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REVIEW

Role of host immune responses in sequence variability of HIV-1 Vpu

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Abstract

Viral protein U (Vpu) is an accessory protein associated with two main functions important in human immunodeficiency virus type 1 (HIV-1) replication and dissemination; these are down-regulation of CD4 receptor through mediating its proteasomal degradation and enhancement of virion release by antagonizing tetherin/BST2. It is also well established that Vpu is one of the most highly variable proteins in the HIV-1 proteome. However it is still unclear what drives Vpu sequence variability, whether Vpu acquires polymorphisms as a means of immune escape, functional advantage, or otherwise. It is assumed that the host-pathogen interaction is a cause of polymorphic phenotype of Vpu and that the resulting functional heterogeneity of Vpu may have critical significance *in vivo*. In order to comprehensively understand Vpu variability, it is important to integrate at the population level the genetic association approaches to identify specific amino acid residues and the immune escape kinetics which may impose Vpu functional constraints *in vivo*. This review will focus on HIV-1 accessory protein Vpu in the context of its sequence variability at population level and also bring forward evidence on the role of the host immune responses in driving Vpu sequence variability; we will also highlight the recent findings that illustrate Vpu functional implication in HIV-1 pathogenesis.

Key words: Human immunodeficiency virus type 1; Vpu; Sequence variability; Immune responses; Human leukocyte antigen class I

Core tip: Viral protein U (Vpu) is a highly polymorphic human immunodeficiency virus type 1 (HIV-1) accessory protein; however factors that are attributable to Vpu sequence variability are not well defined. In this review we have focused on the immune responses both innate (natural killer cells) and adaptive (cellular and humoral) immunity that are directed towards HIV-1 Vpu and we also show the interaction between Vpu and host cellular factors. We also highlight evidence that suggests interaction between the host immune responses and Vpu may contribute to Vpu sequence variability. Finally we have summarized the current knowledge on HIV-1 Vpu functions including Vpu evasion activities from the host immune surveillance.

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) demonstrates a significant genetic diversity due to its high

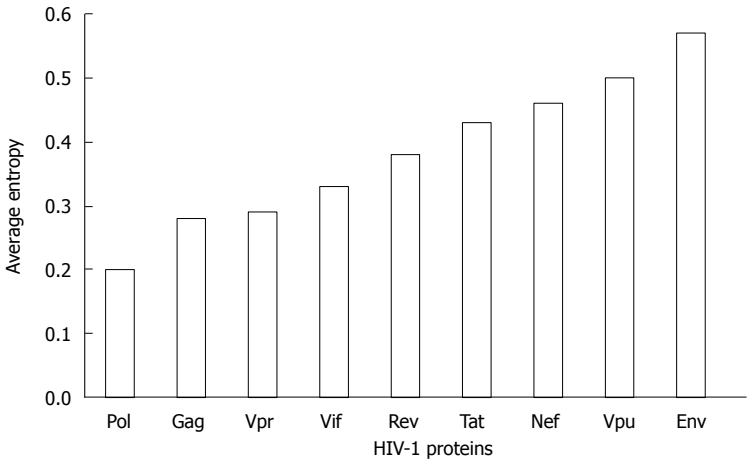

Figure 1 Sequence variability of human immunodeficiency virus type 1 proteins. The sequence variability of nine proteins of human immunodeficiency virus type 1 (HIV-1) shown in the graph was determined by using Shannon entropy approach[24,90]. The full genome clade B sequences of the individual patients were retrieved from Los Alamos database (n = 544). Vpu: Viral protein U.

mutation rate; so far this extraordinary diversity has been a major setback in development of vaccine and antiretroviral drugs. Low fidelity of reverse transcriptase that give rise to error prone replication process, high progeny production, turnover rates and recombination of circulating HIV-1 strains are some of the viral factors that contributes to HIV-1 diversity[1-3]. The adaptive potential of HIV-1 is shaped by both virus and the host immune factors, in other words both the diversifying and purifying selection factors influence HIV-1 diversity. In fact, strong evidence has also indicated that the host immune responses influence HIV-1 diversity by selection of escape mutations[4-6]. Thus a comprehensive analysis of the dynamics of polymorphisms in HIV-1 proteins is a powerful tool to reveal actual interactions between HIV-1 and the host immune system[7-9].

