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Diplom-Agriculture Jingyan Chen

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Functional Comparison of HIV-1 Vpu Alleles Derived from Elite Controller and Chronic Progressor Patients

Referees: Prof. Dr. Stephan Urban

Prof. Dr. Oliver T. Fackler

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Summary

Human Immunodeficiency Virus type 1 (HIV-1) is the major causative agent of the AIDS epidemic. Several independent transmission events from monkeys to humans gave rise to different viral lineages that differ with respect to their ability to encode for accessory gene products that facilitate virus replication in the infected host. In particular, the *vpu* gene is unique to the HIV-1/SIVcpz lineage and not present in HIV-2 and most SIV isolates. Vpu is not essential for HIV-1 replication but intensively modulaties host immune components such as the HIV-1 primary entry receptor CD4, whose cell surface levels are reduced by Vpu. Uniquely, Vpu promotes the release of mature viral particle from infected cells by antagonizing the host restriction factor CD317/tetherin. Moreover, Vpu interferes with NF-ĸB signalling triggered by CD317/tetherin and reduces the cell surface exposure of MHC class I (MHC-I) and natural killer cells ligand NTB-A. While these Vpu activities have been established ex vivo, their relevance for HIV pathogenesis in the infected host remains unclear.

In an attempt to correlate Vpu function with the clinical outcome of HIV-1 infection, we assessed here the functions of *vpu* alleles derived from two distinct clinical groups of treatment-naïve HIV-1 infected patients. While HIV-1 elite controllers (ECs) naturally control virus replication and keep the viral load below detectable level (<50 copies/ml), chronic progressors (CPs) display viral loads of more than 2 000 copies/ml. Both EC and CP Vpu alleles showed conserved and potent capacities to reduce cell surface levels of CD4 and CD317 molecules and to promote viral particle release. In contrast, EC Vpu alleles were less activitive in MHC-I and NTB-A downregulation than CP Vpu alleles and the antagonism of NF-kB signalling was not conserved in both patient groups. Sequence analysis of our Vpu alleles revealed the enrichment of killer-cell immunoglobulin-like receptor (KIR) KIR2DL2-associated footprints in EC Vpus, this polymorphism however did not explain the functional difference between EC and CP Vpus.

These results suggest downregulation of cell surface CD4 and antagonism of the particle release restriction by CD317 as important in vivo functions of Vpu. Since attenuated Vpu alleles were more frequent in ECs than in CPs, at least a subgroup of EC Vpu alleles may be under selection pressure resulting in adaptation of Vpu that is associated with a mild fitness cost. Whether the functional constrains of EC Vpu alleles contribute to the suppression of HIV-1 in these patients warrants further investigation.

Zusammenfassung

Das humane Immundefizienz-Virus 1(HIV-1) ist der Haupterreger der AIDS-Epidemie. Mehrere unabhängige Übertragungsereignisse von Affen zum Menschen sind der Ursprung verschiedener viraler Stämme, die sich in Bezug auf ihre Fähigkeit, für akzessorische Genprodukte zu kodieren, welche die virale Replikation im infizierten Wirt erleichtern, unterscheiden. Insbesondere das *vpu* Gen ist ein spezielles Charakteristikum der HIV-1/SIV cpz Linie, und ist nicht in HIV-2 und den meisten SIV Isolaten vorhanden. Vpu ist zwar für die HIV-1 Replikation nicht essentiell, aber es moduliert intensiv Immunkomponenten des Wirts, wie den Haupteingangsrezeptor CD4, dessen Zelloberflächenlevel durch Vpu reduziert werden. Vpu fördert auf einzigartige Weise die Freisetzung von reifen viralen Partikeln von infizierten Zellen, indem es dem wirtseigenen Restriktionsfaktor CD317/Tetherin entgegenwirkt. Darüber hinaus interferiert Vpu mit CD317/Tetherin vermittelter Signaltransduktion und reduziert die Zelloberflächenexposition von MHC Klasse I (MHC-I) und dem natürliche Killerzellliganden NTB-A. Da diese Aktivitäten von Vpu *ex vivo* etabliert wurden, bleibt ihre Relevanz für die HIV Pathogenese im infizierten Wirt ungeklärt. In einem Versuch, Vpu Funktion mit dem klinischen Verlauf der HIV Infektion zu korrelieren, untersuchten wir hier die Funktion von *vpu* Allelen, welche aus zwei unterschiedlichen klinischen Gruppen von behandlungsnaiven HIV-1 infizierten Patienten stammten. Während sogenannte HIV-1 Elite Controller (EC) die Virusvermehrung auf natürliche Weise kontrollieren und dabei die Viruslast unterhalb der Nachweisgrenze halten (<50 Kopien/ml), zeigen chronische Progressoren (CP) eine Viruslast von mehr als 2000 Kopien/ml. Sowohl EC als auch CP Vpu Allele zeigten ein konserviertes wirkungsvolles Vermögen, die Oberflächenexpression von CD4 und CD317 Molekülen zu modulieren und die Freilassung von Viruspartikeln zu fördern. Im Gegensatz dazu waren EC Vpu Allele im Vergleich zu CP Allelen weniger aktiv in der Herabregulierung von MHC-I und NTB-A von der Zelloberfläche und der Antagonismus der NF-ĸB Signalkaskade war in beiden Patientengruppen nicht konserviert. Sequenzanalysen unserer Vpu Allele zeigten eine Anreicherung von Killerzell Immunglobulin-ähnlichen Rezeptoren (KIR) KIR2DL2-assoziierten Footprints, was jedoch nicht die Funktionsunterschiede zwischen den EC und CP Vpu Allelen erklärt.

Diese Ergebnisse weisen darauf hin, dass die Herunterregulierung von Zelloberflächen-CD4 und der Antagonismus der CD317-vermittelten Hemmung von Partikelfreisetzung eine bedeutsame *in vivo* Funktionen von Vpu darstellen. Dass abgeschwächte Vpu Allele in EC häufiger auftraten als in CP, deutet darauf hin, dass zumindest eine Untergruppe von EC Vpu Allelen unter Selektionsdruck steht, was in einer Anpassung von Vpu auf Kosten von leicht abgeschwächter Fitness resultiert. Es

benötigt weiterer Untersuchungen, ob die funktionellen Einschränkungen von EC Vpu Allelen zur Unterdrückung von HIV-1 in diesen Patienten beiträgt.

1 Introduction of HIV/AIDS

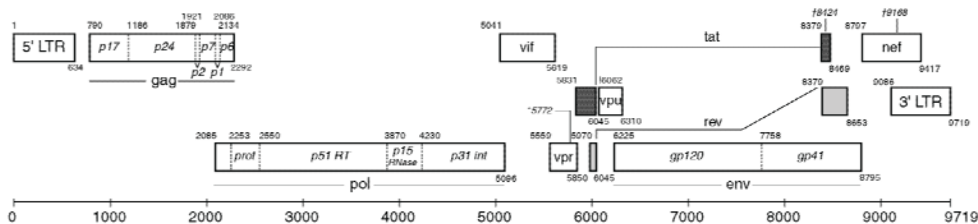
The human immunodeficiency virus (HIV) is a retrovirus that specifically infects cells of the human immune system (Maddon, Dalgleish et al. 1986) (Koenig, Gendelman et al. 1986) (Koppensteiner, Brack-Werner et al. 2012). The most deteriorative stage of the HIV infection constitutes the acquired immunodeficiency syndrome (AIDS). Both HIV-1 and HIV-2 are the causative agents of AIDS. More than 35 million people are living with HIV, and over 1.5 million have died from AIDS within the WHO region by the year of 2012 (WHO HIV/AIDS data and statistics, 2014). HIV/AIDS is considered as one of the major global public health issues (WHO media centre) because of its devastating effect on the human immune system, while still no therapeutic and prophylactic vaccines are currently available (Cohen and Dolin 2013). Since HIV-1 is the leading cause of the global HIV/AIDS pandemic (WHO, health topics HIV/AIDS), the scope of this introduction remains directed toward HIV-1.

