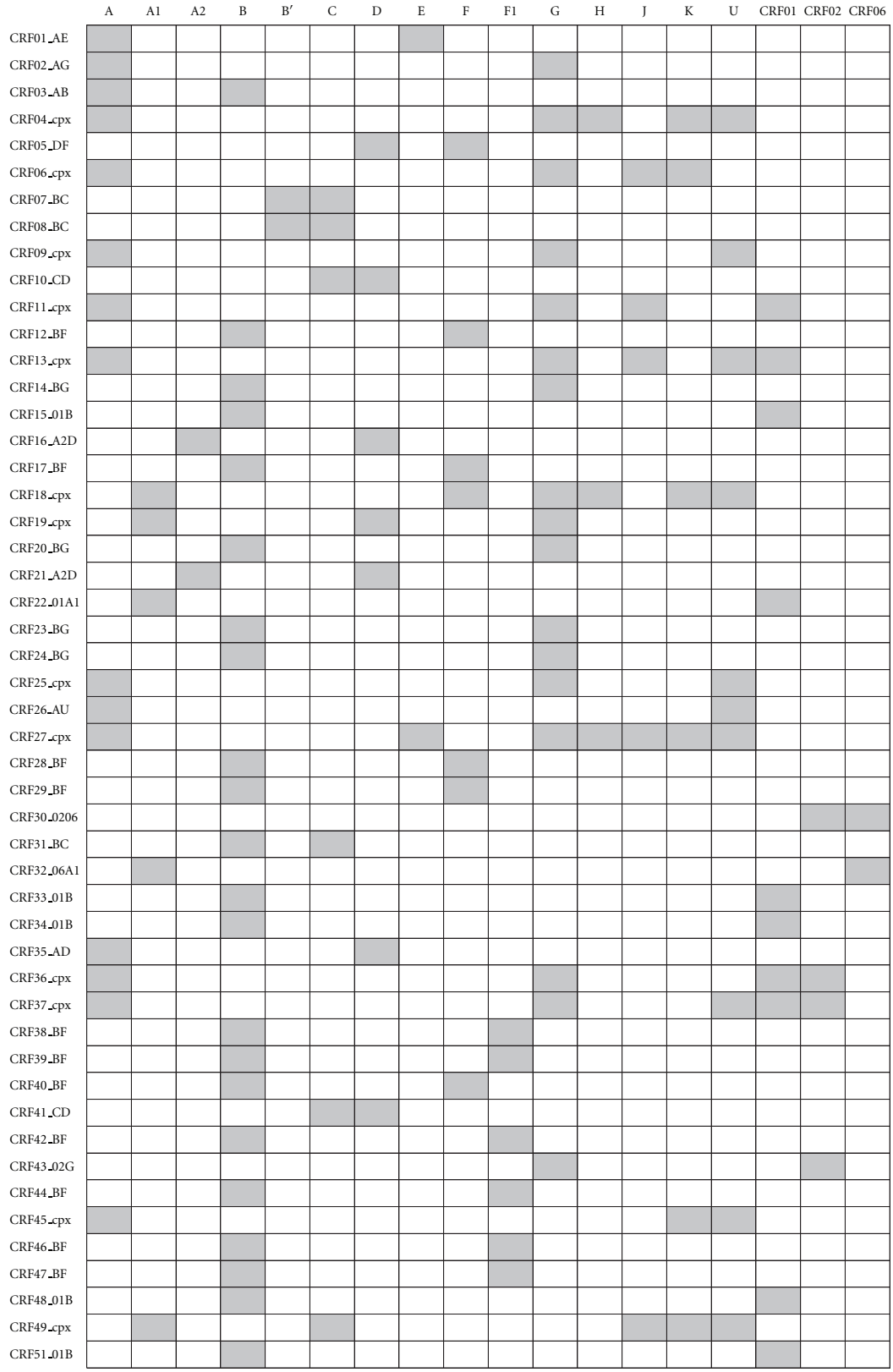



Figure 1: (a) Global distribution of HIV-1 (nonrecombinants) sequences. (b) Global distribution of recombinant HIV-1 sequences. Source: HIV databases [14].

recombination between any of the subtypes and/or CRFs leading to the formation of CRFs such as AE, AG, AB, DF, BC, CD, and other complex forms. Figure 2 provides a schematic representation of composition of 50 CRFs that have been identified hitherto [14]. The subtypes and CRFs attest to the genetic diversity of HIV-1. These M group viruses cause most of the HIV-1 infections, accounting for the current AIDS pandemic. Circulating recombinant forms account for about 10% of all HIV infections [8], and the proportion might increase in future. Different subtypes among HIV-1 viruses impact disease progression differently [15] and may also have differential sensitivity to antiretroviral therapy (ART) drugs [16]. Individuals infected with subtype D viruses are known to experience rapid disease progression [17], whereas those infected with subtype C undergo slow disease progression [18]. During the course of HIV-1 infection, strains that utilize coreceptor CXCR4, emerge during late stages of infection in contrast to CCR5 utilizing M tropic strains that are seen during early stages of infection. The strains that use CXCR4 are known to exhibit cytopathic effect *in vitro* [19]. However, this observation might be an *in vitro* artifact since little cytopathic effects were noticed *in vivo* [20]. There is differential usage of CXCR4 coreceptor among subtype C and D viruses, with subtype C viruses rarely switching to CXCR4 usage and subtype D viruses utilizing CXCR4 receptor earlier and frequently, and this, alongwith other factors like genetic variation in long terminal repeat (LTR) promoter, might account for their varied impact on disease progression [8, 21–24]. The promoter/enhancer activities of LTRs of subtype C viruses were shown to be higher than that of other subtypes A, B, D, E, and G [23], and subtle differences in promoter activity of LTRs might affect HIV replication kinetics substantially [24, 25]. The intrasubtype diversity is substantial among different subtypes of HIV-1. The protein sequence diversity among subtypes of HIV-1 group M viruses for Gag, Pol, and Env are reported to be 15%, 10%, and 24%, respectively [26]. Gag-30 position is phylogenetically important. The sequences of $SIV_{CPZ}Ptt$, from which HIV-1 originated, are marked by presence of Met at Gag-30. In contrast, the ancestors of all the three HIV-1 groups (M, O, and N) are marked by sequences that contain Arg at Gag-30, highlighting potential host-species-specific adaptation [27]. Understanding HIV evolution and the role that the host plays in mediating it and controlling infection will undoubtedly help to determine the effective approaches to combat HIV/AIDS.

## 3. Degree of HIV-1 Variability

HIV-1, with its RNA genome, demonstrates significant genetic diversity due its high mutation rate. It has diversified itself to such an extent, through its ability to form "cloud" of variants or quasispecies, that there is no single wild-type strain. *In vitro* data have shown that RNA viruses generate nonhomogeneous genetic clones that are closely related but genetically diverse, which are known as quasispecies. This phenomenon, which aids viruses to persist in their host, possibly causing disease, is observed in other RNA viruses such as hepatitis C and influenza virus as well [28, 29]. The reverse transcriptase (RT) of HIV-1, which lacks 3′-5′ exonucleolytic proof-reading function, misincorporates 1 in 6900 and 1 in 5900 nucleotides polymerized on the RNA and DNA template, respectively, and hence accounts for larger proportion of mutations seen in HIV-1 [30]. It has been estimated that, after a single round of HIV-1 replication, under




Note: U: unclassified

Figure 2: Schematic representation of composition of HIV-1 CRFs.

the assumption of absence of selection pressure, the resulting progeny viruses will have substitution, frameshift and deletions at 24%, 4%, and 2%, respectively [31]. It is interesting to note that 80% of heterosexual-mediated HIV-1 infections are due to productive infection by a single HIV-1 virion [32–34]. HIV-1 evolves at about 1% per year [35]. Given that HIV-1 faces selection pressures, a gamut of mutations has shaped its genome since its origin, which in turn, ensures its virulence at population level [36, 37], despite the fact that certain mutations in its conserved region impacts its fitness negatively [38]. Further, a recent study that utilized phylogenetic comparative approach revealed that viral genotype, as against the host genetic profile, largely determines the HIV set-point viral load and hence the virulence [39]. A schematic sketch of error-causing machinery involved in HIV-1 mutagenesis and a gamut of selection pressure acting on HIV-1 are provided in Figures 3(a) and 3(b), respectively.

*3.1. Ultra-Deep Sequencing Reveals Vastness of HIV-1 Variability.* With the emergence of new pyrosequencing technology, HIV-1 viral quasispecies are now more rapidly and accurately sequenced and analyzed. Mutation spectra of HIV-1 quasispecies are wide, and traditional sequencing methodologies are limited in their ability to capture minority variants [40]. Next generation sequencing (NGS) methodologies have made it possible to obtain high-throughput sequence data at an unprecedented pace and coverage (e.g., pyrosequencing using GS FLX+ system permit characterizing up to 1000 bp read length with 1,000,000 reads per run at run time of 23 hours and consensus accuracy of 99.997%) [41, 42] and are being employed to decipher HIV-1 evolutionary trajectories [34, 43]. Recently, Liang et al. [44] used 454 pyrosequencing technology and sanger clone-based sequencing to assess the genetic diversity of HIV-1 gag and it was determined that pyrosequencing detected almost four times more variation in gag than sanger sequencing. Ultra-deep sequence sets of HIV-1 allow for deciphering CTL escape variants that are not discernable with the sequences obtained through conventional sequencing strategies [34, 44]. While single genome amplication (SGA) is superior to standard genotyping method [45], ultra-deep sequencing methods offer highest sensitivity to date in relation to those conventional methods as it can detect minor viral variants that comprise lower than 1% of the population. This highest level of sensitivity by ultra-deep sequencing also allows for identifying low-abundance drug resistant variants [40], with potential to interfere with ART outcome. Not only NGS techniques are used to gain insights into the sequence of the viral genome with greater depth, it has also been recently utilized to examine viral diversity after therapy. For example, a study that used deep sequencing technology to examine escape mutations in the V3 loop of HIV-1 that arise as a result of selection by CCR5 antagonist (vicriviroc, a drug that inhibits HIV-1 entry) therapy indicated significantly higher sequence heterogeneity [43]. Knowledge on nature of HIV-1 quasispecies gained through ultra-deep sequencing technologies can aid in progressing HIV research and managing HIV/AIDS clinically better. Finally, with advanced whole genome sequencing technologies, the ability to correlate genome profile of HIV with that of patients could lead to comprehensive understanding of disease process and effective interventions.

*3.2. Factors Driving HIV-1 Variability*

*(a) Inherent Property of the Reverse Transcriptase (RT) and Recombination.* The generation of diverse variants in HIV-1 can be attributed mainly to its low-fidelity RT enzyme, leading to error-prone reverse transcription [30]. RT also accounts for genomic heterogeneity in progeny viruses through its role in recombination. Besides RT, which accounts for larger proportion of mutations observed in HIV-1, the host RNA pol II involved in transcription of proviral DNA can also contribute to mutations, albeit minimally. A study has indicated contribution of cellular RNA pol II to be less than 3% of retroviral frame-shift mutations [46].

HIV-1, with documented dual and triple infections in patients [47, 48], can substantially drive production of viral quasispecies that are endowed with superior fitness through the process of genetic recombination, a time-tested evolutionary strategy to thrive in a changing environment. With an average of $1.38 \times 10^{-4}$ recombination events/adjacent sites/generation *in vivo* [49], HIV-1 ensures its ability to enrich both diversity and fitness. In HIV-1, recombination in genomic regions with high selection pressure, either in the form of host immune response or ART drugs, could lead to selection of more fit genomes, while, in regions under negligible selection, recombination can enhance diversity [50]. HIV-1-infected commercial sex workers in Nairobi, Kenya, were shown to harbor high proportion of recombinant HIV-1 viruses [51, 52]. Recombinants between highly similar HIV-1 strains are formed at highest frequencies while that between very distant HIV-1 strains occur at very low frequencies [53]. Genetic recombination between HIV-1 and HIV-2 is also a potential possibility [54].