HIV-1 viral protein U (Vpu) is a 16-kDa accessory protein[10] responsible for various functions such as CD4 down-regulation[11-13] and enhancement of virion release by antagonizing tetherin/BST2[14-17]. Interestingly, functionally competent Vpu (with respect to BST-2 antagonistic activity) were only found in the pandemic group M subtypes, suggesting that Vpu functional adaptation may confer pandemic spread of this HIV-1 subtype[18]. In general, the host genetic factor is one of the main driving force of sequence polymorphism in HIV-1[18], as evidenced in HIV-1 Nef[17,19-21] and Env[22,23] proteins whose highly polymorphic phenotype is mostly attributed by the host immune responses such as HLA class I-restricted CD8+ T lymphocytes and neutralizing antibodies, respectively. However, it is still unclear to what extent the host immune responses influence Vpu sequence variation. This review focuses on the role of host immune responses in Vpu sequence variability. Briefly, we also discuss the current understanding of Vpu functions including evasion of the immune system and their implication in viral pathogenesis.

SEQUENCE VARIABILITY OF VPU

Vpu exhibit a stable reading frame *in vivo* despite being a highly variable protein, suggesting functional importance of Vpu in HIV-1 replication and persistence. Furthermore, it has evidently been shown that only HIV-1 strains of the pandemic M group evolved a fully functional Vpu that efficiently antagonizes human tetherin/BST-2; this suggests that Vpu evolutional adaptation may be associated with the pandemic spread of HIV-1[18]. Several studies have demonstrated the extent of Vpu sequence variability both at inter- and intra-patient level. By using the 101 aligned amino acid sequences of entire HIV-1 genome, one study showed that Vpu had the highest average entropy score in comparison to other proteins in HIV-1 genome[24]. Another study analyzing the intra-patient diversity and adaptation of non-structural genes in primary HIV-1 subtype C infection reported that *vpu* compared to *vif, vpr, tat exon 1* and *rev exon 1* genes has the highest mean of intra-patient diversity that increased gradually[25]. We retrieved full lengths clade B sequences (n = 544) of HIV-1 proteins (Gag, Pol, Env, Nef, Vif, Vpu, Vpr, Tat and Rev) from Los Alamos database and the average entropy score of each protein was determined. Vpu was observed to be one of the proteins with the highest average entropy score (Figure 1), confirming the highly variable nature of Vpu at population level. However, interestingly, a recent study has shown that despite extensive Vpu sequence variation in HIV-1 infected individuals, Vpu functions (CD4 cell surface downregulation and tetherin counteraction activity) were maintained[26].

IMMUNE RESPONSES TOWARDS VPU

Humoral immunity

Several studies have reported Vpu-specific humoral immune responses during HIV-1 infection[27-31]. However there has been some controversy on correlation between the presence of anti-Vpu Ab responses in HIV-infected patients' sera and clinical outcome. Some studies have indicated that anti-Vpu Ab responses may influence the clinical outcomes in HIV-1 infected individuals[27,28,30,31]; while on the other hand other studies have showed no correlation[29]. These findings indicate that Vpu is indeed a target of antibodies although no evidence yet support that such antibody responses influence the Vpu variability. The epitopic regions for such antibodies reported include 37-50[30] and 68-81[28] of Vpu; nonetheless there

is no specific Vpu activity mapped to these regions so far. However, considering that Vpu is a small protein (81 amino acids); it is intriguing to test whether such Vpu-specific antibodies can inhibit Vpu functions and subvert viral replication.