1.1 HIV-1 Virology

1.1.1 Genome and structure of HIV-1

HIV-1 is classified in the genus *Lentivirus* of the family *Retroviridae* based on its morphological, genetic and biological properties (Karlsson Hedestam, Fouchier et al. 2008). It specifically infects human immune cells including $CD4^{+}$ T cells, macrophages, and dendritic cells (Maddon, Dalgleish et al. 1986) (Cunningham, Donaghy et al. 2010) (Koppensteiner, Brack-Werner et al. 2012). HIV-1 is an enveloped virus possessing two identical copies of positive sense RNA genome. Each copy contains nine open reading frames encoding fifteen proteins, as well as two long terminal repeats (LTR) 5’-LTR and 3’-LTR serving as regulatory elements for the expression of viral RNA and proteins (Frankel and Young 1998) (Sierra, Kupfer et al. 2005) (Watts, Dang et al. 2009). The three largest reading frames of the HIV-1 genome encode three poly-proteins, which can be processed into viral structural constituents. The group specific antigen (Gag) is cleaved by the viral protease into the viral matrix (p17, MA), the capsid (p24, CA) and the nucleocapsid (p7, NC), which

A



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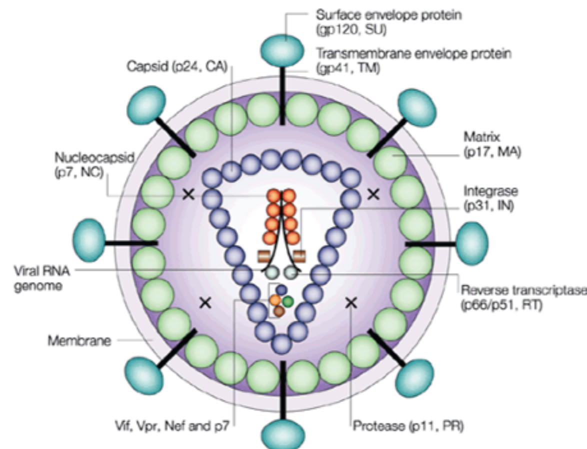

Figure 1: Genome organization and structure of HIV-1. A: Organization of the HIV-1 genome (strain HXB2). HIV-1 RNA contains 9719 base pair nucleic acids (Accession number K03455.1). Nine open reading frames are shown as rectangles and the cleavage sites of Gag, Pol and Env polyproteins are shown as dash lines in each rectangle. *Tat* has two isoforms and *rev* gene is located in two exons. The numbers in the upper left corner represent the starting position of the gene and the numbers in the lower right corner record the ending position of the genes. **B:** Graphical depiction of a mature HIV-1 virion organization. HIV-1 virion consists of a virion core and an envelop membrane. The core is shelled by HIV-1capsid proteins. Two positive RNA strands in association with nucleocapsid proteins, as well as reverse transcriptase, integrase, and protease are encapisudated into the core. And the core is further shelled by HIV-1 matrix proteins, which is located in the interface of host-cell-derived membrane with the insertion of viral spikes composed by Env (SU and gp120) proteins.

(A: adapted from Los Alamos National Laboratory database; B: adapted from Harriet L. Robinson, Nature reviews. Immunology, 2002(Robinson 2002))

are the major structural components of the virion core (Figure 1B). The processing of polymerase (Pol) polyprotein produces the enzymatic proteins including protease (p11, PR), reverse transcriptase (p66/p51, RT) and integrase (p31, IN), all of which remain encapsulated in the virion core, together with the viral RNA genome (Freed 2001) (Robinson 2002). Cleavage of the envelop (Env) protein by cellular protease generates the transmembrane protein (TM, gp40) and the surface glycoprotein protein (SU, gp120), which are required for the formation of the surface spike (Freed 2001). The Env spikes are inserted into the host cell-derived lipidprotein-rich membrane which surrounds the virion core (Brugger, Glass et al. 2006) (Robinson 2002). Beyond the genes common to all retroviruses, HIV-1 possesses two regulatory genes *tat* and *rev* essential for the HIV-1 gene expression, as well as four multifunctional accessory genes *vpr, vif, vpu,* and *nef* being mainly responsible for antagonizing host restriction factors (Strebel 2013). Except for Vpu, the rest are incorporated to HIV-1 particles (Sherman, de Noronha et al. 2003). All HIV-1 gene products have elaborate functions in the proceeding of the HIV-1 replication cycle and in circumventing restriction factors in the host environment.

1.1.2 Replication Cycle of HIV-1

The replication of HIV-1 can be arbitrarily divided into successive steps which are deeply networked. HIV-1 exploits not only the viral components but also a great number of host cellular factors for its own replication. The major events in a step of the replication cycle, together with the critical viral and accompanying cellular factors involved, are briefly summarized on the basis of the relevant literatures and reviews, and rendered in graphic form as shown in Figure 2.

Viral Entry

The entry of HIV-1 into a susceptible target cell is the first step toward a new infection. It is a coordinated multi-step process. Initially the HIV-1 attaches itself to the target cell via specific interactions of Env with adhesion factors such as α4β7 integrin, and non-integrin DC-SIGN, or via unspecific interactions of positively

charged Env with negatively charged cell surface proteoglycans (Kuritzkes 2009) (Friedrich, Dziuba et al. 2011). The attachment is followed by the high-affinity binding of Env protruding protein gp 120 with its specific primary receptor CD4, which leads to the exposure of the chemokine receptor binding sites of gp120. And the binding of gp 120 with chemokine receptor then triggers the membrane fusion machinery in the gp41 subunit which pulls the viral and cellular membranes together termed as fusion (Wilen, Tilton et al. 2012). The main chemokine receptors that HIV-1 utilizes are CCR5 and CXCR4 (Deng, Liu et al. 1996) (Feng, Broder et al. 1996), and HIV-1 can be classified into R5 HIV, X4 HIV and R5X4 HIV based on the chemokine receptor usage (Berger, Doms et al. 1998) (Berger 1997) (Bleul, Wu et al. 1997). In addition to the receptor-mediated fusion at the plasma membrane, HIV-1 could also infect cells via endocytosis and fuse with intracellular compartments (Miyauchi, Kim et al. 2009) (Pritschet, Donhauser et al. 2012). At the site of fusion occurs, a virion core is released into the host cell cytoplasm and then proceeds into the next step of its life cycle.

Reverse Transcription

After the viral core is released into the cytoplasm, the viral capsid first needs to be dissociated, a process termed as uncoating. It is known that uncoating is a dynamic process and is critical for the reverse transcription of viral RNA, while the timing and cellular factors remain poorly understood mainly due to the lack of available methods to label capsid molecules without perturbing the function of the viral capsid (Arhel 2010) (Hulme, Perez et al. 2011). Uncoating ultimately results in the rearrangement of the virion core to form the reverse transcription complex (RTC), which contains genomic RNA, viral proteins MA and Vpr and various host factors such as tRNA(Lys)3 primers (incorporated into the virion during assembling) and the cAMP-dependent protein kinase (Lanchy, Isel et al. 2000) (Arhel 2010) (Karn and Stoltzfus 2012) (Giroud, Chazal et al. 2013). RTC is associated with actin microfilaments of the host cell cytoskeleton where the reverse transcription mainly occurs (Bukrinskaya, Brichacek et al. 1998). The reverse transcription is initiated with the binding of denatured primer tRNA(Lys)3 and the viral RNA (Wakefield, Wolf et al.

1995) (Mak and Kleiman 1997), then followed by a transcription of the viral RNA from the binding site to the LTR into a small fraction of DNA, while simultaneously the transcribed region of the template RNA is degraded by the RNase H activity of HIV-1 RT. Then the resulting DNA fraction is transferred to the complementary region of the other RNA template thereby completing the synthesis. This RNA template is again degraded once it is copied by RNase H, but as the RNA polypurine tract (PPT) is resistant to the degradation it primes the other DNA synthesis by using the first strand of RNA as the template. All the remaining RNA is removed by RNase H. The double strands of DNA are then processed to be integrated into the host chromosomal DNA or form stable DNA circles that are not integrated into the host genome (reviewed in (Warren, Warrilow et al. 2009; Warrilow, Tachedjian et al. 2009; Arhel 2010) (Sloan and Wainberg 2011)).

Integration

Once the viral DNA is synthesized in the cytoplasm, it stably associates with the integrase (IN), which form the pre-integration complex (PIC) together with other viral and cellular proteins (Turlure, Devroe et al. 2004) (Suzuki and Craigie 2007). It is a prerequisite for the integration that PIC is transported into the nucleus to encounter the cellular DNA (Marchand, Johnson et al. 2006) (Craigie and Bushman 2012) The nuclear envelope lamina together with the associated proteins such as the well characterized emerin and barrier-to-auto-integration factor (BAF) mediate the entry of PIC into the nucleus and the proper localization within the nucleus (Jacque and Stevenson 2006) (Li and Craigie 2006). The integration of viral DNA into the host chromosomal DNA occurs precisely at the termini of the viral DNA but can also take place at many other locations in the host genome (Di Nunzio 2013). The linkage of viral integrase (IN) with the host chromatin via the co-activator lens epithelium derived growth factor (LEDGF) is essential for the integration (Llano, Saenz et al. 2006) (Shun, Raghavendra et al. 2007). Two main actions are involved in this process. Firstly, IN catalyzes the removal of two nucleotides from each of the 3′ ends of the viral DNA in the region of LTR and generates the CA_{OH} 3'-hydroxyl, and this provides the nucleophiles to the 5′-phosphates of the chromosomal DNA which is

then captured and cut by IN as well (Suzuki, Chew et al. 2012). Subsequently each junction with the un-ligated nucleotides is repaired by the cellular DNA repair machinery, which is considered as being a limiting step for integration (Van Cor-Hosmer, Kim et al. 2013) (Brady, Kelly et al. 2013).