*(b) Swift Turnover Rates of HIV-1 In Vivo.* HIV-1 virions are produced and cleared at extremely rapid pace. HIV-1 turnover is high at $10^{11}$ virions and $10^8$ infected cells per day [45]. Studies have estimated that free HIV-1 viral particles have an half-life of less than 6 hours, while the productively infected cells possess an half-life of about 1 day [55]. This rapid turnover has been considered as the major factor underlying pathogenesis of HIV/AIDS, wherein there is greater destruction of $CD4^+$ T helper lymphocytes.

*(c) Drugs of ART Drive Changes in HIV Genetic Makeup.* Antiretroviral drugs as well as associated drug resistance mutations could influence *in vivo* HIV-1 mutation rates. The drug 3′-azido-3′-deoxythymidine (AZT) can enhance HIV-1 mutation rate by a factor of seven per round of replication, and HIV-1 variants harbouring AZT resistant RT can incur higher mutation rate as much as threefold relative to wild-type RT bearing HIV-1 [56]. The V106A is a nevirapine (nonnucleoside RT inhibitor) resistance mutation that has been consistently shown to affect viral fitness severely [57]. Mutations associated with HIV-1 drug resistance could be

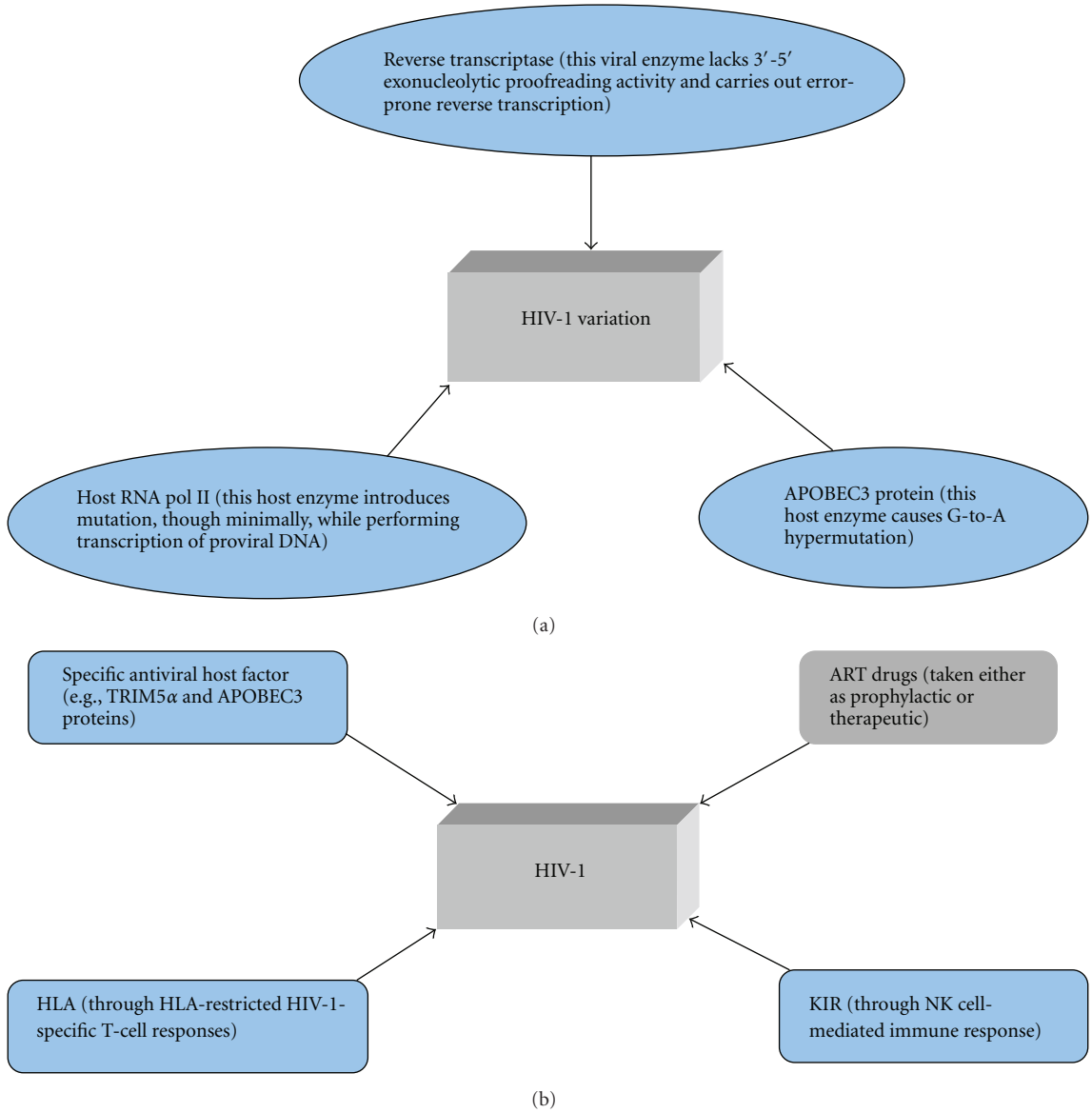



Figure 3: (a) Error-causing machinery involved in HIV-1 mutagenesis. (b) A schematic sketch of selection pressures acting on HIV-1. Note: ART drugs block is shown in grey colour to differentiate from others, as such drugs exert influence over HIV-1 indirectly in patients undergoing ART.

present in drug-naïve patients at low frequencies, often interfering with outcome of ART [58]. Metzner et al. [59] have reported occurrence of M184V (RT) and L90M (protease) mutations as minority populations in patients undergoing structured treatment interruptions. A comprehensive list of ART drug-associated mutations is being maintained in Stanford HIV Drug Resistance Database [60]. Further discussion on ART drugs is covered in Section 6.

*(d) Selective Immune Pressures from Host.* There is heterogeneity in disease progression among HIV-1-infected patients. Host genetic variants have been shown to account for at least 15% of the observed differences in disease progression [61]. Human leukocyte antigen (HLA) system, residing in the sixth chromosome, is the most polymorphic loci in human genome and this extensive polymorphism is the result of evolving with millions of pathogens that the human species has faced throughout its existence [62, 63]. This elaborate polymorphic nature of HLA alleles enables them to recognize various protein fragments of pathogens and present to T cells for generation of appropriate immune responses. Among these host genetic factors, HLA class I molecules, which present peptides to cytotoxic T cells, have been shown to exert profound influence over controlling the HIV-1. The protective alleles, HLA-B*57, -B*27, and -B*51, through presentation of highly conserved HIV-1 epitopes to cytotoxic T lymphocytes (CTL) and subsequent immunodominant

immune response, drive formation of specific CTL escape mutants, that are compromised in their replicative fitness [64–68]. These CTL escape mutants, depending on nature of HLA-mediated immune pressure from subsequent hosts in the transmission chain, may get either fixed in the population or get reverted to their wild type form [38, 69]. Selection pressure exerted by host on HIV-1 is discussed further under the subsequent Section 4.

## 4. Host Genetic Factors Influence HIV-1 Evolution

HIV-1 adapts to host immune pressure, and this is revealed through studies of positively selected amino acid changes in different proteins of the virus [70–74]. An immunoinformatic analysis that looked at envelope sequences across clades from varied geographical regions has indicated differences in frequency of positive selection (PS) sites, suggesting that viral clades prevalent in various geographically distinct parts of the globe evolve in response to the characteristic immunogenetic profile of the host population [70]. Evolutionary pathways of HIV-1 appear to be vast with occurrence of positive selection sites not only in epitopes of $CD4^+$ and $CD8^+$ T cells and antibodies, wherein HLA impacts profoundly, but also in other regions which are likely to suffer selection pressure via effectors of innate arm of the immune system such as KIR and HIV restriction factors TRIM5$\alpha$, APOBEC3G [75]. In contrast, HIV-2 that causes less severe form of disease face significant negative selection pressure [76].

Several AIDS restriction genes have been identified [77, 78]. Among these HLA, killer-cell immunoglobulin-like receptors (KIRs), chemokine receptors and intrinsic antiviral factors like TRIM5$\alpha$, APOBEC3 are known to exert substantial influence over HIV/AIDS and affect HIV-1 evolution. TRIM5$\alpha$, for example, has the ability to recognize the capsid protein of the incoming virus and disassembling it upon entry [79]. APOBEC3 proteins are another group of host restriction factors that play a role in reducing viral infection, including HIV-1 [80]. These host proteins are cytidine deaminases that catalyze the deamination of cytidine to uridine, which results in guanosine to adenosine hypermutation in the concerned opposite strand, favouring inactivation of the virus [79, 81]. HIV-1 negates the antiviral effects of APOBEC3G (A3G) through its Vif (viral infectivity factor). Viral infectivity factor promotes proteosomal degradation of A3G in an incomplete fashion, as a result of which there is generation of hypermutated viral population that could still survive, aiding HIV-1 evolution and possibly favouring emergence of drug resistant forms [82]. Fourati et al. [83] showed that HIV patients exhibiting virological treatment failure often possessed K22H point mutation in Vif, which resulted in inability of Vif to counteract APOBEC3 proteins, ultimately leading to G-to-A hypermutation in HIV. Several other studies have also demonstrated role of APOBEC3 proteins in HIV-1 evolution [84–87]. Recently, study by Norman et al. [88] indicated that HIV-1 also employs viral protein R (Vpr) to negate A3G antiviral properties by diminishing the incorporation of uridines in the deamination process. Interestingly, this act by Vpr results in favour of host as DNA damage response pathway got triggered and NK cell-activating ligands got expressed, making the virus vulnerable to attack by NK cells [88]. Human tetherin also acts to prevent the spread of HIV-1 infection to other cells. Tetherin, a cell surface host protein, is able to trap virions that are being released from the surface of the infected cell [79, 89]. Differential adaptation of HIV-1 viruses to antiviral activity of tetherin has been recently confirmed. Yang et al. [90] demonstrated that while Vpu of both group M and N HIV-1 viruses had activity against human tetherin, Vpu and Nef from group O and P viruses lacked such anti-tetherin activity. A very recent study by Liu et al. [91] reported identification of 114 intrinsic host factors with significant ability to inhibit HIV-1 infection and this illustrates the tremendous pressure HIV-1 is subjected to, upon entry into its host. It could be inferred that various host genetic factors might additively contribute to controlling the virus. The complex HIV-1 host interactions are being dissected using genome-wide and large-scale strategies to map virus-host interactions comprehensively [92].

*4.1. HLA Leaves Footprints on HIV-1.* The impact of HLA diversity on HIV evolution has been documented in several studies. Different HLA alleles have been shown to be associated with different rates of HIV disease progression. For example, patients who possess HLA-B*27 and -B*57 alleles normally have low viral loads and progress to AIDS at a much slower rate, while those possessing HLA-B*35 progress to AIDS defining illnesses rapidly [64]. HIV-1 is under pressure from HLA-mediated CTL responses quite early in the infection as CTL escape mutations have been shown to arise as early as 14 days of postinfection [93]. CTL epitopes, are reported to be more conserved compared to $CD4^+$ T helper and monoclonal antibody epitopes and this conservation of CTL epitopes has been suggested as a host strategy to constrain HIV-1 adaptation [94]. Evidence of HLA footprint on HIV-1 genome is demonstrated by studies that have investigated the mutation profile of original infecting HIV-1 strains. Leslie et al. [95] analyzed the mutation of clade B and C HIV-1 in patients with HLA-B*57/58 : 01 allele, which are associated with slow progression to AIDS. It was observed that positively selected amino acids had accumulated and, once transmitted to HLA-B*57/B*58 : 01 negative individuals, the virus reverted back to its wild-type form [95]. This illustrates the ability of HLA alleles to drive the necessary mutation in HIV, as part of controlling the infection. In another study, the carriage of HLA-B*57 allele in patients infected with HIV-1 and its impact on viral control was assessed. It was demonstrated that individuals expressing the HLA-B*57 allele controlled viremia without therapy at levels <5000 copies/mL of virus for upto 29 months, and a stronger and broader response was generated by HLA-B*57 allele than other HLA class I alleles [96]. A Swiss HIV cohort study, that reported similar transcriptome profile of $CD4^+$ and $CD8^+$ T cells among rapid progressors and pathogenic SIV-infected rhesus macaques, also found underrepresentation

of protective alleles and overrepresentation of risk alleles at HLA loci in rapid progressors [97]. HLA selection pressure on HIV-1 is so fine-tuned that micropolymorphism seen among subtypes of a particular allele could exert differential pressure on virus. This phenomenon has been recently demonstrated for the HLA-B*57 alleles [98]. The support for extensive HLA associated selection in HIV-1 is also evident in the recent study by Dong et al. [99], wherein they followed a narrow-source HIV-1 outbreak, that occurred through a plasma donation scheme in a Chinese village and found 24–56% of the polymorphic sites across Gag, reverse transcriptase, integrase, and Nef had HLA footprints. A comprehensive genomewide association analysis has revealed that amino acids at positions 67, 70, and 97 in HLA-B play a major role in determining HIV-1 control, given their involvement in peptide binding within the peptide binding groove [100].