Cellular immunity

A growing number of clinical evidence has suggested that HLA-restricted, HIV-specific CD8+ cytotoxic T lymphocytes (CTL) is mainly involved in controlling HIV-1 replication[32-34]. CTL responses have been well appreciated in SIV-infected macaque's model[32,33] and in HIV-1 infected patients of both acute[35,36] and chronic[37] phases as well as in elite controllers who spontaneously suppress viral replication below detection limit[38,39]. HLA-restricted CTL responses are thought to be the main driving force of HIV-1 control and viral evolution[40-43]. The viral polymorphism in response to immune selective pressures follows predictable patterns and kinetics at the population and these immune "footprints/landscape" could be predictable based on the autologous viral sequences and the host immune genetics[9,42,44]. However, Vpu has been reported to be a poor target for CD8+ T cells as revealed by interferon (IFN)-γ Elispot assay[45], because only some few epitopes were identified and less than 3% of patients showed detectable Vpu-specific CD8+ T cell responses. Although several HLA-restricted CTL epitopes of Vpu are reported[45-49], this protein is less targeted by CTLs at least compared to the Nef protein. Consistently, our previous study showed only three HLA-associated polymorphisms in Vpu at Glu-5 with HLA-C*03 and Arg-37, Lsy-37 with HLA-A*3303 in a chronic HIV-infected patient cohort in Japan (n = 216), indicating that the HLA class I has minor contribution (2% of the total codons) towards Vpu variability[50]. The increased numbers of subjects to 516 showed similar results (DK, ZH, and TU: unpublished observation). Furthermore, an international large IHAC cohort (International HIV Adaptation Collaborative, n = 1888) identified that only 26.3% of the highly variable Vpu codons exhibited statistically significant HLA class I associations[20]. Although the HLA class I -associated viral polymorphisms observed in the two cohorts suggested to be influenced by several factors such as the host genetic profiles, mixture of multiethnic populations, studied sample size, geographical location and circulating HIV-strains, these results suggest that HLA-associated polymorphisms are only partly attributable to the Vpu variability (Figure 2). However, it is of note that the low CTL responses observed in the previous studies[45,51] and subtle numbers of HLA-associated polymorphisms[20,50] may be an underestimation due to the current technical limitation toward a highly variable protein, even though a number of studies reported a plenty of CTL targeting[52,53] and HLA-associated polymorphisms in Nef[19,20,42], which showed comparable variability with Vpu at a population level (Figure 1).

Natural killer cells

A number of evidence suggests that natural killer (NK) cells have an important role in control of HIV-1 infection[54-56]. Assuming that NK cells may act as a selective force, as similar to CTLs, HIV-1 may leave footprints as viral polymorphisms in association with polymorphic NK cell ligand such as killer-cell immunoglobulin-like receptors (KIR). In fact, one study identified 22 amino-acid polymorphisms within the HIV-1 clade B sequence that are significantly associated with the expression of specific KIR genes in chronically HIV-1 infected, treatment naïve patients (n =91)[44]. Three (13.6%) of these KIR associated polymorphisms were located in Vpu at positions Ser-3 and Vpu-Env overlapping region (Met-71 and His-74) (Figure 2)[44]. In addition, the HIV-1-specific antibody-dependent NK cell cytotoxicity is identified towards a 13-mer Vpu peptide (^{69}EMGHHAPWDVD-DL^{81})[57]. Such responses are also observed toward Env[58] and Nef[59] in HIV-1 infected patients as well. However, there is no evidence at the moment that show antibody-dependent NK cell cytotoxicity associates with viral polymorphisms.

VPU FUNCTIONALITY INCLUDING IMMUNE EVASION ACTIVITY

In order to conquer the hostile host environment, viruses need to evolve and develop critical interactions with the host cellular factors. Vpu does not only play important role in HIV-1 pathogenesis through CD4 receptor degradation[11] and enhancement of virion release from infected cells by antagonizing tetherin/BST-2[60-62]; but Vpu has also evolved to interact with and modulate other host surface receptors and factors (Figure 3).

Vpu induces CD4 receptor degradation

Vpu induces the rapid degradation of newly synthesized CD4 receptor molecules that are retained together with Env precursor protein (gp160) in the endoplasmic reticulum[13]. The cytoplasmic domain of Vpu and the DSGxxS motif are critical in interaction with and degradation of CD4, respectively[12,63] (Figure 2). The degradation process is achieved by Vpu recruiting β-TrCP and then interacts with CD4 cytoplasmic domain and subsequently subject CD4 to degradation by the ubiquitin-proteasome pathway[11,64]. In doing so Vpu contributes to the suppression of HIV-1 primary receptor at the surface of the infected cell.

Vpu enhances virion release

Enhancement of virion release by Vpu has been shown to be achieved through antagonizing tetherin/BST-2, an IFN regulated host restriction factor. BST-2 directly binds to virions and hence retains them on the surface of infected cells[61,62]. Vpu through AxxxAxxxA motif in transmembrane domain directly interacts with BST-2 transmembrane domain, the Vpu DSGxxS and [D/E]XXXL[L/I/V] motifs in the cytoplasmic domain also play crucial role in ensuring BST-2 downmodulation[15,65,66] (Figure 2). Previous studies indicated BST-2