Transcription

The proviral DNA can be transcribed into RNA by using the host cellular machineries during cycles of cell division along with the chromosomal DNA (Karn and Stoltzfus 2012). The transcription is initiated by the binding of HIV-1 LTR with the host RNA polymerase II (RNAP II). HIV-1 Tat and the cellular transcriptional elongation factor P-TEFb play a critical role in enhancing the transcription efficiency via a mechanism, in which Tat binds with the transactivation responsive region (TAR) located downstream of LTR and directs P-TEFb to the nascent RNA polymerases for the transcription elongation (Frankel 1992) (Marshall and Price 1995). The transcription efficiency is tightly regulated via the interaction of the various binding sites embedded in LTR with corresponding cellular factors such as NF-ßB (Burnett, Miller-Jensen et al. 2009) (Krishnan and Engelman 2012).

mRNA Processing and Nucleus Export

Along with the generation of nascent HIV-1 pre-mRNA transcripts, they are modified by capping 5'LTR with 7meGpppG and adding poly-adenyl at 3'LTR which are mediated by cellular enzymes and HIV-1 Tat (Chiu, Ho et al. 2002) (Wilusz 2013). Further, the modified pre-mRNA transcripts undergo extensive and complex alternative splicing to convert the premature mRNAs into the full range of mature multiple-spliced RNA species which include three major classes: 1) unspliced RNAs functioning as genomic RNA packaged into virions and the mRNAs for the Gag and Gag/Pol polyprotein precursors; 2) partially spliced mRNAs encoding Env/Vpu, Vif, Vpr and a truncated 72 aa form of Tat; 3) completely spliced mRNAs translating into the HIV-1 regulatory proteins Tat, Rev, and Nef (Purcell and Martin 1993; Stoltzfus and Madsen 2006) (Karn and Stoltzfus 2012). The processing and regulation of HIV-1 mature mRNAs are dependent on a number of *cis*-acting elements within the HIV-1 transcript and complex cellular factors referred to as the spliceosome (Kim and Yin

2005) (Chen and Manley 2009). The splicing patterns are distinct among different HIV-1 strains and clinical isolates, so the mRNAs splicing has a critical role in HIV-1 infectivity (Purcell and Martin 1993; Ocwieja, Sherrill-Mix et al. 2012).

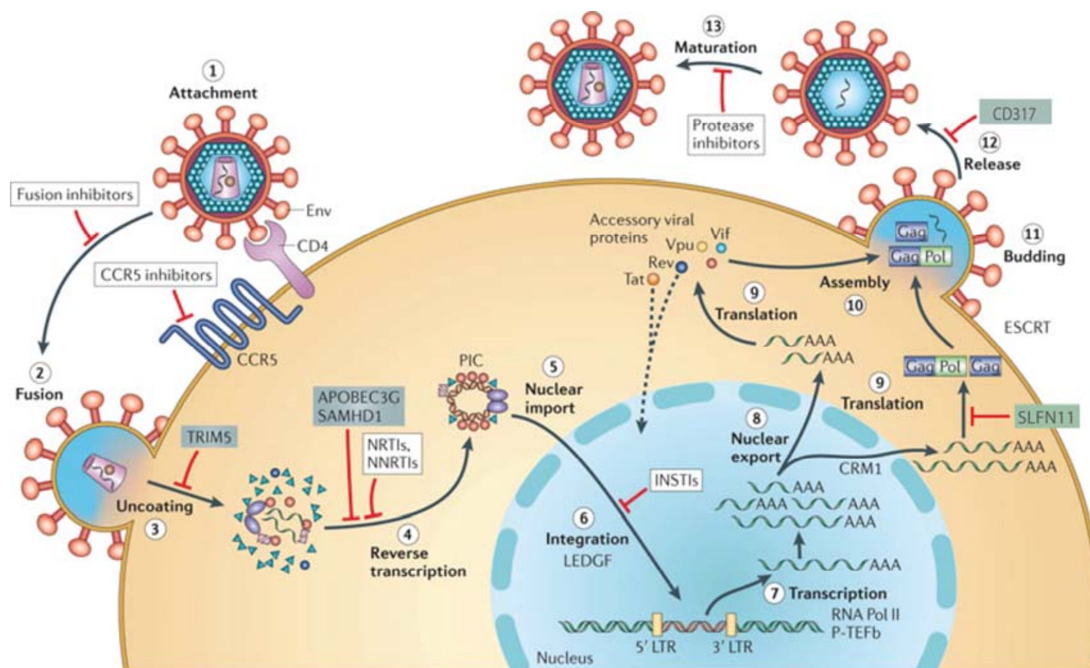

Figure 2 Schematic view of HIV-1 replication cycle, critical accompanying cellular factors and host restriction factor of HIV-1 replication and HIV-1 drugs. This graph contains four sets of information: 1) Each step of the HIV-1 replication cycle is marked as numbers in order; 2) The critical accompanying cellular factors for the HIV-1 replication are indicated: CD4 and CCR5 for viral entry, LEDGF for integration, RNA Pol II for transcription, and ESCRT for viral protein transport; 3) Host restriction factors: TRIM5, APOBEC3G, SAMHD1, CD317 and SLFN11. 4) Drugs against HIV-1: inhibitors for fusion and CCR5, revers transcription inhibitors (NRTIs and NNRTIs), integrase inhibitor (INSTIs) and protease inhibitors.

(Adapted from Alan Engelman & Peter Cherepanov, Nature reviews. Microbiology, 2012 (Engelman and Cherepanov 2012))

To translate the mature mRNA into viral proteins, they need to be transported from the nucleus to cytoplasm. The completely spliced mRNAs are first exported constitutively to the cytoplasm via the endogenous cellular pathway used by host cellular mRNAs as well. However, the transport of HIV-1 unspliced and partially spliced mRNA species relies on the HIV-1 Rev by interacting with the Rev-responsive element (RRE) located in the *env* gene (Sodroski, Goh et al. 1986). This transport pathway is regulated by the level of Rev and the unspliced and incompletely spliced mRNAs in the nucleus (Malim, Hauber et al. 1989). Overall the maturation of HIV-1 mRNA transcripts and the following transport are dynamically and tightly regulated.

Translation of HIV-1 mRNAs

After entering the cytoplasm, HIV-1 exploits the cellular machineries to translate mRNAs to viral proteins (Freed 2001) (Friedrich, Dziuba et al. 2011). This process basically includes three phases: initiation, elongation and termination, which require the concerted interactions of the three classes of RNA (ribosomal RNA, transfer RNA and messenger RNA) with cellular translation factors in a timely manner (Chamond, Locker et al. 2010). The initiation is considered as the rate-limiting step, and there are distinct pathways according to the ribosome scanning model (Bolinger and Boris-Lawrie 2009). The conventional way is dependent on the recognition of the 5'cap structure by the eukaryotic initiation factor 4E (eIF4E) and the recruitment of 43S pre-initation complex which is consisting of the 40S ribosomal subunit, eIF3 and the ternary complex (eIF2, GTP, Met-tRNA)(Kozak 1989; Merrick 2004). The alternative initiation pathways involve the internal ribosome entry segment (IRES) and posttranscriptional elements (PCEs) in the viral gene sequence (Pelletier, Kaplan et al. 1988; Hellen and Sarnow 2001) (Chamond, Locker et al. 2010). The ratio of translated proteins needs to be regulated for the successful HIV-1 virion production.

HIV-1 utilizes the ribosomal frame shifting program to produce the balanced ratio of Gag/ Pol polyproteins, which is generated when translating the ribosome shift into the -1 reading frame at a site near the 3′ end of the *gag* open reading frame, and then go on to translate the *pol* gene (Parkin, Chamorro et al. 1992) (Brakier-Gingras, Charbonneau et al. 2012).