HLA-B loci, the most rapidly evolving class I region, contains alleles that exert strong selection pressure over HIV-1 through their allele-restricted $CD8^+$ T cell responses and contributes to shaping of HIV-1 evolution [101, 102]. HLA-B might predominantly shape HIV and vice versa, a coevolution scenario [67, 101] as exemplified by Red Queen Hypothesis [103]. Also rapid selection for HLA alleles that protect against HIV-1 infection has been found to correlate significantly with declining incidence of HIV-1 in an East African sex worker cohort of Kenya, which suggests that natural selection might eventually play a vital role in containing the HIV-1 epidemic [104]. It may be plausible that mutome of HIV and human is being shaped by each other in a very delicate dynamic process of virus-human partnership.

Intrinsic and adaptive immunity might work synergistically to contain HIV-1. This can be inferred from the studies that have dissected role of HLA and KIR compound genotypes over HIV disease progression [105]. Further, recently CTL escape mutations in Gag have been shown to enhance sensitivity of HIV-1 to TRIM5$\alpha$ [106]. It is plausible that HIV-1 could suffer a double whammy attack-one, fitness cost due to mutation in a highly conserved region and, second, increased vulnerability to attack from TRIM5$\alpha$. In order to survive host immune pressure, which is predominantly dictated by HLA, HIV mutates at specific epitopic region and this escape variant to survive further without compromising its fitness might undergo compensatory mutations in regions away from the concerned epitope. Though HIV-1 mutates rapidly as a stochastic process, its mutational strategies are relatively predictable. The studies employing HIV-1-infected identical twins suggested the presence of a relatively narrow window period in HIV infection, wherein the immune responses, viral evolution as well as disease progression are somewhat reproducible and hence predictable [107–109]. Moreover, recently Dahirel et al. [110] have elegantly carried out coordinate linkage analysis employing a physics concept to highlight multidimensionally constrained regions of HIV-1 proteome. They have identified HIV sectors that is, distinct sets of amino acids whose mutations are collectively coordinated and indicated that among the five sectors of Gag, sector 3, which plays vital role in assemblage of multiprotein structures for formation of HIV-1 capsid, is the most immunologically vulnerable multidimensionally constrained and also is the sector most targeted by elite controllers of HIV-1, who harbour protective HLA alleles. These studies bear potential clues for designing successful anti-HIV immunogens. While role of HLA class I alleles in attenuating HIV-1 is vastly studied and supported by several findings, part played by HLA class II alleles has been scarcely investigated [64, 111, 112]. A recent study that investigated the correlation between HLA class II alleles and *in vitro* replication capacities of recombinant viruses encoding Gag-protease from HIV-1 subtype C infected chronic patients failed to detect any association of alleles with lower fitness [113]. However, earlier studies have demonstrated potential role of HLA class II alleles in exerting selection pressure on HIV-1 [114, 115]. More studies are warranted, given the reported significant genetic associations of alleles belonging to HLA-DR, -DQ, and -DP loci with HIV infection and disease [64, 111, 116–120], to delineate degree of immune pressure exerted by different HLA class II alleles, the players in generating the essential T helper cell responses. T-cell-based vaccine strategies that could address HIV-1 diversity issues better are being tested [121, 122].

*4.2. KIR Footprints on HIV-1.* Killer-cell immunoglobulin-like receptor (KIR) encoding genes are located on chromosome 19, and their major role is to control the activation or inhibition of Natural Killer (NK) cells, which belong to the innate arm of the immune system. KIRs are quite polymorphic, and thus they are able to generate a diverse response to a variety of pathogens. KIRs mediate their effects using HLA molecules as ligands [123, 124].

HIV-1, like other viruses, down-regulate HLA class I molecules, specifically HLA-A and -B, and hence escapes from those HLA-mediated CTL effectors. However, in order to escape attack by NK cells, which destroys target cells lacking expression of HLA class I molecules, HIV-1 avoids downregulating KIR-interacting HLA-C or the nonclassical HLA-E molecules [125]. KIRs are known to impact HIV-1 disease outcome both independently and synergistically through its interaction with HLA ligands [126, 127]. A recent study has demonstrated role of copy number variation in KIR genes in influencing HIV-1 control [128]. Alter et al. [129] have shown that HIV-1 evades NK cell-mediated immune response by selecting for viral variants that modulate recognition of infected cells by KIR to their advantage. Specifically they identified 22 KIR-associated polymorphisms in HIV-1 from a cohort of 91 untreated chronically HIV-1-infected patients. HIV-1 viruses with Vpu (71 M/74 H) (Env (17 W/20 M)), Gag (138I), and Nef (9 K) were found to be significantly enriched in individuals possessing KIR2DL2, and these KIR footprints enhanced the binding of inhibitory KIR to infected cells, due to which inhibition of NK cell function ensues and HIV-1 escapes attack [129].

## 5. Problems Posed by HIV-1 Diversity

*5.1. Search for a Broadly Cross-Reactive Anti-HIV Neutralizing Antibody.* HIV-1 diversity is one among several challenges that needs to be addressed while attempting to design an

effective anti-HIV vaccine. Generating broadly neutralizing antibodies (bnAbs) that can effectively inactivate or neutralize HIV variants remains elusive [130]. Broadly neutralizing antibodies are rare and undetectable in most HIV-1-infected individuals. Several hypotheses exist that attempt to explain the rarity of bnAbs. For example, one reason that has been proposed is that highly immunogenic epitopes may trigger nonneutralizing antibodies instead of activating required specific responses [131, 132]. However, nonneutralizing antibodies could be functional against HIV-1, as observed in study subjects of RV144 Trial [133], and have potential to mediate protection against HIV-1. Studies have also shown that antibodies sometimes select for escape mutations [134, 135]. There are four regions of HIV-1 Env that could serve as targets for bnAbs: gp120 CD40 binding site, quaternary V2/V3 loop epitopes, gp41 Membrane proximal external region (MPER), and Env carbohydrates [132]. Human immunoglobulin, VRC01, is capable of neutralizing 90% of the HIV-1 isolates [136]. Recent studies have delineated both evolutionary course and nature of VRC01-like antibodies [137, 138], and this knowledge has opened up new avenues for strategies to attack HIV-1 better.

*5.2. Correlates of Protection Obscured by HIV-1 Evolution.* Certain host genotypes known to be favourable prior to ART might turn out to exert a detrimental or neutral effect upon initiation of treatment. This intriguing observation is being reported frequently, and yet the mechanism underlying the association is unclear. Rauch et al. [139] reported that Bw4 homozygosity, associated with protection in untreated patients, predicted impaired CD4 T-cell recovery upon commencement of combination ART. Another study revealed strong association of HLA-B*57:01 and -B*58:01, both exhibiting Bw4 motifs, with failure to control HIV replication following HAART initiation [140]. While HAART exhibits the potential to suppress HIV replication profoundly irrespective of the genotype of the individual, documented association of specific highly protective alleles with differential outcome over treatment may have an unidentified functional immunological basis and warrants extensive investigation. Antiretroviral therapy-induced selection pressure on *pol* could lead to generation of HIV-1 quasispecies with significant changes in epitope profile, including loss of protective epitopes. Furthermore, HLA-KIR interaction might contribute to outcome of ART [139, 140] and may explain the conundrum of what is good before ART is not so after ART.

HLA-B*51 has been associated with protection against HIV-1 in Asian population [67, 68]. This allele has been able to confer a multilayered defence against HIV/AIDS through presentation of highly conserved immunodominant epitopes in Gag region, that rarely undergo mutation, and, if at all gets mutated, it is only at the cost of fitness. However, it has been noted that, over a period of time, at population level, the circulating viruses, as they evolve, tend to lose the epitopes targeted by the protective alleles such as HLA-B*51 [67, 68, 141], such that documented protective association is obscured and these evolutionary strategies by which HIV changes its genomic/proteomic landscape to stay ahead, pose tremendous challenge for scientists as they search for true correlates of protection against HIV/AIDS and venture into developing a stable and effective intervention-prophylactic/therapeutic vaccine [142, 143]. Interestingly, a mathematical modeling study has predicted that the rate of generation of escape mutants and the transmission of escape mutants may have only a weak impact on the epidemic outcome over the first 25 years after the introduction of a nonsterilizing anti-HIV vaccine [144]. However, search for a sterilizing vaccine for HIV/AIDS, a holy grail, remains vital aim in fight against HIV [145].

Characterizing immunological profile in both elite controllers and HIV-exposed seronegatives could lead to better understanding of correlates of protection [143, 146–151]. Additionally, understanding immunobiological basis of benign nature of disease induced by HIV-2 can provide clues into virus-host interaction and aid in tackling HIV-1. Expanding HIV-1 diversity might pose problems at diagnostic level given its impact on viral load testing assays [152].

## 6. Scope of ART in HIV-1 Control

According to UNAIDS world AIDS day report 2011, at least 6.6 million people in low- and middle-income countries are receiving HIV treatment and this has resulted in prevention of 2.5 million AIDS deaths since 1995 [2]. Also ART prevents infection, as it reduces viral load and infectiousness of an infected individual [153]. While this is an encouraging sign towards combating the HIV/AIDS epidemic, it is to be emphasized that current drugs in the prescribed regimen are unable to attack and eradicate the viruses hiding in reservoirs such as seminal vesicles [154] and tissue macrophages of HIV infected patients [155]. Given the evidences that suggest continual on-going replication of HIV-1 in such reservoirs [156, 157], it is plausible that quasispecies that are immune to current combination ART drugs can emerge upon treatment interruption. HIV-1 occupies variety of anatomic compartments such as central nervous system (CNS), gut-associated lymphoid tissue (GALT), and genitourinary tract [158, 159]. The CNS, endowed with blood-brain-barrier, is a pharmacologically "privileged" site, and the virus inside CNS thus gets shielded from attack by some ART drugs [159–161]. Genotypic diversity of HIV-1 is not uniform across different compartments [162]. This can be inferred by the fact that majority variant seen in blood is not always so in semen [163, 164]. Further, *env* sequences from blood and male genital tract compartments differ [165]. Venturi et al. [166] have observed different drug resistance mutation profile between HIV-1 isolates from cerebrospinal fluid and plasma in patients under nonsuppressive ART drug regimens. Indeed selective drug pressure has been shown to result in multiple drug-resistant HIV-1 quasispecies [167]. Viral rebound in patients who cease to continue with the ART is an added concern [168].