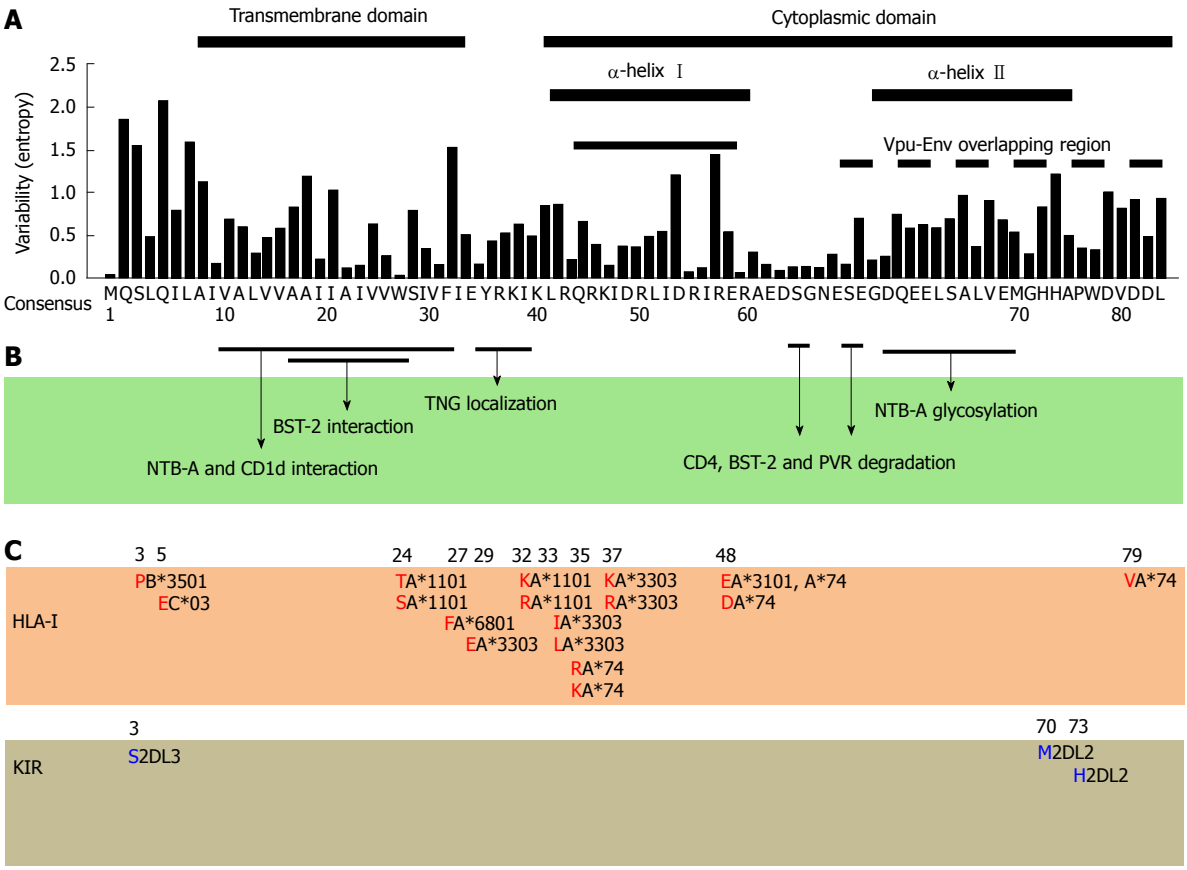

Figure 2 Correlation among amino-acid codon variability, functional regions, and host-mediated immune escape map of human immunodeficiency virus type 1 viral protein U. A: Amino acid codon variability is assigned to each position of Vpu using Shannon entropy approach[24,90]. Sequences were retrieved from Los Alamos database (*n* =1139), the consensus subtype B sequence is indicated as a reference; B: Interacting positions and domains responsible for the indicated functions of human immunodeficiency virus type 1 (HIV-1) Vpu are shown[14,62,91]; C: Immune escape map shows amino acid codons and residues (red and blue) associated with HLA-I alleles[20,45,50] and killer-cell immunoglobulin-like receptors (KIR)[44], respectively. The specific alleles are indicated in black adjacent to the amino acid.

downmodulation is through β-TrCP-dependent proteasomal degradation pathway[67] while others suggested the β-TrCP-dependent endo-lysosomal pathway[65,68]. In contrast, recent studies showed that BST-2 antagonistic activity by Vpu takes place in the trans-Golgi networks (TGN)[14]. Vpu interferes with anterograde transport of BST-2 to the cell surface subsequently leading to BST-2 trapping in the TGN[15-17,69].

Vpu modulation of other cell surface receptors and host factors

Recent studies have indicated that Vpu is emerging as a viral factor with a range of activities devoted to counteracting host innate and adaptive immunity including the modulation of NK cell co-activation ligand NK-T and B cell antigen (NTB-A)[70], PVR activating ligand of NK cells[71], and CD1d[72,73] (Figure 3).