Assembly, Budding, and Maturation

The viral proteins, together with the viral genome RNA, must be directed to the site of virion assembly. HIV-1 virion assembly occurs at the plasma membrane and is fundamentally mediated by the HIV-1Gag/Pol polyprotein precursor (Gheysen, Jacobs et al. 1989) (Adamson and Freed 2007). The main events involved during this process are as follows: Gag N-terminal MA region binds the plasma membrane and recruits the viral Env protein. The central CA domain mediates the protein–protein interactions for the creation of the conical shell of the viral core. NC captures the viral genome via direct interactions with the RNA packaging sequence. The Gag p6 region recruits the late assembly motifs which could recognize the endosomal sorting complexes required for the transport machinery (ESCRT) of the host cell and therefore facilitates the budding of the immature assembled virion from the lipid membrane (Morita, Sandrin et al. 2007) (Bieniasz 2006) (Van Engelenburg, Shtengel et al. 2014). Along with the budding, the viral protease embedded in the Gag/Pol polyproteins is activated and cleaves Gag/pol into their own constituents. The proteolysis of Gag/Pol triggers conformational changes of both the virion structure and the RNA genome, which therefore leads to viral maturation (Adamson and Freed 2007) (Ganser-Pornillos, Yeager et al. 2008) (Ivanchenko, Godinez et al. 2009; Sundquist and Krausslich 2012). To this point, the virus completes its replication cycle and new infectious viruses are generated.

1.1.3 Host restriction factors of HIV-1 replication

In contrast to the accompanying cellular machineries adding HIV-1 replication, five host cellular proteins were identified as the HIV-1 restriction factors which can intrinsically suppress HIV-1production and can be antagonized by HIV-1 proteins, as shown in Figure 2. The first identified one is the tripartite-motif-containing 5α (TRIM5α), a component of cytoplasmic body, has an effect on the uncoating of viral capsid and reverse transcription (Stremlau, Owens et al. 2004). HIV-1 employs its MA to overcome this restriction (Stremlau, Owens et al. 2004) (Huthoff and Towers 2008).

APOBEC3, one of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like family proteins, could be encapsidated into viral particles and restrict reverse transcription by editing sense-stranded DNA in the absence of HIV-1 Vif (Sheehy, Gaddis et al. 2002) (Goila-Gaur and Strebel 2008). The membrane protein CD317 acts on the release of mature virions, which is counteracted by the accessory protein Vpu. Another two host restriction factors are recently identified. SAMHD1 restricts the ability of HIV-1 to infect the dendritic and other myeloid cells and this restriction can be counteracted by SIVsmm/HIV-2 lineage which encodes the accessory protein Vpx (Lahouassa, Daddacha et al. 2012). The target step is again the reverse transcription by lowering the concentrations of intracellular deoxynucleoside triphosphates (dNTPs) to below the threshold needed for the synthesis of the viral DNA by reverse transcriptase (RT) (Lahouassa, Daddacha et al. 2012) (Ayinde, Casartelli et al. 2012). Last but not least, SLFN11, whose viral antagonist is not identified yet, acts at the point of virus protein synthesis by exploiting the unique viral codon bias towards A/T nucleotides (Razzak 2012).

1.1.4 HIV-1 Latency

One striking feature of HIV-1 infection is that it can fall into the latency stage during the life cycle. There are two situations of latency depending on the afferent or the efferent stage of the replication cycle. The afferent one exists as the pre-integration complex which is generated after transcription in the cytoplasm and mainly locates in the resting $CD4^+$ T lymphocytes. Once the $CD4^+$ T lymphocytes become activated, the latent pre-integration complex produces infectious viruses and so they are considered as the major HIV-1 reservoir. The efferent one refers to the integrated HIV-1 provirus of which the transcription is ceased regardless of the activation status of the infected cell (Butera 2000) (Jordan, Bisgrove et al. 2003). HIV-1 latency limits the benefits from the highly active antiretroviral therapy (HAART) and therefore puts a formidable obstacle for eradiation of HIV-1 in resting T cells. However, the

mechanisms behind need to be further elucidated for developing the therapeutic strategies.

1.1.5 HIV-1 Genetic Diversity and Subtypes

Clinical HIV-1 isolates represent great genetic diversity which is up to 30%, and this sets a formidable obstacle to control HIV/AIDS (Spira, Wainberg et al. 2003) (Santoro and Perno 2013). There are four types of HIV-1: main (M), outliner (O), non-M and non-O (N), and proposed (P), which have different geographic distributions but show similar symptoms (Robertson, Anderson et al. 2000) (Vallari, Holzmayer et al. 2011) (Santoro and Perno 2013). The M group which is responsible for the globe HIV/AIDS epidemic can be split further into nine clades (A-J) (Perrin, Kaiser et al. 2003) (Wainberg and Brenner 2012). Besides, over 58 HIV-1 circulation recombinant forms (CRF) have been identified (HIV lanl data base; http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). Several factors contribute to the high variability. First of all, HIV-1 RT is an error-prone enzyme lack of proofreading functions. It generates much mutation in the HIV-1 genome at a rate of 1:2 000-10 000 (Mansky 1998). And further, the recombination of the mutated genome increases the genetic variability (Hu and Temin 1990) (Moutouh, Corbeil et al. 1996). Last but not least, the rate of HIV-1 replication being high, this gives rise to the immune escape mutations due to selection pressure from the host. However, the rate of genetic diversity varies among the different regions of HIV with a relatively low level in *pol* but a much higher level within the region of *vpu/env* (Santoro and Perno 2013). Overall, the genetic diversities between HIV-1 isolates, especially specific regions, lead to changes of antigenicity with impediments for the development of HIV-1 vaccines (Santoro and Perno 2013).

1.2 HIV/AIDS Epidemics

Since the recognition of AIDS in 1981 and the identification of its causative agents HIV-1 and HIV-2 in 1983, HIV/AIDS becomes a worldwide HIV/AIDS pandemic.

According to the latest data the world health organization (WHO) released by 2012, approximately 36 million people live with HIV, including about 2.0 million adolescents. 1.6 million people died of HIV/AIDS related diseases worldwide by 2012. Currently the global prevalence is around 0.8%, with the highest ratio in Africa reaching up to 4.8% (WHO, http://www.who.int/topics/hiv_aids/en/). More than four decades of intensive basic and translational research make the treatments against HIV/AIDS available. However, the development of vaccines remains confined mainly due to the complicated properties of HIV-1. With an increase of preventive interventions and HIV-1 testing and counselling, as well as the global cooperation between nations, the new HIV-1 incidents have declined with the result, that the quality of life for people with contracted HV-1 is seen to improve.

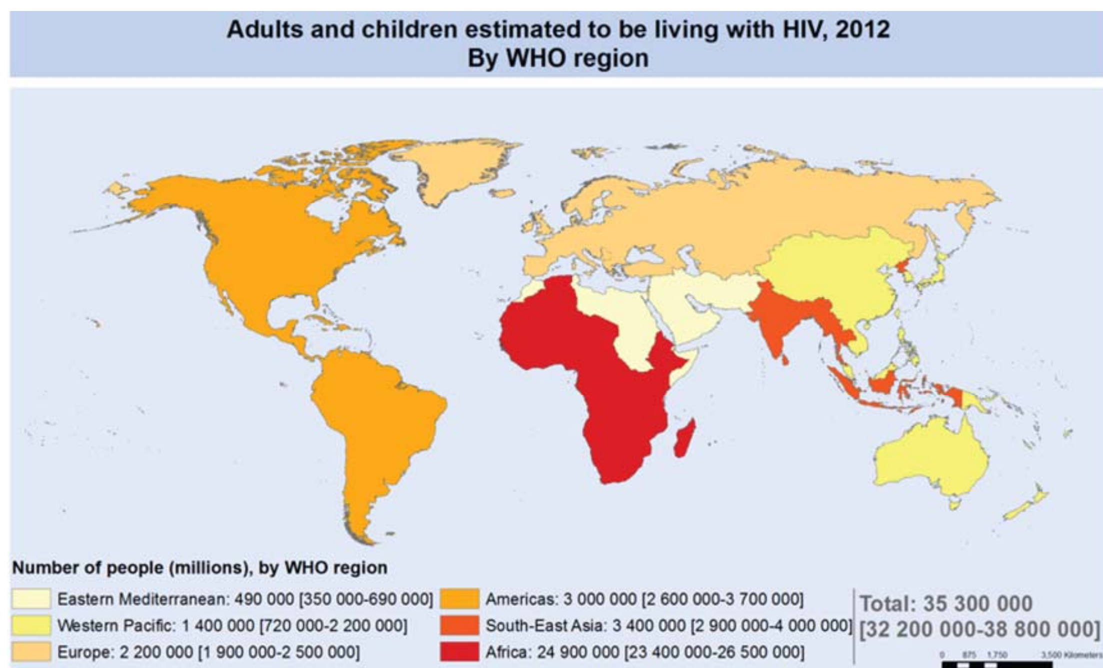

Figure 3: HIV/AIDS distribution in WHO region. The number of people living with HIV is recorded corresponding to different regions marked with different colours in the map (The colours do not apply into any opinion.) (**Adapted from WHO, 2013**)