A recent study that evaluated the correlation of preexisting drug-resistant HIV-1 minority variants with risk of first-line nonnucleoside reverse transcriptase inhibitor (NNRTI)

based antiretroviral virologic failure, by reviewing 10 different studies, has suggested significant association of low-frequency drug resistance mutations with a dose-dependent increased risk of failure to control the virus [169]. Another cause for concern is the differential persistence of transmitted HIV-1 drug resistance mutation classes as reported by Jain et al. [170], wherein they indicated long-term persistence of NNRTI and protease inhibitor mutations which might facilitate person-to-person propagation. Adherence to drug regimen among HIV-1 patients is threatened by the fact that certain prescribed combination ART drugs could induce unfavourable side effects among patients with specific genotypes. For instance, hypersensitivity reactions (HSRs) are seen in HLA-B*57 : 01 positive HIV-1 patients receiving Abacavir (ABC), an nucleoside reverse transcriptase inhibitor (NRTI) drug [171]. While there are similar effects documented for other ART drugs [172], the association of ABC with HSR in HLA-B*57 : 01 patients is robust enough that screening for this allele has been made routine prior to prescription of combination ART regimen containing ABC [173]. More insights into pathophysiology of drug-induced HSR in HIV patients can ensure case-specific recommendation of combination ART drugs, averting compliance issues and emergence of drug resistant viral population. He et al. [174], by conducting a 7-year follow-up study on 437 HIV-infected Chinese patients undergoing HAART, suggested that two NRTIs and one NNRTI regimens could persistently suppress HIV viremia and enhance $CD4^+$ T-cell population with good safety and tolerance. The study also reported 19.2% of the participants changed to other first-line drug due to drug-related side effects and 10.2% switched to second-line regimens because of viral resistance. As UNAIDS and WHO advocate Treatment 2.0 [175], many such prospective studies analyzing outcome of HAART regimens are essential and antiretroviral pharmacovigilance [176] will attain greater importance.

HIV drug resistance could be either acquired or transmitted. According to recent initial survey conducted in low- and middle-income countries, WHO has reported acquired HIV drug resistance rate to be 6% while 3.7% rate for the transmitted HIV drug resistance [177]. The 2009 Surveillance Drug Resistance Mutation (SDRM) list has indicated 93 mutations including 34 NRTI-resistance mutations (at 15 RT positions), 19 NNRTI-resistance mutations (at 10 RT positions), and 40 PI-resistance mutations (at 18 protease positions), and this suggests the vast number of mutations linked to antiretroviral drug resistance [178]. The fitness landscape of HIV-1 RT and protease has been shown to be under strong epistasis [179]. Epistasis refers to a situation wherein action of one genetic locus masks the allelic effects at another locus, and the locus thus masked is referred to be "hypostatic" to the other locus [180, 181]. This phenomenon of epistatic interaction complicates comprehensive understanding of viral variants and their relationship to drug resistance. Even though today there are more than 20 different antiretroviral drugs to treat HIV-infected patients, a major global public health concern is the emergence of new strains that develop resistance to these drugs and subsequent transmission to other hosts [176].

Antiretroviral therapy has been quite successful which can be attributed to its ability to control HIV replication and preserve optimal $CD4^+$ T helper cell population, due to which many of the opportunistic infections associated with abnormal low $CD4^+$ T-cell counts are averted. However, tuberculosis (TB), caused by *Mycobacterium tuberculosis*, can occur at any stage of the disease, irrespective of $CD4^+$ T-cell counts, in HIV-1-infected patients [182] and poses a tremendous public health challenge in regions plagued by dual epidemic of HIV and TB. HIV-associated TB and hepatitis complicate the clinical management of individuals suffering from such coinfections, with potential for development of immune reconstitution inflammatory syndrome and drug-drug interactions are not clearly understood [183, 184].

HIV-1 latency presents challenges for the attempts directed at eradicating it. The half-life of the latent, replication-competent HIV-1 in resting $CD4^+$ T cells is roughly six months, which necessitates compliance to effective ART regimen for several years to clear the virus from reservoir [157]. Strategies are being contemplated to activate the HIV residing in latent reservoirs, in such a manner that does not allow wide-spread infection of uninfected cells [185, 186], and, in this regard, current and future ART drugs could play vital role in eradicating resilient virus.

## 7. Conclusion

The studies on biology of HIV-1 variation by characterizing emerging quasispecies population carries prognostic value as they impact rate of development of AIDS defining illnesses [187] as well as effectiveness of therapy [188]. Different recombinant forms of HIV-1 emerge, and they are seen to predominate in particular environments [189–191]. Given this scenario, more comprehensive epitope mapping studies with focus on CTL epitope escape mutants of CRFs, in addition to characterizing epitope profile of major HIV-1 clades, are warranted, and such findings will augment the efforts to curb spread of HIV-1, a virus nonpareil in medical history due to its ever elusive tricks inflicting damage to global health. The advent of HAART has made HIV/AIDS a life-threatening fatal into a potential chronic disease. However, HIV patients under long-term treatment are likely to have higher risk for medical complications like heart, liver, and neurodegenerative diseases, and hence there is an increasing need to deal with these additional health issues effectively [192]. A vaccine that could elicit sterilizing immunity against HIV/AIDS is much desired. Recent findings, RV144 Trial, with its finding that prime-boost vaccine combination of ALVAC-HIV and AIDSVAX® B/E offering 39.2% protection against HIV [193]; a 1% tenofovir gel inhibiting HIV sexual transmission by 39% [194]; a person with 12 years of infection considered to be cured of HIV as part of fighting acute myeloid leukemia via haematopoietic stem cell transplantation from a CCR5 $\triangle 32$ homozygous donor [195–197] are quite encouraging and serve as testimonial to the fact that HIV-1 can be conquered through further research which includes dissecting mechanisms of underlying protection and moving forward with those anti-HIV immunobiological clues [198–201]. While debate on attenuation of HIV-1, as it

evolves continues [36, 37, 202, 203], focused and concerted efforts by scientists, employing multidisciplinary approaches to attack HIV, might enable achieving UNAIDS mission of "zero new HIV infections, zero discrimination and zero AIDS-related deaths" earlier.

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*Review Article*

# Antibody-Dependent Cellular Cytotoxicity and NK Cell-Driven Immune Escape in HIV Infection: Implications for HIV Vaccine Development

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The HIV-1 genome is malleable and a difficult target tot vaccinate against. It has long been recognised that cytotoxic T lymphocytes and neutralising antibodies readily select for immune escape HIV variants. It is now also clear that NK cells can also select for immune escape. NK cells force immune escape through both direct Killer-immunoglobulin-like receptor (KIR)-mediated killing as well as through facilitating antibody-dependent cellular cytotoxicity (ADCC). These newer finding suggest NK cells and ADCC responses apply significant pressure to the virus. There is an opportunity to harness these immune responses in the design of more effective HIV vaccines.

## 1. Introduction

The human immunodeficiency virus (HIV-1) pandemic is causing substantial morbidity and mortality across the globe, particularly in developing countries. Antiretroviral drug therapy for HIV is highly effective in controlling disease; however, eradication of HIV-1 is currently not feasible so treatment is life long and is both expensive and leads to considerable toxicity and drug resistance. A vaccine is widely viewed as being essential to controlling the epidemic. Several advanced efforts to develop an effective vaccine have failed or shown only marginal efficacy to date [1–4]. One of the greatest challenges in developing a vaccine against HIV is to overcome its ability to constantly mutate and escape anti-HIV immune responses. This high mutation rate is a direct result of the presence of the virus' low fidelity RNA polymerase enzyme as well as the high levels of recombination it undergoes [5, 6].

A measure of the pressure immune responses apply is through their ability to force viral mutations that result in escape from immune recognition. Both CTLs and Nabs have long been reported to select for immune escape variants during the course of HIV-1 infection [7, 8]. Much effort in vaccine development centers on inducing broad and potent CTL (cytotoxic T lymphocyte) and Nab (Neutralizing antibody) responses to conserved viral epitopes and restricting opportunities for viral escape. However, it is now also recognised other immune responses, such as antibody-dependent cellular cytotoxicity (ADCC) and NK cells, select for immune escape variants, suggesting additional immune responses apply significant pressure to the virus [9]. ADCC responses mediated by effector NK cells may be useful responses to induce by vaccination. This paper summarizes current thinking on immune escape from anti-HIV immune responses.

## 2. CTL Escape and the Road to Reduced Viral Fitness

Immune escape from HIV was first demonstrated for CTL-based immunity in 1991 [8]. Considerable work since then has shown CTL escape is typically regulated by the effect of the escape mutation on comparative viral fitness, a complex parameter illustrating the overall contribution of all mutation-related advantages and losses (Table 1). Even though the evasion of immune responses presented by escape mutations presents a definite fitness benefit to the virus, the HIV-1 proteome is not infinitely malleable hence the same

Table 1: Key escape papers.

| Immune response | Hypothesis | Result | Ref. |
|---|---|---|---|
| CTL based | HLA-B*57/B*5801 CTL escape mutations in Gag impacts viral replication *in vivo* | Reductions in relative replication capacity reduce "viral fitness" | [20] |
| | CTL escape mutations in Env do not result in reduced viral fitness | Escape mutations within Env-specific CTL are epitopes evident but no correlation with reduced SIV replication | [25] |
| | Step HIV-1 vaccine trial exerts selective CTL pressure on HIV-1 | Extended sequence divergence for vaccine recipients who become infected suggests vaccine-induced CTL imparted significant immune pressure<br>Gag-84 most significant signature site | [36] |
| Nab based | Evolving "glycan shield" mechanism prevents Nab binding | Env gene mutations in escape virus sparse<br>Escape mutations did not map to known epitopes<br>Efficient neutralization requires potent, high titres | [54] |
| | Continual selection of Nab escape variants occurs | All previous viral isolates, but not concurrent isolate, are recognised by concurrent Nab | [7] |
| | Passive transfer of human neutralizing monoclonal antibodies delays HIV-1 rebound post-antiretroviral therapy | 2G12 monoclonal was crucial for transient *in vivo* effect of Nab cocktail but immune escape resulted | [55] |
| ADCC based | Immune pressure from HIV-specific ADCC results in immune-escape variants | ADCC causes escape in multiple epitopes and evolves over timeADCC antibodies forcing immune escape can be non-eutralizing | [9] |
| | NK cells apply immunological pressure on HIV-1 through direct killing of infected cells | HIV-1 selects KIR2DL2+ virus mutations that result in reduced antiviral activity of NK cells | [85] |

mutations can result in fitness costs. Some CTL immune escape variants have reduced replicative capacity of the virus (reduced "fitness") that slows the progression of disease [10, 11]. Studies have demonstrated that certain viruses composed of immune escape mutations are associated with lower viral loads within subjects [12, 13]. It has also been suggested that the rate of viral escape likely reflects the strength of the immune pressure and the fitness cost of the mutant virus [14]. Fitness costs are most dramatically illustrated *in vivo* by the reversion of transmitted escape mutations during acute and early HIV-1 infection [15–19].

Several CTL escape mutations have been confirmed to disrupt normal virus protein structure and/or function [20–23]. More than half of deleterious escape mutations have been verified in the relatively conserved Gag protein, whereas Troyer et al. [24] recently presented that CTL escape mutations in Env did not commonly transfer an associated fitness cost and indeed in a number of cases strengthened competitive viral fitness. This result is consistent with the lack of reversion of Env CTL escape mutations *in vivo* [25, 26]. Macaque and human studies have also demonstrated that escape from T-cell immunity leads to ongoing HIV or SIV infection [27]. The latest investigation of the effect on viral replication of twenty CTL escape mutations in Gag epitopes established only three escape mutations that resulted in substantial reductions in viral replication capacity, indicating that high-cost escape mutations are rather rare [28]. More importantly, these three highly effective CTL escape mutations appeared in epitopes primarily targeted during acute infection by protective HLA class I alleles [29]. This demonstrates that the protection allowed by certain HLA class I alleles may arise because the barrier to viral escape in the targeted epitope is high leading to either maintenance of a dominant and effective CD8+ T-cell response, and/or attenuation of virus replication from selection of high-cost escape mutations. Examination of viruses derived from HIV-1 controllers (individuals who maintain long-term control of HIV-1 viremia) reveal evidence of a role for rare or novel CTL escape-associated fitness costs in control of HIV-1 replication [30–33].