NTB-A triggering is necessary for induction of efficient lysis of target cells upon engagement of the activating receptor NKG2D[74]. The Ser-52 and Ser-56 residues important for CD4 and BST-2 degradation did not affect NTB-A expression, indicating that the down modulation of NTB-A by Vpu is mediated by different domains[70]. A recent study has shown that downmodulation of NTB-A is achieved by Vpu interfering with the anterograde transport of NTB-A by retaining it within the Golgi compartment and hence affects its glycosylation pattern that subsequently reduces surface expression of NTB-A[75].

PVR (CD155, Necl-5) is a ligand for the activating receptor DNAM-1 (CD226) expressed by NK cells[76,77]. PVR downmodulation by Nef and Vpu is another strategy evolved by HIV-1 to avoid NK cell-mediated lysis of infected cells[71]. PVR downregulation alters multiple important PVR-mediated innate cellular immune processes such as adhesion and migration, and therefore may influence HIV-1 pathogenesis.

CD1d molecules are important in dendritic cells for lipid antigen presentation to CD1d-restricted NKT cells[78,79]. CD1d and CD1d-restricted NKT cells are present at pathogen entry sites thus play a crucial role in early immune responses[80]. Vpu has been shown to be the major viral factor that inhibit recycling of CD1d from the endosomal compartment back to cell surface through retaining CD1d in early endosomes[72].

Vpu has also been implicated in inhibition of ubiquitination and degradation of p53 (a substrate of $SCF^{\beta\text{-}TrCP}$ ligase complex). The successful interaction of $SCF^{\beta\text{-}TrCP}$ complex with β-TrCP binding motif ($DS_{52}GNES_{56}$) present in Vpu has been shown to be essential[81]. It was observed that Vpu mutants with alanine substitutions