1.3 HIV-1 Pathogenesis

1.3.1 Acquisition of HIV-1 Infection

HIV-1 can be transmitted from an infected person to another via several channels: 1) mucosal surfaces of the oropharynx, rectum, and genital. They can trap antigens and virus particles for they are rich in langerhans cells; 2) accidental blood transfusion from HIV infected donors; 3) mother-to-infant. A number of factors contribute to the risk of HIV infection. The risk of infection via the blood transfusion approaches 100%. Trans-mucosal infection risks vary according to the site of exposure, with the highest risk through the rectum and the lowest risk across the oral mucosa. Beyond that, the high levels of plasma HIV RNA enhance mother-to-infant transmission of HIV. The acquisition of HIV infection is also influenced by the viral load of infected donor individual. The viral load is defined as the viral titres in the blood and described as the number of copies of HIV genetic RNA or DNA per millilitre (copies/ml) (CDC, HIV/AIDS; http://www.cdc.gov/hiv/default.html). Especially, the viral set point, defined as the stable level of viral loads after acute phage of HIV-1 infection, is good marker to predict the probability of transmission and the disease progression (Fraser, Hollingsworth et al. 2007).

1.3.2 General Clinical Courses of Untreated HIV-1 Infection

HIV infection is typically classified as a three-stage process ranging from an initial infection to the development of AIDS based on the criteria put forward by the Centers for Disease Control and Prevention (CDC).

Acute Stage

The acute stage of HIV-1 infection is defined as the time period from the initial acquisition of HIV-1 to the development of an antibody response detectable by standard tests (McMichael, Borrow et al. 2010). This stage may last from a few days to several weeks, with the mild or severe flu-like symptoms of fever fatigue, lymphadenopathy, headache, and rash. During the early period of the stage, the viruses replicate extensively in activated $CD4^+$ T lymphocytes which are then

destructed, and this leads to high viral load and a widespread dissemination of HIV-1 to the lymphoid organs and other sites including the dramatic fall of $CD4^+$ T lymphocytes counts in the blood. Meanwhile, a reservoir of HIV-1 infected resting $CD4^+$ lymphocytes is established. During the later stage, the virus titer in the blood falls and $CD4^+$ lymphocyte counts begin to increase, with the development of HIV-1-specific $CD8^+$ cytotoxic T cells (CTLs). After this stage, the HIV-1 set point is reached. This value varies widely between patients and is reversely correlated with the breadth and strength of HIV-1-specific CTL response. Therefore, it serves as a significant mark for predicting the progression of the disease and the probability of HIV-1transmission (Mellors, Munoz et al. 1997) (Fraser, Hollingsworth et al. 2007) (Huang, Chen et al. 2012).

Asymptomatic Stage

After the acute stage of HIV-1 infection, there is usually a relatively long period that is characterized by little or no clinical manifestations, and we refer it as the asymptomatic stage or clinical latency (Ford, Puronen et al. 2009). The time duration varies among infected individuals, averaging around about 10 years, even in the absence of treatments. This stage is characterized as the persistent immune activation which is fueled by the translocation of microbial products because of the CD4+ T lymphocytes loss resulted from the acute HIV-1 infection at the gut sites (Ford, Puronen et al. 2009). The immune activation has profound influences on HIV-1 replication and the host immune system because it speeds up HIV-1 production by stimulating the tremendous naïve and central memory CD4+ T cell repertoire (Zhang, Wietgrefe et al. 2004) (Klatt, Villinger et al. 2008). The enormous HIV-1 replication, with its high viral mutation propensity, leads to the accelerated viral evolution, which is mainly responsible for the alterations in the target cellular trophism from CCR5-trophic to dual trophic or dominantly CXCR4 trophic strains with increased virulence and broader target cell trophism (Grossman, Meier-Schellersheim et al. 2006) (Mogensen, Melchjorsen et al. 2010). From another aspect, the immune activation is disadvantageous to the host because of the damage to the adaptive immune system and the dysregulated innate immune defence. HIV-1 infection induces

resting T cells into increased T-cell turnover because of the enhanced immune activation by antigens and inflammation (Ho, Neumann et al. 1995) (Sachsenberg, Perelson et al. 1998) (Grossman, Meier-Schellersheim et al. 2002), as well as abnormalities of B-cell activation and immunoregulation (Lane, Masur et al. 1983) (Moir, Malaspina et al. 2001). HIV-1 infection could also causes the hyperactivation of innate immune system, hence leading to the production of a range of cytokines and chemokines, which in turn contributes to the persistent immune activation (Boasso, Hardy et al. 2008) (Boasso and Shearer 2008). All of these contribute to the progressive loss and ultimate depletion of $CD4^+$ T lymphocytes. As summarized in the review of Ford, "The asymptomatic chronic phase of HIV infection is a dynamic balance between host and virus, the outcome of which determines an individual's course of disease" (Ford, Puronen et al. 2009).

AIDS

AIDS is the final and most deteriorate stage of the HIV-1 infection. The median time from initial infection to the development of AIDS among untreated patients ranges from 8 to 10 years (Vergis and Mellors 2000). This clinical stage is defined primarily on the $CD4^+$ T lymphocytes count of below 200 cells/mm^3 (CDC criteria). In the absence of anti-retroviral treatments (ART) to fight off the opportunistic infections and neoplasms the patients would die because of the HIV-induced immune deficiency. The initiation of ART is highly recommended to start when $CD4^+$ T lymphocyte-counts remain lower than 350 cells/mm^3.

1.3.3 Variable Disease Progression of HIV-1 Infection

In the absence of antiretroviral therapy, various patterns of HIV-1 related disease progression are recognized among HIV-1 infected individuals (Langford, Ananworanich et al. 2007) (Hogg, Yip et al. 2001). A variety of viral and host factors are associated with the rate at which the disease progresses (Shankarappa, Margolick et al. 1999) (Bello, Casado et al. 2005) (Fellay, Shianna et al. 2007) (Pereyra, Addo et al. 2008). Because of the variations of the related factors, the plasma viral load (VL)

CP	• Asymptomatic phase duration up to 10 years • Viral set point above 2000 copies/ml in the absence of ART
RP	• CD4+ T lymphocytes below 350/mm^3 within 3 years • AIDS-related symptoms within 3 years
LTNP-EC	• Asymptomatic phase duration up to 10 years • Plasma VL below 50 copies by ultrasensitive PCR in the absence of ART
LTNP-VC	• Asymptomatic phase duration up to 10 years • Plasma VL below 2000 copies/ml by ultrasensitive PCR in the absence of ART
LTNP-NC	• Asymptomatic phase duration up to 10 years • Plasma VL above 2000 copies/ml by ultrasensitive PCR in the absence of ART

Table 1: Categories of HIV-1 infected clinical groups and the major parameter(s)
CP: chronic progressors; RP: rapid progressors; LTNP-EC: long-term nonprogressors, elite controllers; LTNP-VC: long-term nonprogressors, viremic controllers; LTNP-NC: long-term nonprogressors, viremic non-controllers. (**Adapted from Concepcion Casado et al. PLoS ONE, 2010**)

and CD4+ T lymphocytes count are the most widely used ones for predicting the stage of disease and initiating anti-retroviral treatments, which are well established (Langford, Ananworanich et al. 2007) (Hessol, Lifson et al. 1989) (Goujard, Bonarek et al. 2006). Based on the asymptomatic duration, the measurement of vial load and CD4+ T lymphocyte counts, HIV-1 infection is categorized into five classes as shown in table 1: rapid progressors (RP), chronic progressors (CP) and long-term nonprogressors which can be further divided into elite controllers (LTNP-EC), viremic controllers (LTNP-VC) and viremic non-controllers (LTNP-NC) (Casado, Colombo et al. 2010) (Hunt 2009). CPs progress toward AIDS within 10 years and a maintained viral set point above 2000 copies/ml in the absence of ART, a portion of around 70-80% of patients is affected. RP indicates a dramatic decline of CD4+ T lymphocytes below to 350/mm^3 and the occurrence of AIDS-related events within a few years after infection. The LTNP are rare, especially for the LTNP-EC, which are found around 0.1% of the patients. They could naturally suppress HIV-1 replication to

a level below standard detection (< 50 copies/ml by ultrasensitive PCR), and therefore do not ultimately progress toward AIDS (Deeks and Walker 2007) (Hubert, Burgard et al. 2000). Shown below are the clinical evidences for CP and EC patients (Figure 4). The HIV-1 Vpu alleles are derived from the HIV-1 isolates circulating in these two clinic groups.