CTL responses and the immune escape variants induced are also important in HIV transmission scenarios. Two recent reports detail early clinical correlates related with the transmission of viruses expressing a number of CTL escape mutations known to weaken *in vitro* replication capacity [12, 34]. As the transmitted escape mutations revert to wild type [23], these enhanced results associated with such transmissions have been perceived to decline and the long-term clinical outcome of these transient effects remains unforeseeable. With improved characterization of the virology of acute

HIV-1 infection differences in founder virus, replication compared with viral escape strains caused by dominant CD8+ T-cell responses is becoming easier to model. The distinctions between the founder virus and viral escape strains may contribute to observed variability in the immune control of HIV-1 replication, which may be caused by carry-over mutations and variation in the rate of escape related to fitness costs from key CD8+ T-cell responses, in return influencing set-point viral load and early clinical disease course [35]. Elite control of viral replication may in part be due to the transmission of a virus attenuated by accumulated carryover mutations from hosts with such principal CD8+ T-cell responses to escape associated epitopes resulting in high fitness costs. Rolland et al. recently illustrated the first evidence of selective pressure from vaccine-induced T-cell responses on HIV-1 infection by analyzing HIV-1 genome sequences from 68 volunteers who participated in the STEP Adenovirus-vector HIV-1 vaccine efficacy trial [36]. Comparison of T-cell epitopes in the founder sequences to epitopes in the vaccine distinguished greater breadth for sequences from vaccine recipients than from placebo recipients, suggesting the vaccine imparted important immune pressure to the selection of the infecting isolates. Vaccine-induced fitness-impaired virus could, if sufficiently potent, translate into a reduction in viral loads and attenuation of disease progression.

## 3. The Great Escape from Neutralizing Antibodies

Considerable data exist illustrating the effect of neutralizing antibodies in protecting against HIV-1 infection *in vitro* [37, 38] and *in vivo* using animal models [39–46]. Although antibodies are made to all HIV proteins within a few weeks, only those to the envelope glycoproteins can prevent or neutralize HIV infection. These neutralizing antibodies (Nab) take considerably longer to develop than binding antibodies, generally months to years [47]. HIV-infected subjects almost always develop Nab to their own virus (autologous neutralization), although Nabs typically respond to earlier viral isolates, with the subject's contemporaneous virus having escaped. Some subjects eventually develop Nabs able to cross-neutralize additional viruses (heterologous neutralization), but their concurrent virus is still usually escaped from their autologous Nab. This highlights many of the difficulties involved in controlling HIV replication by Nab and the ability of HIV to escape antibody pressure through a process of genetic change [38]. The envelope gene presents the highest ratio of genetic diversity, most likely as a direct result of Nab pressure. However, for the virus to remain infective, portions of the envelope gene that encode regions essential for functional activity, such as CD4 and coreceptor binding, need to be conserved, and hence escape from Env Nabs probably results in little fitness cost. Individuals who do develop outstanding Nab responses generally have antibodies directed towards such crucial functional regions [48]. Long-term nonprogressors who have remained symptom-free for many years without antiretroviral therapy in general have broader and more potent responses compared to persons who show progressive disease [49–53].

Escape from neutralizing antibody responses often involves serial changes in glycosylation patterns and small insertions and deletions [7]. Richman and colleagues found that 9 of 12 untreated patients with detectable neutralizing antibody had the highest neutralising antibody titer towards against the baseline virus (month 0) whereas only three subjects showed higher titers of neutralizing antibody against viruses that appeared later in infection [7]. Wei and colleagues clearly illustrated the inhibition of HIV-1 by Nabs when successive populations of resistant virus were completely substituted by neutralization-sensitive virus [54]. Furthermore, they showed escape virus contained infrequent mutations in the *env* gene, generally mapped to unknown neutralization epitopes, and involved changes mainly in N-linked glycosylation sites. This pattern of escape led to the hypothesis of an evolving "glycan shield" mechanism of neutralization escape which selected differences in glycan packing preventing Nab binding but not receptor binding. Mutational substitution assays showed that Nab-selected alterations in glycosylation presented escape from both autologous antibody and epitope-specific monoclonal antibodies. Thus a new mechanism was presented contributing to HIV-1 persistence in the presence of an antibody repertoire.

Viral escape regardless of the presence of neutralizing antibodies could demonstrate either that antibodies were ineffective *in vivo*, in which case antibody-sensitive viral strains would remain, or otherwise that the virus escaped the pressure applied by the antibody. Trkola et al. illustrated that passive transfer of Nabs in humans with established HIV resulted in immune escape by comparing the inhibitory activity of 3 monoclonal Nabs (2F5, 4E10 and 2G12) against virus isolates derived before the passive transfer trial and to sequential isolates after antibody treatment [55]. There was a strong association between development of 2G12-resistant viral strains and emergence of escape mutants to this antibody, failure to respond to treatment and loss of viremia control. While evidence of virus escape implies Nab selective pressure to a certain extent [7, 54, 55], it has been speculated that postinfection Nabs could exert only a limited suppressive effect on primary HIV replication [45, 56, 57]. Prevention of primary SIV or SHIV replication in monkeys by passive Nab immunization prior to or very early after infection is achievable [40, 58–60]. Taken together, this suggests that HIV control by potent Nabs is most likely to be effective prior to infection. Immune escape is likely to compromise the role of Nabs after infection is already manifested.

The role of neutralizing antibodies in preventing or limiting HIV-1 infection is becoming clearer with a better understanding of the structure of the envelope glycoprotein as well as passive immunization studies in animals showing that antibodies can indeed control infection. Further insights into neutralization-sensitive epitopes on the envelope glycoprotein are needed that will enable us to design better vaccine immunogens in vaccines. Ultimately this should allow the ability to induce neutralizing antibodies in conjunction with additional antibody-mediated protective mechanisms such

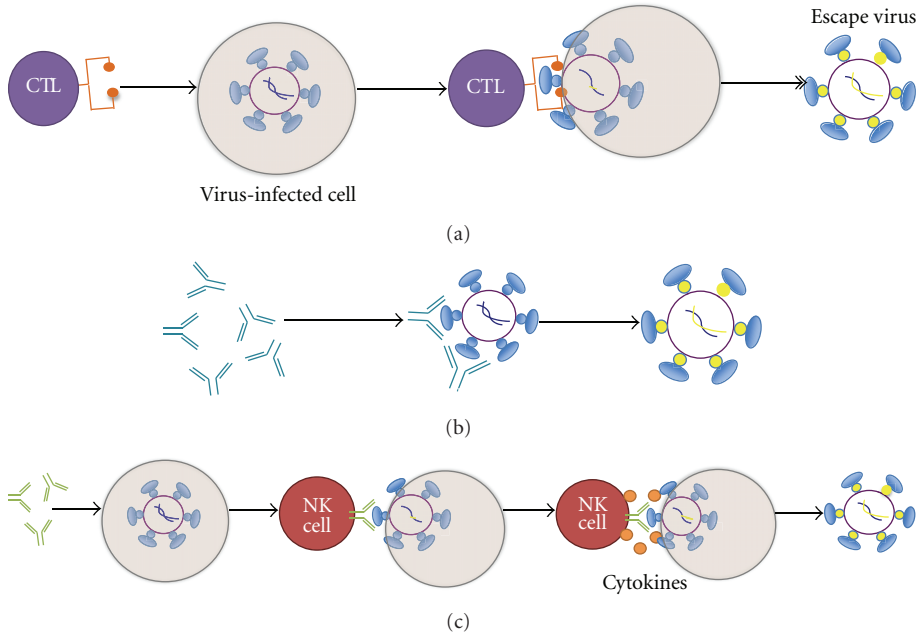


Figure 1: HIV-specific immune responses force immune escape. The mechanism of immune pressure applied by Cytotoxic T lymphocytes (a), neutralizing antibodies (b), and ADCC antibodies (1) is illustrated. Escape from immune responses shows results once free virus (Nab responses) or viral particles are presented either via the MHC class pathway (CTL responses) or possibly on the surface of the infected cell by virus budding (ADCC).

as antibody-dependent cell-mediated cytotoxicity (ADCC) in the fight against HIV.

## 4. Escape from ADCC

Sequencing single HIV genomes from subjects with acute HIV-1 infection reveals that multiple mutations are acquired during the first months of infection and most align with sites of CTL or Nab escape mutations [61, 62]. However, some mutations do not clearly map to known sites of CTL or Nab escape, suggesting there may be other immune responses, such as ADCC responses, sufficiently potent to select immune escape strains. ADCC antibodies bind to viral antigens on the surface of infected cells and engage Fc receptors on innate immune cells such as NK cells, macrophages, and neutrophils, which in turn lyse the HIV-infected cell (Figure 1(c)).

ADCC is an area relatively poorly explored in HIV immunology in recent years. Very few ADCC epitopes have been identified to date within HIV. The majority of these identified ADCC epitopes are within Env glycoproteins, gp120 [63–66] and gp41 [67–69], Tat [70] and Nef [71, 72].

Several recent studies in both humans and macaques are now suggesting that ADCC antibodies can be effective in controlling HIV or SIV [4, 73, 74]. The potent immune pressure that can be applied by HIV-specific ADCC antibodies has only recently been brought into sharper focus. Importantly, Hessell and colleagues performed experiments on Nab mutated in the Fc region, which showed a reduced ability of the Nab to mediate killing of infected cells *in vitro*. When they administered the mutant (Fc defective) Nab to macaques, they were also markedly reduced in their ability to prevent SHIV infection [73]. It perhaps should be no surprise that ADCC antibodies are also implicated in viral escape as the HIV genome is able to make multiple changes to avoid CTL, Nab, and antiretroviral drug pressure.

ADCC responses forcing immune escape had until recently not previously been demonstrated. Demonstrating viral escape from ADCC responses would strongly suggest that ADCC responses exert significant pressure on the immune system [75]. Stratov et al. described a novel assay which allowed the mapping of a series of HIV-specific ADCC epitopes in subjects infected with HIV, using a set of consensus HIV peptides [76]. The identified epitopes within the subjects HIV strains were then further cloned and sequenced across the relevant epitopes and analyzed as to whether their ADCC responses were able to recognize their own virus strain. Evidence of immune escape was found against multiple HIV-specific ADCC epitopes studied in the Env protein of HIV-1 [9]. Evolution of escape over time was detected in contemporary plasma samples, which differed significantly from initial viral sequences at areas targeted by ADCC.

ADCC antibody responses are generally thought to target viral surface proteins presented on the surface of infected cells. Our group has also identified ADCC responses to viral peptides derived from internal HIV-1 proteins such as Vpu and Pol [75, 76]. Interestingly, we also identified possible immune escape to an epitope of the highly conserved protein Pol [77]. It is not immediately apparent how these epitopes would be presented on the surface of cells to ADCC antibodies and force viral escape and much more work needs to be

done to define whether ADCC antibodies to internal proteins can recognize HIV-infected cells *in vitro*. We speculate that it may also be possible that ADCC recognition of viral debris on the surface of healthy neighboring cells may trigger noncytolytic activity from NK cells that could limit HIV-1 spread in a local environment. Such a mechanism would also be susceptible to immune escape.

Using ADCC peptide epitopes and an NK-cell activation assay, the hypothesis that ADCC plays a major part in the immune response against HIV was confirmed. This work likely underestimates the number of ADCC epitopes targeted by each HIV+ subject, since linear epitopes are readily mapped and dissected and consensus B overlapping peptides are used for screening. Conformational ADCC antibodies are likely to elicit escape also but to map such responses and identify escape patterns will be more difficult and require large numbers of mutant whole Env proteins.

The partial efficacy shown by the recently reported canarypox prime/protein boost vaccine trial conducted in Thailand [78] could possibly be associated with ADCC antibodies. Recent conference presentations have correlated non-neutralizing antibody responses to vaccine efficacy, although much work remains to be done to understand this fully [79]. It is conceivable, by analogy with results on CTL responses in the STEP trial [36], that vaccinated subjects in the Thai trial who still became infected may have become infected with HIV variants already "preescaped" at the ADCC responses induced by their vaccination.

ADCC-forced mutations could theoretically incur some "fitness cost" to viral replicative capacity, similar to that observed for CTL escape variants [12]. Constructing replicating viruses with ADCC-induced mutations will allow testing of this hypothesis. More potent ADCC antibodies are likely to target conserved or functional domains of viral proteins. The Env protein is highly diverse and readily escapes CTL and Nab responses with apparent minimal fitness costs [7, 24, 26, 80]. It is possible that any fitness cost of ADCC escape in Env could also be small. ADCC antibodies targeting conserved non-Env proteins such as Vpu and Pol may be more potent, although it needs to be assessed whether these antibodies recognize HIV-infected cells as noted above. It is also likely that compensatory mutations may emerge which repair any fitness cost of primary mutations [81]. Further studies on the patterns of ADCC escape and the specific cellular components involved in ADCC should allow a finer understanding of how to either limit ADCC escape or force larger fitness costs.