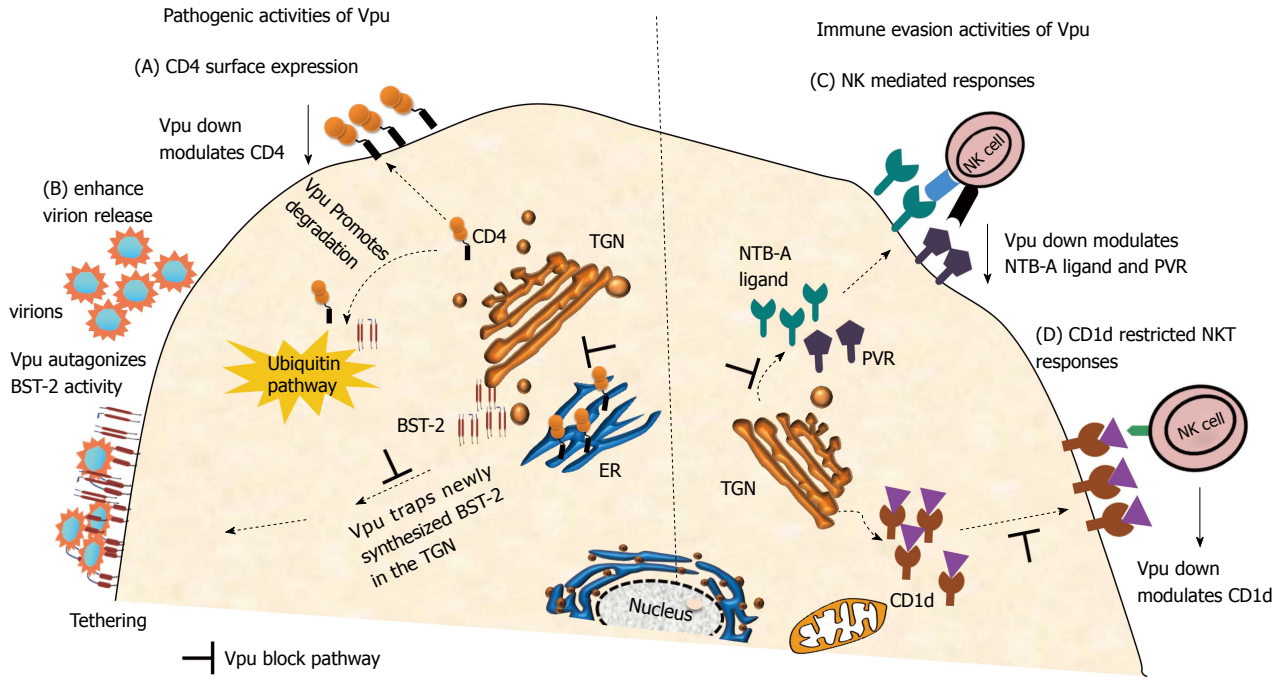

Figure 3 Viral protein U functionality including immune evasion activity. The schematic representation of the cell illustrates some key functions of viral protein U (Vpu) including immune evasion activities. (A): Panel A illustrates CD4 down regulation by Vpu through degradation in a β-TrCP dependent ubiquitination pathway[11,12,64]; (B): Panel B demonstrates enhancement of virion release by Vpu through antagonizing BST-2, which is achieved through direct interaction with BST-2 which subsequently leads to trapping of BST-2 in the trans-Golgi networks[14-16] and also indicates β-TrCP dependent ubiquitination of BST-2[62,65,66]; (C): Panel C demonstrates Vpu evasion of NK cell recognition through down modulation of NTB-A ligand[70] and PVR[71]; D: Panel D shows down modulation of CD1d from cell surface hence avoid CD1d-restricted NKT cell responses[72,73]. NK: Natural killer; NKT: Natural killer T.

(DA$_{52}$GNEA$_{56}$) failed to stabilize p53 and did not prevent its ubiquitination. This suggested that Vpu is able to achieve modulation of p53 through competing efficiently with p53 protein for the β-TrCP subunit of the SCF complex and hence inhibits subsequent ubiquitination of p53 protein. The modulation of p53 positively correlated with apoptosis during the late stages of HIV-1 infection[81].

Finally, although Vpu showed multiple functions *in vitro* and *ex vivo*, it is yet clear how and what functions of Vpu are important in viral pathogenesis *in vivo*.

CONCLUSION

The current knowledge on factors that are attributed to Vpu polymorphism has not been quite sufficient; therefore this prompt for further analysis to reveal the unresolved questions of why Vpu is so variable and what factors drive Vpu polymorphism. In order to define the complex dynamics of HIV-1 Vpu evolution, immune escape patterns, and functional adaptation during the course of infection, further insight is needed on the role of host genetics and other immune selection pressures towards shaping HIV-1 Vpu diversity. The emergence of advanced DNA sequencing technologies such as ultra-deep sequence which is superior and more sensitive than Sanger sequence methods has made it possible to accurately detect and analyze minor variants of HIV-1 within a host[82-85]. Furthermore, the establishment of different contemporaneous cohorts of HIV-1-infected individuals worldwide enables us to examine to what extent the host immune components play a role on viral adaptation and/or evolution at both intra- and inter-patients' level.

So far the current studies have indicated that the host immune responses directed towards Vpu is not entirely attributable to HIV-1 Vpu variation (Figure 2), it is therefore crucial to apprehend other factors that may explain Vpu variation. Of note previous studies have identified immune responses directed towards Vpu, using peptides of HIV-1 consensus sequences[45,57]. However, ironically due to Vpu polymorphic nature itself, these results may mask the exact extent to which immune responses contribute to Vpu sequence variation. Alternatively, HIV-1 like other RNA viruses has evolved to shorten its genome length through overlapping its genes[86]. The overlapping region of Vpu and Env is one of promising aspect to consider when we focus on Vpu variation. Because host immune responses (neutralizing antibodies) contribute to Env polymorphic nature[87,88], it is enticing to assume that immune responses directed towards Env may influence Vpu polymorphisms through Vpu-Env overlapping region. KIR associated polymorphisms within Vpu-Env overlapping region have been reported previously[44]. Although it is still unknown whether NK cells recognize Vpu or Env protein, nonetheless these findings indicate the importance of this region for Vpu variability. Furthermore, it is reported that X4- and R5-tropic HIV-1 showed differential amino acid polymorphisms in Vpu[89], suggesting that cellular compartment influences Vpu variability.

The current increase in number of new findings of

Vpu from pandemic HIV-1 group M strain and other HIV-1 strains, enlighten us the precise role or mechanisms of how Vpu degrade the viral receptor CD4, antagonize tetherin/BST-2, enhance p53 stability and modulate NK-cell activities through modulation of PVR, NTB-A and CD1d receptors (Figure 3). Understanding the mode of action of Vpu and association of the immune factors certainly open plenty of new windows to deciphering the intricate mechanisms associated with HIV-1 immune pathogenesis *in vivo*. Also, understanding pathways of Vpu intra- and inter-patients sequence variability and adaptation may provide us with an alternative approach for prospects of viral persistence and Vpu contributions *in vivo*.

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