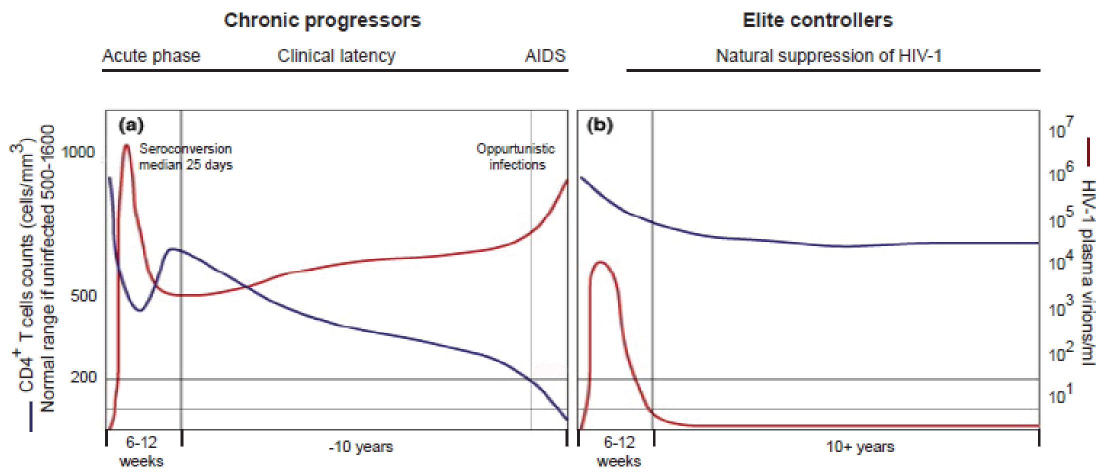

Figure 4: Clinical records of chronic progressors and elite controllers. Viral loads (in red) and $CD4^+$ T cells count (in blue) are shown. (a) Chronic progressors. three typical clinical stages including acute phase, clinical latency and AIDS. (b) Elite controllers. Following the acute phase, the natural suppression of HIV-1 takes place. (**Adapted from Karen A. O'Connell et al. Trends in Pharmacological Sciences, 2009**)

1.3.4 Treatments of HIV/AIDS

No prophylactic and therapeutic vaccines against HIV are on the horizon due to its inherent plasticity and capacity for mutation to escape the host immune clearance and surveillance (Cohen and Dolin 2013). In the course of the discovery of the association between HIV-1 plasma viral RNA level and the progression of the disease together with the unmasking of the molecular mechanisms of HIV-1 replication, the development of drug interventions, targeting different steps of its life cycle as shown

in Figure 2, have been advancing. To date, an arsenal of 24 Food and Drug Administration (FDA)-approved small molecule anti-retroviral drugs are used for HIV-1 treatment regimes, which have evolved into cocktail therapies from the mono-therapy in the early 1900s (Arts and Hazuda 2012). The drugs target different steps of HIV-1 replication cycle (Figure 2), they are categorized into six classes according to the different interactions they target: 1) CCR5 inhibitors; 2) fusion inhibitors; 3) nucleotide reverse transcriptase inhibitors, NRTIs; 4) non-nucleotide inhibitors reverse transcriptase inhibitors, NNRTI; 5) integrase inhibitors; 6) protease inhibitors (PIs) (Arts and Hazuda 2012). The drug-based treatments can, in the best cases, control the HIV viral loads down to undetectable levels, however, not all people can bear the side effects and afford the high cost and, moreover, ART cannot undo the HIV infection and eliminate the risk of transmission. Efforts to decipher and fight HIV are still on the way.

1.4 Natural Control of HIV/AIDS: Elite Controllers

A rare and striking phenotype of HIV-1 infection is the natural control of HIV-1 replication by around 0.1% of infected individuals, referred to as HIV-1 Elite controllers (EC) (Deeks and Walker 2007) (Hubert, Burgard et al. 2000). ECs are different from other patients because of three parameters: 1) Their plasma viral RNA load is lower than 50 copies/mm^3 in the absence of anti-retroviral therapy (ART), a range below the detectable level of traditional PCR; 2) The CD4 counts remain normal and stay at a stable level with respect to these infected individuals; 3) They do not progress toward AIDS for at least 10 years and typically there is no need to apply the antiviral therapies for them (Deeks and Walker 2007) (Hubert, Burgard et al. 2000) (O'Connell, Bailey et al. 2009).

ECs bring the hope that HIV-1 can be controlled without antiviral therapies, which are expensive and require a life-time commitment. Understanding the mechanisms of natural HIV-1 control could contribute to the development of the vaccine design. Based on different cohort studies, no single virological or immunological factor is

defined to explain the virus control status. It's universally admitted that heterogeneous factors are responsible for the natural suppression of HIV-1, including viral elements, host genetic backgrounds, and host immune-system properties.

1.4.1 Viral Genetics in Different EC cohorts

One possibility to cause asymptomatic HIV-1 infection and thus EC status is of infection with less pathogenic and attenuated viruses. Indeed, *nef*-deficient HIV-1 isolates were found and the infected individual do not progress or progress very slowly to AIDS (Brambilla, Turchetto et al. 1999) (Casartelli, Di Matteo et al. 2003) (Geffin, Wolf et al. 2000). While the infection with less fit HIV-1 could not explain all the ECs. HIV-1 isolates from our cohorts and other groups showed there are no common mutations or significant deletions in the plasma RNA sequences compared with the HIV-1 isolates from chronic progressors (CPs) (Miura, Brockman et al. 2008). Very recently, a paper proposed that 33% of the variation in disease progression is attributed from the viral genetic by summarizing the published literatures. They proposed that the evolutionary capacity of HIV-1 had an underscored impact in its virulence and pathogenesis which is critical for the set point in the early stage of infection (Fraser, Lythgoe et al. 2014).

1.4.2 Heterogeneous Host genetic Factors Determine the Spontaneous Control

Host genetics strongly influences the susceptibility and resistance of an individual to HIV-1. The scanning of single-nucleotide polymorphisms (SNPs) on whole genome scale dissected two major genetic determinants for the viral set point level in a consortium of nine cohorts including hundreds of patients with a clear background. The polymorphisms located at the HLA-B and HLA-C loci could explain around 15% of the variation in HIV-1 set point (Fellay, Shianna et al. 2007). The following large-scale epidemiological studies confirmed the enrichment of HLA-B*57, HLA-B*27, HLA-B*13 and HLA-B*58:01 in HIV-1 elite controllers compared with HIV-1 progressors (International, Pereyra et al. 2010) (Limou, Le Clerc et al. 2009).

By contrast, certain HLA alleles, particularly subtypes of HLA-B*35 and HLA-B*07, are highly enriched in HIV-1 rapid progressors (Carrington, Nelson et al. 1999) (Gao, Nelson et al. 2001) (International, Pereyra et al. 2010). Not only the special *HLA* genotype but also its epistatic interactions with killer immunoglobulin-like receptors (KIRs) on natural killer (NK) cells are also correlated to disease progression. The combined pairs including KIR3DS1&HLA-Bw4-80I, KIR3DL1&HLA-Bw4-80I, KIR3DL1&HLA-B*57 were shown to have effects on the HIV-1 plasma RNA level (Flores-Villanueva, Yunis et al. 2001) (Martin, Gao et al. 2002). In addition to the special protective genotypes, the high expression level of HLA-C is independently associated with low viral set points and control of HIV-1 (Fellay, Shianna et al. 2007) (Thomas, Apps et al. 2009). The spontaneous control of HIV-1 can also result from the reduced susceptibility of the infection for new target cells in the population of a mutant chemokine receptor CCR5 with a 32-basepair (bp) deletion (Martinson, Chapman et al. 1997).