Natural killer (NK) cells are the key effector cells mediating ADCC function. Virally infected cells are identified through a range of activating and inhibitory receptors [82] as well as both activating and inhibitory killer immunoglobulin-like receptors (KIRs) [83, 84]. Alter and colleagues recently clearly demonstrated that NK cells can directly mediate antiviral immune pressure *in vivo* in humans [85]. They showed that the binding of inhibitory KIRs to HIV-1-infected CD4+ T cells is amplified and the antiviral activity of KIR-positive NK cells is diminished by KIR-associated HIV-1 sequence polymorphisms. Similar to immune pressure applied by virus-specific T cells and neutralizing antibodies, it seems plausible to state that KIR-positive NK cells can place immunological pressure on HIV-1 and that the virus can evade such NK-cell-mediated immune pressure by selecting for sequence polymorphisms.

## 5. Conclusions

CTL and Nab immune responses are pivotal drivers in immune escape and viral variability. It is now clear that the role of NK cells in viral selection, both through direct killing and ADCC mechanisms, is likely to have been previously underestimated. Other effector cells of the innate immune system, including macrophages and neutrophils, may also be important in driving HIV evolution. Evidence of the pressure applied by ADCC antibodies now provides challenges to inducing the most effective ADCC antibodies by vaccination. A better understanding of the immune responses to HIV is required to fully harness the potential of a vaccine to both prevent viral entry and ongoing infection.

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*Research Article*

# Resistance of Subtype C HIV-1 Strains to Anti-V3 Loop Antibodies

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HIV-1's subtype C V3 loop consensus sequence exhibits increased resistance to anti-V3 antibody-mediated neutralization as compared to the subtype B consensus sequence. The dynamic 3D structure of the consensus C V3 loop crown, visualized by *ab initio* folding, suggested that the resistance derives from structural rigidity and non-$\beta$-strand secondary protein structure in the N-terminal strand of the $\beta$-hairpin of the V3 loop crown, which is where most known anti-V3 loop antibodies bind. The observation of either rigidity or non-$\beta$-strand structure in this region correlated with observed resistance to antibody-mediated neutralization in a series of chimeric pseudovirus (psV) mutants. The results suggest the presence of an epitope-independent, neutralization-relevant structural difference in the antibody-targeted region of the V3 loop crown between subtype C and subtype B, a difference that we hypothesize may contribute to the divergent pattern of global spread between these subtypes. As antibodies to a variable loop were recently identified as an inverse correlate of risk for HIV infection, the structure-function relationships discussed in this study may have relevance to HIV vaccine research.

## 1. Introduction

Subtype C infections now represent the majority of HIV-1 infections worldwide [1], suggesting greater *in vivo* or host-pathogen fitness. By contrast, in direct *in vitro* competition assays, R5 subtype B isolates outcompete R5 subtype C isolates [2], suggesting greater *in vitro* infective fitness. Thus, more rapid *in vivo* spread of subtype C infections may be occurring despite an apparent greater *in vitro* fitness of subtype B.

Differential susceptibility to human antibody-mediated neutralization could result in differing extents of global spread between different subtypes. The V3 loop is often referred to as the principal neutralizing determinant of HIV-1 viruses as several of the early and recent studies describing human antibodies that could neutralize HIV-1 were dominated by anti-V3 loop antibodies [3–6]. Indeed, several observations suggest a conformational or functional difference between subtype B and subtype C V3 loops [7], but the nature of the difference has not been elucidated. The V3 loop is also the site of CCR5 and CXCR4 engagement, a necessary determinant of virus entry [8–13]. Thus, antibody neutralization determinants and infective determinants coincide to the same location on the HIV-1 envelope glycoprotein surface, and disturbances to one are likely to affect the other.

A comparison of antibody-mediated neutralizations of SF162 chimeric psVs carrying the consensus subtype C V3 loop sequence (conC) to those for the consensus subtype B V3 loop sequence (conB) by two different broadly neutralizing anti-V3 loop monoclonal antibodies (mAbs) previously demonstrated that ConC has substantially more resistance to neutralization mediated by both antibodies (Figure 1) [14]. Each of those mAbs (i.e., 2219 and 447-52D) has been crystallographically confirmed to have distinct V3 loop binding modes [15, 16]. Poorly characterized and variable V3 loop surface exposure features were controlled in these experiments using a previously established approach in which psVs are constructed to express different sequences of the V3 loop within the same SF162 Env background [17]. SF162 is sensitive to antibody-mediated neutralization and in this setting

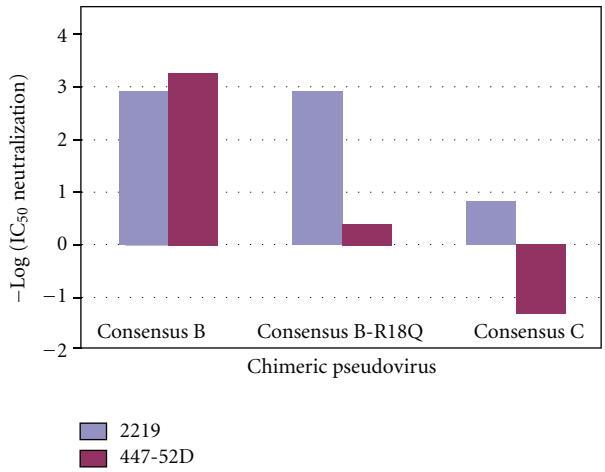


Figure 1: Neutralization of chimeric psVs by antibodies 447-52D (purple) and 2219 (blue). The psV consist of the SF162 strain with its V3 loop replaced by the indicated V3 loop sequence. Consensus B-R18Q is a psV consisting of the consensus subtype B V3 loop sequence with position 18 in the V3 loop mutated from a subtype B consensus arginine to a subtype C consensus glutamine. $IC_{50}$ is the amount of antibody in ug/mL required to achieve 50% neutralization. The negative base 10 logarithm of the $IC_{50}$ has been plotted for easier comparison: higher, positive bars towards the top of the graph indicate strong neutralization by the antibody. Antibodies were only tested to a concentration of 20 ug/mL for neutralization, so the negative value of 1.3 on the plot is maximal and indicated no detectable neutralization by the antibody. Adapted from [14].

provides a relatively constant level of V3 loop exposure and a minimal Env variability background across the different chimeric psVs. Differences in neutralization between two mutants differing only in their V3 loop sequences should therefore be caused by structural differences in the V3 loop itself. Thus, some portion of the subtype C virus resistance to anti-V3 antibody-mediated neutralization maps to the V3 loop itself and not to structural effects outside the V3 loop or to surface exposure differences between subtype B and subtype C.

Previous analysis of the 3D interaction surface of 2219 and 447-52D bound to the V3 loop divides the V3 loop into amino acid positions that comprise the Ab "epitope" and those that do not [14]. The 3D structure of the epitope or Ab-bound surface can be divided into two functionally distinct categories. The first category is amino acids that were found to comprise the neutralization epitope: these are defined as tight (usually electrostatic), substitution-intolerant complementarities between pockets on the antibody surface, and specific V3 amino acid side chains that restrict the neutralization activity of the antibody. These amino acids were identified as R18 for 447-52D and K10-I12-Y21 for antibody 2219 [14], where the number following each amino acid single-letter designation refers to the position of that amino acid in the V3 loop starting with the disulfide-bonded cysteine as number 1. Mutation of any of these amino acid side chains disrupt Ab-mediated neutralization strongly.

The second category is the rest of the epitope or Ab-bound 3D molecular surface: mutation-tolerant contacts between loosely bound V3 side chains or the V3 loop backbone and the antibody binding surface that depend on the overall tertiary shape of the bound V3 loop conformation. We define these areas as mutation insensitive, "epitope-independent" locations for the purposes of this study, although technically all atoms contacting the Ab comprise the epitope and these locations can indirectly influence the epitope.

The conC sequence differs from the conB sequence in the former, substitution-intolerant type of contact. Thus, with a Q18 present in ConC versus R18 in ConB, the chimeric psV carrying the conC loop was shown to be predictably resistant to antibody 447-52D (up to 20 ug/mL tested, Figure 1). However, when this important contact was abolished in the conB sequence (by an R-to-Q mutation at position 18 (R18Q), antibody-mediated neutralization was reduced but not abolished, indicating that the observed resistance to antibody-mediated neutralization does not depend completely on this specific neutralization epitope contact (Figure 1). Conversely, the conC sequence *does not* differ from the conB sequence in neutralization epitope contacts for antibody 2219 (K10, I12, and Y21), but conB is sensitive to 2219 antibody-mediated neutralization while conC is partially resistant. The magnitude of this epitope-independent resistance for 2219 is approximately of the same magnitude as that inferred for 447-52D. Thus, for both antibodies, a substantial epitope-independent structural mechanism, present in conC but not in conB, appears to underlie resistance to anti-V3 antibody-mediated neutralization of the subtype C virus.

We therefore hypothesized that we could visualize the 3D structural basis of the epitope-independent differential resistance observed between the subtype B and subtype C V3 loops by correlating previously published psV neutralization measurements with dynamic 3D structural visualization of several carefully constructed V3 loop mutants. In order to do this, we needed a minimum of 2 Abs with crystallographically confirmed differences in their targeted V3 crown epitopes, so Abs 447-52D and 2219 were chosen, but we expect these results to apply generally to all V3 crown-targeted Abs.

## 2. Results

*2.1. The Segment at Positions 12 to 14 of the Consensus C V3 Loop Crown Has a Rigid, Non-β-Strand Conformation.* Several recent studies have visualized snapshots of the 3D structure of the V3 loop by NMR and crystallography [15, 16, 25]. One common feature of mAbs 2219 and 447-52D is that both bind positions 12 to 14 of the V3 loop in an antiparallel $\beta$-sheet fashion. This result may be applicable to most broadly neutralizing anti-V3-loop antibodies, as their linear epitopes overlap, for the most part, at the N-terminal $\beta$-strand (positions 12 to 14) of the V3 loop (M. Gorny, personal communication). The $\beta$-strand at positions 12–14 of the V3 loop appears to be a common structural feature required for recognition and function of anti-V3 antibodies. Since this region makes mostly backbone (non-side-chain) contacts with the V3 antibodies, the protein backbone conformation of this region (i.e., $\alpha$-helical, $\beta$-strand, etc.) is likely to influence anti-V3 antibody-mediated neutralization.

We therefore examined this region carefully for backbone conformational differences between neutralization-resistant conC and neutralization-sensitive conB. *ab initio* peptide folding algorithms have previously been shown to be capable of predicting the flexibility and conformational preferences of the V3 loop crown, recapitulating crystallographic forms (See Supplementary Figure 1 in the Supplementary Material available online at doi:10.1155/2012/803535) and demonstrating that the V3 loop crown from positions 10 to 22 of the V3 loop behaves as an autonomously folded, but flexible, domain [18–20]. Folding a peptide identical in sequence to the conC V3 crown from positions 10 to 22 shows that the peptide backbone prefers a rigid, non-$\beta$-strand structure at positions 12 to 14 (Figure 2). In contrast, a peptide identical in sequence to amino acids 10–22 of the conB V3 crown backbone adopts a flexible conformation with clear $\beta$-strand character at positions 12 to 14 and overall a clear $\beta$-hairpin fold (Figure 2). The key positions in the conB sequence consistently adopt $\phi$-$\varphi$ angles typical of a Type II beta-hairpin at the V3 GPG sequence, while these are lost in conC. The rigidity and non-$\beta$-strand structure of the 12–14 V3 segment in the subtype C V3 crown may present an energetic barrier to peptide deformations required for antibody-mediated neutralization, specifically bending of the structure into and out of a neutralization relevant $\beta$-strand conformation. Thus, these results suggest the following epitope-independent structure-activity relationship: anti-V3-loop-antibody-mediated neutralization depends on a flexible $\beta$-strand at positions 12 to 14 in the V3 loop.

*2.2. Substitutions in the V3 Antibody Binding Site Affect the Rigidity and β-Strand Conformation of This Area.* We began by focusing on the structural effects of mutations at the key positions 13 and 14 of the subtype C V3 loop by folding a series of V3 loop crowns with point mutations at these positions (Table 1). Position 12 was avoided because it is part of the neutralization epitope for 2219 and such mutants would therefore obviate a standardized assessment of the non-epitope-dependent effects across mutants. *ab initio* folding of conC mutated in the 14th position of the V3 loop from Ile to Met (I14M) mildly increased the flexibility of the V3 crown but retained a strong $\beta$-hairpin conformation. *ab initio* folding of an I14V conC mutant restored full flexibility and 2/3 $\beta$-strand character to this local region. Folding of I14L demonstrated a non-$\beta$ strand, partly $\alpha$-helical conformation. Interestingly, we previously demonstrated that $\alpha$-helical conformations in this region are sufficiently disruptive to abolish infectivity of the virus [20], suggesting that I14L has the strongest resistance to antibodies in this dataset but may also be the least infective construct. I14F re-established two-thirds of the $\beta$-strand in the 12 to 14 region, but did not preserve a $\beta$-hairpin as the C-terminal strand of the V3 crown did not contact the N-terminal strand and the overall structure remained somewhat rigid.

Comparison of this folding data with neutralization data from *in vitro* chimeric psVs with the same mutations showed that loss of 2219 antibody-mediated neutralization correlated with the loss of both $\beta$-strand character and structural flexibility, while reduction of 2219 neutralization is associated with the loss of either factor independently (Table 1). As noted previously, all tested chimeric psVs from Table 1 preserve the key 2219 binding epitope side chains as a control feature, so increased resistance to antibody-mediated neutralization appears to correlate with loss of $\beta$-strand or $\beta$-hairpin conformation in the V3 loop and/or loss of structural flexibility at positions 12 to 14 in an epitope-independent manner.

Mutations anywhere in the V3 crown can affect the folding of the whole crown, and as such simulations were performed for mutations outside of positions 13 and 14. *In silico* mutations at positions 18 and 22 in conB did not alter the folding significantly (Table 1). A conC T19A *in silico* mutation did alter overall folding somewhat, resulting in partial restoration of flexibility and full restoration of $\beta$-strand character at 12–14. These results also correlated with the previously noted resistance of *in vitro* chimeric psVs to 2219 antibody-mediated neutralization, so changes outside the key Ab-targeted region can indirectly affect folding, and

Table 1: antibody-mediated neutralization of psVs constructed from SF162 with the V3 loop replaced by the consensus C or consensus B V3 loop sequence with and without the indicated point mutations in the "Sequence" column. *In vitro* measured strong, weak, or no neutralization is indicated along with the $IC_{50}$ (ug/mL) in the "Neutralization" column on the right. Numbering of mutated residues is from the beginning of the V3 loop with the starting cysteine being residue number 1 so that D25E (V3 loop numbering) is the same as D322E (numbering of residues from N-terminus of gp120). The "Flex" column is the structural flexibility of the V3 crown from positions 10 to 22 as assessed by *ab initio* folding: +++ indicates no energy gap and many conformations near the energy minimum suggesting a flexible structure; ++, +, and −indicate a spectrum of energy gaps of <2 U slightly more than the standard error of the energy function suggesting a partly flexible structure; −− indicates an energy gap >2 U indicating a rigid conformation. The "$\beta$-hairpin" column is the $\beta$-strand character of positions 12 to 14 as assessed in the same *ab initio* folding: +++ indicates that all three residues from 12 to 14 adopt canonical $\beta$-strand $\phi$ and $\Psi$ angles in the lowest energy structure; ++ indicates that two of the three residues from 12 to 14 adopt canonical $\beta$-strand $\phi$ and $\Psi$ angles; + indicates that two or more of the residues from 12 to 14 adopt canonical $\beta$-strand $\phi$ and $\Psi$ angles, but that the overall structure does not form a $\beta$-hairpin. – and −− indicate that residues from 12 to 14 adopt canonical non-$\beta$-strand $\phi$ and $\Psi$ angles.

| Sequence | Flexibility | $\beta$-Hairpin | Naturalization ($IC_{50}$ $\mu$g/mL) |
|---|---|---|---|
| Consensus B | +++ | +++ | Strong (0.001) |
| B-R18Q | +++ | +++ | Strong (0.001) |
| B-T22A | +++ | ++ | Strong (0.002) |
| C-I14V | ++ | +++ | Strong (0.01) |
| C-T19A | + | +++ | Weak (0.02) |
| C-I14M | ++ | −− | Weak (0.03) |
| C-I14F | − | ++ | Weak (0.1) |
| Consensus C | −− | −− | None (0.15) |
| C-D25E | −− | −− | None (0.15) |
| C-I14L | − | −− | None (0.15) |

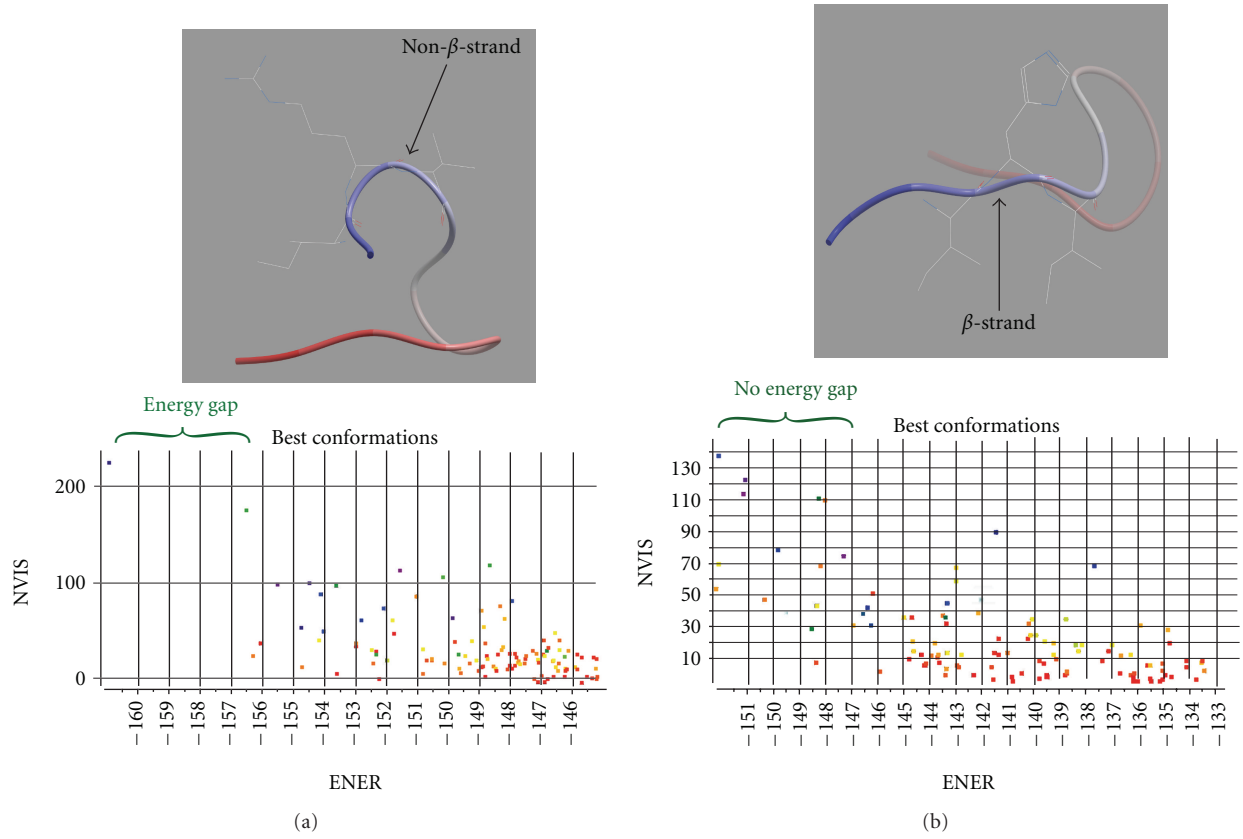


Figure 2: (a) *Top:* lowest energy conformation of the subtype C V3 loop crown from V3 loop amino acid positions 10 to 22. The structure is shown in ribbon representation colored in a gradient from the N-terminal residue at position 10 (dark blue) to the C-terminal residue at position 22 (colored dark red). The side chain conformations of Ile12, Arg13, and Ile14 are shown in full-atom wire representation. Their backbone curvature and fanned orientation are inconsistent with $\beta$-strand secondary structure. *Bottom:* plot of 180 lowest energy conformations from the folding simulation. $x$-axis (ENER): energy score ranging from lowest at the left to higher at the right. $y$-axis (NVIS): number of visits by the simulation to the indicated conformation. For example, the lowest energy conformation was found again and again by the search over 150 times. The presence of a 4 kcal simulation energy unit gap in the subtype C simulation indicates a rigid structure as a 4 kcal energy barrier prevents exit from the lowest energy conformation most of the time on the biological time scale. (b) *Top:* lowest energy conformation of the subtype B V3 loop crown from positions 10–22 depicted as in A. The extended linear conformation of Ile12, Arg13, and Ile14 with alternating directions for the side chains is typical of canonical $\beta$-strand structure. *Bottom:* plot of 180 lowest energy conformations from the folding simulation as in A. Many conformations are present near the lowest energy conformation in this subtype B simulation predicting a flexible structure that flickers between ~10 conformations all the time (is flexible) on the biological timescale.

the observed effect is tertiary and not specifically dependent on any amino acid position.

*2.3. The Epitope-Independent Effect May Be General to a Wide Variety of Anti-V3 Antibodies.* When tested with 14 broadly neutralizing anti-V3 antibodies derived from donors infected with subtypes A and B, the conC chimeric psV was neutralization resistant to all of the mAbs to a much greater degree than the conB chimeric psV (Table 2). A non-V3 Ab—b12—did not show the same magnitude of effect. In the panel, 447-52D and 2219 are known to have distinct epitopes, and it is likely that many of the other mAbs have distinct epitopes as well. The common resistance of conC to all these different antibodies suggests an epitope-independent structural resistance to neutralization residing in the V3 loop.