1.4.3 Adaptive Immune Responses in ECs

Strong and effective HIV-1-specific $CD4^+$ and $CD8^+$ T cell responses are generally regarded as the best weapons of antiviral immune activity, and they might define the biological characteristics of an effective T cell response against HIV-1 (Ogg, Jin et al. 1998) (Oxenius, Price et al. 2004). Currently available data suggest stronger $CD8^+$ T cell-mediated antiviral response is the backbone for the EC status because $CD8^+$ T cells from ECs have higher capacity to proliferate and synthesize greater amounts of cytotoxic granule components (Migueles, Osborne et al. 2008) (Hersperger, Pereyra et al. 2010) (Chen, Ndhlovu et al. 2012). Moreover, HIV-1-specific $CD4^+$ T cells from ECs seem to have higher functional avidities and they secrete multiple cytokines, such as IL-2 and IL-21 that increase the antiviral activities of HIV-1-specific $CD8^+$ T cells (Chevalier, Julg et al. 2011). It's generally recognized, although controversial with respect to early studies, that neutralizing antibody (NAb) has no obvious role in viral

suppression (Pereyra, Addo et al. 2008) (Doria-Rose, Klein et al. 2010) (Bailey, Lassen et al. 2006) (Lambotte, Ferrari et al. 2009).

1.4.4 Role of Innate Immunity in ECs

Natural Killer Cells

In addition to the effectiveness of CD8+ T cell-mediated responses, the relevance of natural killer (NK) cells was reported to have an impact on the control of HIV-1 disease progression. There are two major phenotypes of NK cells in the peripheral circulation. The majority are CD56^{dim} NK cells which can directly lyseHIV-1 infected cells via cytolytic granules. The less population are poorly cytotoxic CD56^{bright} NK cells which secrete cytokines and shape the adaptive immune responses. The functions of NK cells are delicately regulated by the in interaction of its activating or inhibitory receptors on the surface with ligands on target cells or (Fauci, Mavilio et al. 2005) (Alter and Altfeld 2009). NK cells can also be activated directly through binding of specific antibody to particular Fc-receptors on NK cells, a process known as antibody dependent cellular cytotoxicity (ADCC). The role of NK cells in HIV-1 disease control has been observed. One cohorts study showed that NK cells from ECs displayed higher levels of interferon-gamma, activation markers, and cytolytic activity than NK cells from CPs (Lichtfuss, Cheng et al. 2012). Another group displayed NK cells from ECs had increased level of activating receptor NKG2D which is critical for the lytic function. Very strikingly, these NK cells had higher capacity to maintain the stable level of one receptor NKp44 which is related to CD4 maintenance (Marras, Nicco et al. 2013). Further another group characterized the CD38 expression in the cytolytic CD56^{dim} NK cells, and its association with HIV-1 disease progression in treatment naïve ECs, CPs and AIDS subjects, and they claimed that the activation of NK cells was reversely associated with HIV-1 disease progression (Kuri-Cervantes, de Oca et al. 2014). This is inherent with a former study showing the the strong NK cell-mediated inhibition of viral replication is not necessary for the immunological control of HIV-1 in all ECs (O'Connell, Han et al. 2009). The results about NK cells

role in suppress HIV-1 infection so far are quite conflicting in different cohorts. In some studies, ECs had the trend to maintain higher cytolytic effects via higher activation level, while in some ECs, they maintain the similar activation level as healthy donors. These facts just reflect that the mechanism to suppress the viral replication in ECs can be different among each individual.

Antibody Dependent Cellular Cytotoxicity (ADCC)

ADCC is carried out by the effector cells of innate immune system bearing Fc receptors, such as NK cells and macrophages, through their interaction with antibody coated cells. ADCC is considered as one of the efficient protective mechanisms and regains the attraction in the HIV-1 field mainly because its level inversely correlates with infection risk in the ALVAC-HIV/AIDSVAX-B/E RV144 vaccine trial (Haynes, Gilbert et al. 2012). Higher levels of ADCC antibodies were found in ECs, especially in HLA-B57 positive controllers (Lambotte, Ferrari et al. 2009). Other cohort studies also showed that the broader ADCC response was correlated with disease progression (Baum, Cassutt et al. 1996) (Wren, Chung et al. 2013). Very strikingly, the Vpu-derived epitopes could stimulate ADCC, and moreover, they were only recognized by long term slow progressors but not CP patients (Wren, Chung et al. 2013).

Overall, the spontaneous control of HIV-1 in ECs could be established via virological, genetic and immunologic factors. The mechanisms for the natural control of HIV-1 replication among all patients are heterogeneous. Nevertheless, research of this rare group could provide valuable implications for the control of HIV-1(Fraser, Lythgoe et al. 2014).

2 Review of HIV-1 Vpu

2.1 General Properties of HIV-1 Vpu

2.1.1 *Vpu* Gene and its Role in HIV-1 Pathogenesis

HIV-1 has a set of accessary genes including *vpu, vif, vpr, and nef* that are dispensable for the replication in the cell culture system but are required for optimal infectivity *in vivo* (Malim and Emerman 2008). The gene of *vpu* is only present in HIV-1/SIVcpz lineages but absent in HIV-2 and the most of SIV such as isolated rhesus macaque and sooty mangabeys (Cohen, Terwilliger et al. 1988) (Strebel, Klimkait et al. 1988) (Dazza, Ekwalanga et al. 2005) (Jia, Serra-Moreno et al. 2009). *Vpu* gene is overlapped at its3'end with the *env* gene, and so Vpu is encoded from the bicistronic *env* mRNAs that contains the Rev-responsive element. And the expression level of Vpu is regulated by the abundance of different isoforms of *env* mRNAs (Schwartz, Felber et al. 1990) (Karn and Stoltzfus 2012).

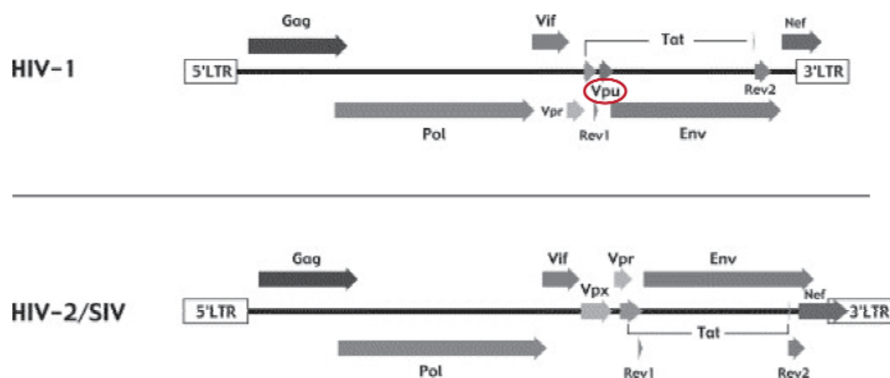

Figure 5: Gene maps of HIV-1 and HIV-2/SIV. The genes to encode prototypical proteins (Gag, Pol Env) for retroviruses are present in HIV-1 and HIV-2/SIV. HIV-1 dese not possess *vpx*. *Vpu* gene is unique to HIV-1, marked in red. (**Adaped from Stephan Bour and Klaus Strebel, Microbes and Infection, 2003**)

Vpu is not incorporated into the mature viral particles, and it is not required for the viral replication in the cell culture system as other accessory proteins. It has two classic two functions: down-regulating the primary HIV-1 receptor CD4 molecules

and enhancing the viral particle release (Willey, Maldarelli et al. 1992) (Bour, Perrin et al. 1999) (Strebel, Klimkait et al. 1989) (Neil, Eastman et al. 2006). The contribution of Vpu to HIV-1 pathogenesis is a central issue which is not elucidated yet. The main obstacle is due to the lack of a suitable animal model because the *vpu* gene is not present in most SIV viruses especially SIVmac which could infect the widely used animal model for HIV-1 study-rhesus macaque. Nevertheless, the animal model researches using the HIV-1/SIV chimeric virus strongly suggest that Vpu plays a role in HIV-1 pathogenesis. It was shown that macaques infected with *vpu*-negative simian-human immunodeficiency virus chimeras (SHIV) had lower virus loads than *vpu*-positive virus (Li, Halloran et al. 1995). Another group demonstrated that deletion of Vpu significantly affects virus infectivity of HIV-1 by using severe combined immunodeficient mouse (SCID) model (Jamieson, Aldrovandi et al. 1996).

2.1.2 Molecular Aspects of HIV-1Vpu

Vpu is composed of around 80 amino acids, with the molecular weight around 16kDa (Cohen, Terwilliger et al. 1988) (Strebel, Klimkait et al. 1988). Is a type I integral trans-membrane protein capable of homooligomerization (Hussain, Das et al. 2007), consisting of a short luminal N-terminal hydrophobic domain (NTD), a trans-membrane domain (TMD) serves as the membrane anchor and a hydrophilic C-terminal domain (CTD) protruding into the cytoplasm. The CTD contains two amphipathic helixes (α-helix) opposite polarity, and they are separated by an unstructured region containing two conserved serine residues at the sites of 52 and 56 amino acids (DSGxxS), which are phosphorylated by protein kinase CK-II and essential for its basic functions which will be introduced in the next sections (Cohen, Terwilliger et al. 1988) (Strebel, Klimkait et al. 1988) (Schubert, Henklein et al. 1994) (Marassi, Ma et al. 1999).