## 3. Discussion

The experimental results described here indicate that a significant fraction of the anti-V3 antibody-mediated neutralization resistance of the conC sequence maps directly to the antibody-binding domain of the V3 crown. Furthermore, the epitope-independent structural feature by which the subtype C V3 crown resists neutralization by a variety of anti-V3 antibodies appears to be a rigid N-terminal non-$\beta$-strand conformation at positions 12 to 14 of the V3 loop. This effect is exclusive of the more commonly observed mechanism of antibody escape, that is, mutations of key neutralization epitope side chains, such as R18Q for 447-52D which we have shown results in a distinguishable, antibody-specific resistance. The combination of the loss of key neutralization epitope amino acid side chains with rigidity or non-$\beta$-strand structure results in total resistance of the psV bearing

Table 2: $IC_{50}$s (ug/mL) of 15 different antibodies (columns) derived from subtype B and subtype A infected patients neutralizing the infectivity of psVs containing the subtype C and B V3 sequences in the SF162 Env backbone. For comparison, the $IC_{50}$ values for a non-V3 Ab (b12) are as follows: clade B cons. (JR-FL) = 0.009 ug/mL; clade C cons. = 0.02 ug/mL, all others untested; which does not show as dramatic a difference in neutralization between the two psVs. 135 MPL23a is a subtype C primary isolate and is included as an example of $IC_{50}$ values in non-neutralization-sensitive ("masked") backgrounds. $IC_{50}$ values are font-type coded as follows: bold >0.1 ug/mL; italic <0.1 ug/mL; bold/italic <0.01 ug/mL.

| V3 sequence | Anti-V3 mAbs from clade B infected patients | | | | | | | Anti-V3 mAbs from clade A infected patients | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2412** | **4117** | **2442** | **4148** | **2456** | **447** | **2191** | **2219** | **2128** | **3074** | **2557** | **2558** | **3019** | **3224** | **2601** |
| c1. B cons (JR-FL) | ***0.0009*** | ***0.0055*** | ***0.0018*** | ***0.0053*** | ***0.0039*** | ***0.00054*** | ***0.0023*** | ***0.0012*** | ***0.0013*** | ***0.0055*** | ***0.0037*** | ***0.0049*** | ***0.0027*** | ***0.0051*** | 0.12 |
| clade A1 cons | 2.81 | 14.4 | *0.027* | *0.068* | *0.083* | 0.58 | *0.043* | *0.029* | **>20** | *0.015* | *0.025* | *0.038* | *0.032* | *0.044* | *0.029* |
| clade C cons | **>20** | **>20** | **>20** | 2.5 | 0.44 | **>20** | 0.59 | 0.15 | **>20** | 0.2 | 0.16 | 0.2 | 0.25 | 1.8 | 0.17 |
| $IC_{50}$ratios B/C | >22,000 | >3,600 | >11,000 | 470 | 113 | >37,000 | 257 | 125 | >15,000 | 36 | 43 | 41 | 93 | 345 | 1.4 |
| 135 MPI 23a* | **>20** | **>20** | **>20** | **>20** | **>20** | **>20** | **>20** | **>20** | **>20** | 8.0 | **>20** | 77.0 | 93.0 | **>20** | **>20** |

* Primary subtype C isolate.

these V3 loop properties to neutralization by the antibody in question. Since these are intrinsic features of the V3 loop sequences, this phenomenon would apply to circulating viruses bearing these properties in their V3 loops as well, specifically subtype C viruses. Our dissection of the effects of single V3 loop point mutations shows that the effects of each point mutation is complex, and the backbone effect is combinatorial to all of the V3 loop positions simultaneously. Thus, no single amino acid position is solely responsible for the conC structural phenomenon. The convergence of three completely independent sets of data—(1) known crystallographic structures of V3 peptides bound to antibody, (2) patterns of psV neutralization, and (3) validated *ab initio* folding simulations—strongly supports these conclusions.

Our observation suggests that a flexible, $\beta$-strand structure at positions 12 to 14 is required for anti-V3 antibody-mediated neutralization, and indeed this region is bound by many anti-V3 antibodies. Nevertheless, it cannot be concluded that antibody binding alone underlies this structure-activity relationship. Neutralization is a multistep process with antibody binding being only one step. One can imagine a rigid V3 loop crown shape that is perfectly complementary to an antibody-combining site and therefore binds the antibody *in vitro,* but the virus may nevertheless be neutralization resistant due to the effects of this selfsame rigidity at other steps in the neutralization process. For example, neutralization-relevant V3 loop interactions with several other surfaces of gp120 may be affected by the rigidity in the V3 loop crown. For this reason, it is possible that structural rigidity in the V3 loop crown may also influence neutralization by non-V3-targeted antibodies by inhibiting intermediate conformations involving the V3 loop in the series of conformational changes that likely comprise the overall neutralization process. Indeed, the conC psV exhibits mildly increased resistance to the non-V3 Ab b12 (Table 2).

The unique resistance of conC to a wide variety of subtype A and subtype B derived anti-V3 antibodies may be informed by the observation of low variability of the consensus C sequence in circulating subtype C strains [26]. If neutralization via the V3 loop is a strong selection pressure on circulating subtype C viruses, then infective V3 loop sequences harboring resistance to anti-V3-loop-antibody mediated neutralization might be observed at a higher rate and exhibit fewer escape mutations (vary less in sequence). As a corollary, subtype A and B derived anti-V3 antibodies may not be very effective as vaccine tools to combat subtype C, an observation that has previously been suggested for subtype B [27]. Different strategies for interrogation of subtype C infected HIV+ sera may be required to uncover novel, effective neutralizing antibody responses to this subtype. On the other hand, a diversity of V3 loop sequences are present in subtype C in addition to the dominant conC and conC-like sequences. It is unlikely that the effect we have observed is universal in subtype C, and some subtype C strains may exhibit V3 loop flexibility or encode compensatory changes in other parts of gp120 to afford efficient neutralization by anti-V3 antibodies.

The psV neutralization data correlates strongly with the results of the folding simulations. This correlation extends previous observations suggesting that the 12 $\beta$-hairpin residues (positions 10 to 22) of the V3 crown—including the currently known anti-V3 antibody combining sites—are sufficiently flexible in situ in the V3 loop to behave essentially as free peptides or as an autonomously folded subdomain [18–20]. *Ab initio* folding simulations of the V3 loop crown may therefore visualize at low resolution the dynamic structural ensemble of some V3 loop crowns *in silico*, a potentially important high-throughput capability for mapping structure-(neutralization) activity relationships in the V3 loop crown. It should be noted that the identification of two properties that are more easily assessed in a dynamic ensemble of many conformations—secondary structure and rigidity—facilitated the comparative interpretation of our library of folding data. Observing the structural tendency at a very specific location—positions 12 to 14 in the V3 loop—also facilitated the study. More subtle and global structural patterns, including overall fold assignment and absolute energetic stability for the whole domain, may be more

difficult to discern and assess across different foldings for the purpose of correlations with experiments.

The strong correlation of the psV neutralization measurements with observed structural features in the folding simulations also establishes the SF162 chimeric psV system as one that provides, even to fine resolution, a consistent virologic background across multiple experiments and V3 loop sequence variations. The combination of chimeric psV neutralization measurements with *ab initio* folding simulations allows detailed quantitative *dynamic* structure-neutralization activity relationships to be mapped out for the V3 loop. Such studies would be difficult with crystallography, which is low throughput and does not evaluate dynamic structure.

Antibody epitopes in the V3 loop may occur broadly in HIV-1 viruses, but antibodies appear to be limited in accessing these epitopes presumably due to the effects of "masking" glycans and nearby variable domains [17]. In this work, the comparison of neutralization tests with folding simulations may have revealed an obscure structural explanation for epitope-independent variations in antibody-mediated neutralization. The fact that some of the resistance to antibody-mediated neutralization maps directly to the antibody binding area of the V3 loop influences the view of masking: some of the observed masking of V3 loop epitopes may be intrinsic to the V3 loop itself and not due to outside factors such as glycans and the other variable loops of gp120, although both factors are likely operative to different degrees in any given strain. The approach described here could potentially be modified to "localize" the masking of the V3 loop epitope in primary HIV-1 isolates, at least to V3 loop or "outside-V3 loop" locations, by identifying anti-V3 loop antibody-resistant primary isolate sequences in which this "local masking" is present.

HIV-1 strains that evolve to partially mask receptor interacting surfaces in order to hide those vulnerable surfaces from the immune system trade a loss of infective efficiency for a gain in camouflage protection. The coincidence of determinants for infection (chemokine receptor binding surfaces) and immune detection (broadly neutralizing epitopes) in the V3 loop likely requires such a tradeoff for best viral fitness. Since a wide variety of anti-V3 antibodies appear to adhere to the antibody resistance mechanism described here, the work suggests that this tradeoff is accomplished in subtype C partly by the adoption of V3 loop structural shapes that are inefficient for both antibody-mediated neutralization and coreceptor binding, that is, rigid non-$\beta$-strand conformations. As the same viral mechanisms that produce this feature of the V3 loop beget the structural features of the other four variable loops, it is likely that the same tradeoff is exploited by viral evolution for functional regions of the V1/V2, V4, and V5-loops. If anti-variable loop antibodies play a significant enough role in the human protective response to circulating HIV-1 strains, the phenomenon we have described here may explain the concurrent diverging observations of decreased fitness (poorer receptor usage) and increased natural spread (successful immune evasion) in subtype C. Indeed, the recent identification of anti-V2 loop Abs as the only known inverse correlate of risk for HIV infection suggests that antibodies to variable loops indeed play a significant role in the human protective response from circulating HIV-1 strains [28].

## 4. Methods

*4.1. Dynamic Structural Characterization of the V3 Crown.* In order to efficiently correlate 3D structure with neutralization patterns, the visualization of the dynamic conformational landscape of various mutants of the V3 loop crown *in silico* is needed. Interestingly, the identical sequence—$V3^{MN}$—has been solved in complex with the two different antibodies and adopts different $\beta$-strand structures in the two different environments despite the identical sequence. These essentially represent biologically relevant snapshots of two conformations out of many in $V3^{MN}$'s dynamic conformational ensemble. We previously reported that a state-of-the-art *Ab initio* peptide folding algorithm could accurately reveal the conformational landscape and dynamic tertiary structure of the V3 loop crown by analyzing whether the two different forms of $V3^{MN}$ seen in the crystallographic structures were recapitulated [18]. *ab initio* folding of residues 10 to 22 of the V3 crown recapitulated the two forms and demonstrated the relationship between them (the 2219 form is the lower energy more prevalent form). Since the input to the folding is only the amino acid sequence, this algorithm can thus at least faintly visualize *in silico* the biologically relevant dynamic ensemble of any V3 loop crown sequence from position 10 to 22. This capability was later verified by an independent group [19]. This result also suggests that this portion of the V3 crown behaves similarly to an autonomously folded, free, unconstrained peptide, since the folding simulation used no constraints on the two stems of the peptide and the folding qualitatively recapitulated a form seen experimentally in the V3 loop in situ in gp120 [6]. Finally, this technology was used to engineer an $\alpha$-helical V3 loop crown and demonstrate that it loses infectivity [20]. *Ab initio* folding of residues 10 to 22 of the V3 crown of all the psVs used in this study was thus used to evaluate the flexibility and conformational preferences of V3 loop crown structures *in silico*.

All folding simulations and analysis were performed with ICM software (MolSoft LLC, La Jolla, CA, USA) as previously described [21]. This algorithm has previously been shown to predict experimentally verified peptide structures up to 23 residues in length within the error accuracy limit [22]. As a starting point for each folding run, fully extended conformations of full-atom (including hydrogens) models of each indicated V3 loop crown sequence (positions 10–22) were generated. The total number of energy evaluations during the MC run was based on the number of free variables (number of free standard torsion angles). Each MC run took 2-3 hours of CPU time on a 3.00 Ghz Dual-Core Intel Xeon Processor. The exact script for the foldings and all the folding data are available upon request from the corresponding author.

All the data on chimeric psV construction and neutralization assays were previously reported in the literature [14, 17], except those repeated for confirmation in this study or those in Table 2. In all cases, the same method was used. Briefly, each chimeric psV was constructed to contain a different V3 loop sequence grafted in to replace the V3 loop

in the SF162 Env, where the V3 loop is relatively accessible ("unmasked") [17]. Thus, the observed differences in psV neutralization elicited by each mAb maps to differences in the V3 loop sequences. The V3 chimeric SF162 psVs were constructed with V3 mutations introduced as indicated in the text in the consensus subtype B or consensus subtype C V3 loop, or V3 chimeric SF162 psVs were constructed in which the SF162 V3 loop was replaced with the V3 loop consensus sequences from subtypes A1, B, C. The neutralization by mAbs 447-52D and 2219, as well as other Abs, of each of these psVs was assessed using methods previously described [23]. Briefly, neutralizing activity was determined with a single-cycle infectivity assay using psVs generated with the *env*-defective luciferase-expressing pNL4-3.Luc.R-E- plasmid v [24] pseudotyped with the SF162 V3 variants described above. The psVs were incubated with serial dilutions of mAbs for 1.5 hour at 37°C and then added to CD4+CCR5+U87 target cells plated in 96-well plates in the presence of polybrene (10 mg/mL). After 24 hrs, cells were refed with RPMI medium containing 10% FBS and 10 $\mu$g/mL polybrene, followed by an additional 24–48 hr incubation. Luciferase activity was determined 48–72 hrs postinfection with a microtiter plate luminometer (HARTA, Inc.) using assay reagents from Promega, Inc. Geometric mean titers for 50% neutralization ($GMT_{50}$) were determined by interpolation from neutralization curves and are averages of at least three independent assays.

## Abbreviations

HIV: Human immunodeficiency virus.

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