The localization of HIV-1 varies among different subtypes. The subtype B Vpu is accumulated in the and trans-golgi-net (TGN), and this is critical for enhancing the virion release. However, the subtype C Vpu was found to localize at the plasma

membrane and TGN (Dube, Roy et al. 2009) (Pacyniak, Gomez et al. 2005). The determinants for raft association and TGN localization (R30 and K31) are embedded in the region of TMD (Fritz, Tibroni et al. 2012) (Dube, Roy et al. 2009; Bruce, Abbink et al. 2012). The conservation of TMD, especially the alanine residues, together with the subcellular trafficking motif ExxxLV present in the second α-helix and the tryptophan (W) in the end of CTD are critical for the virion release enhancement (Petit, Blondeau et al. 2011). (Vigan and Neil 2010) (Kueck and Neil 2012) (Jafari, Guatelli et al. 2014). The determinants for multifaceted functions of Vpu are spanned from its N- to C-terminus.

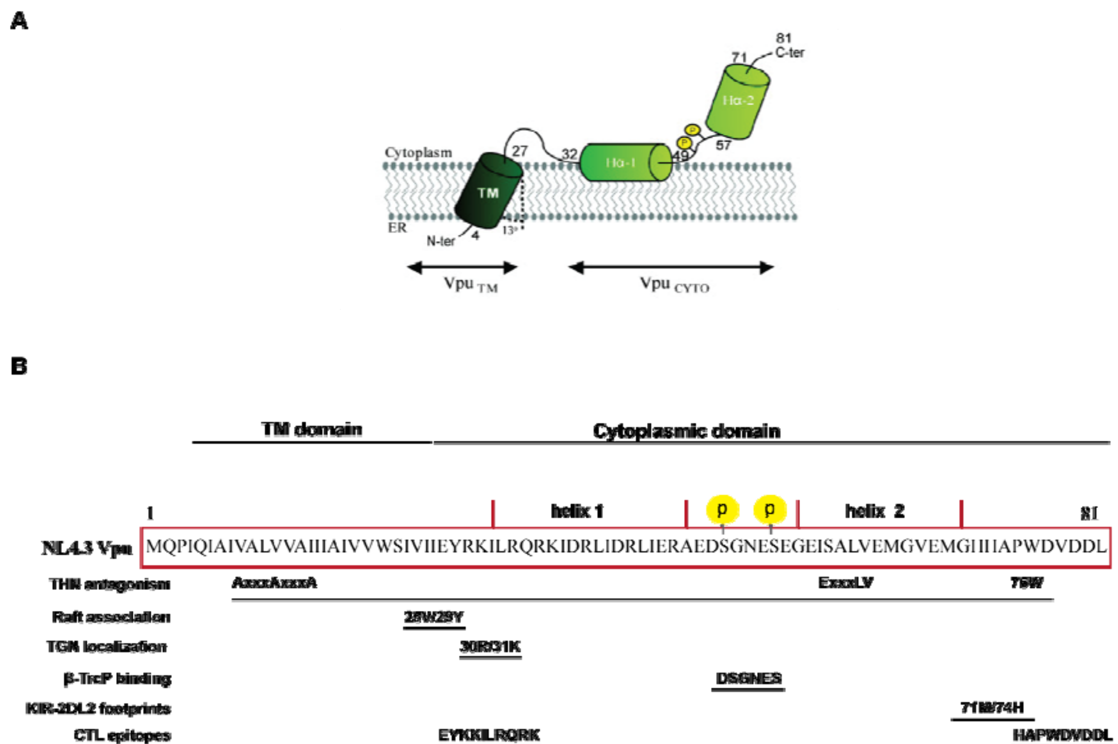

Figure 6: Schematic presentation of HIV-1 NL4.3 Vpu. A: Secondary structure and predicted tertiary fold of Vpu. Vpu is composed of a short N terminal domain (1-4 amino acids), a transmembrane domain spanning from the 4 to 27 amino acid residue, and a relatively long cytoplasmic domain containing two α-helixes. The two phosphorylation sites for recruiting β-TrCP are marked in yellow. **B:** Sequence presentation of Vpu and important motifs. The prototypic NL4.3 Vpu consists of 81 amino acids. The functional motifs for antagonizing CD317 (or tetherin), raft

association, trans-Golgi localization, β-TrCP binding, KIR-2DL2 recognition and two major CTL epitopes are indicted. (A: adapted from Mathieu Dube, et al. Retrovirology, 2010)

2.2 Vpu-Mediated Removal of the HIV-1 Receptor CD4

Vpu is not required for the replication of HIV-1 in the cell culture system, but it modulates a broad array of cellular factors. Strikingly it is one of proteins HIV-1 exploits to reduce the surface level of the primary receptor CD4 molecules in infected cells. The modulation of CD4 seems critical for HIV-1 because it utilizes three of its proteins (Vpu, Nef and Env) to interfere with the surface level of CD4 via distinct mechanisms. Vpu retains newly synthesized CD4 molecules at endoplasmic reticulum (ER) and consequently induces proteasomal degradation (Buonocore and Rose 1990) (Willey, Maldarelli et al. 1992) (Piguet, Chen et al. 1998) (Crise, Buonocore et al. 1990). Vpu-mediated CD4 downregulation starts with the interaction of the cytosolic domains of both proteins and relies on the phosphorylation of Vpu at the two serine of the DSGxxS motif (Bour, Schubert et al. 1995) (Schubert, Henklein et al. 1994). The phosphorylated Vpu binds with β-TrCP, leading to the recruitment of the cytosolic $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex, which then results in the poly-ubiquitination of CD4 at its cytosolic tail (Margottin, Bour et al. 1998). The ubiquitinated CD4 is directed for degradation by cytosolic proteasomes (Schubert, Anton et al. 1998).

The physiological relevance of CD4 down-modulation is partially understood. One benefit for HIV-1 is to prevent super-infection and premature cell death in order to expand the period of effective virus production (Benson, Sanfridson et al. 1993). Another benefit was proposed based on the propensity of Vpu in that it targets newly-made CD4 and therefore results the release of HIV-1 Env protein to enhance viral production (Lama, Mangasarian et al. 1999). More recently study revealed a role of Vpu in the evasion of killing by NK cells via removing CD4 molecules from the HIV-1 infected cells (Pham, Lukhele et al. 2014).

2.3 HIV-1 M Vpu: Competent Antagonist of CD317/tetherin

2.3.1 Dual-faced CD317/tetherin: Viral Restriction Factor and Viral Sensor

The initial biological phenotype of *vpu*-deficient HIV-1 was that the virion particles were retained on the plasma membrane of infected cells (Strebel, Klimkait et al. 1988) (Bour, Perrin et al. 1999). This phenotype was only observed in some cell lines such as human T cell lines but not in African green monkey cell line COS-7 (Geraghty, Talbot et al. 1994), which raised the searching and identification of the specific cellular factor. Finally in 2008 the IFN-stimulated *BST-2* gene product (known as bone marrow antigen 2) was identified as the factor for the cell type dependent phenotype, designated as CD317/tetherin (Neil, Zang et al. 2008). CD317/tetherin is a lipid raft-associated type II integral membrane glycoprotein, consisting of 180 amino acids with the molecular weight of 30-36 kDa (Chiang, Wang et al. 2010) (Kawai, Azuma et al. 2008) (Kupzig, Korolchuk et al. 2003). It is of an unusual topology containing an N-terminal cytoplasmic tail, a conventional trans-membrane helix and a C-terminal glycosylphosphatidylinositol (GPI) membrane anchor connected by a coiled-coil extracellular domain. It further forms stable parallel dimers in the extracellular domain (Kupzig, Korolchuk et al. 2003) (Andrew, Miyagi et al. 2009) (Evans, Serra-Moreno et al. 2010). CD317/tetherin is constitutively expressed on various cells of the immune system including T lymphocytes, macrophages, and plasmacytoid dendritic cells (pDCs) at low level. Its expression can be strongly increased upon the stimulation of type I IFNs (Erikson, Adam et al. 2011). Since its identification as a restriction factor for HIV-1 release, CD317/tetherin was found to block the release of a variety of enveloped viruses (Jouvenet, Neil et al. 2009). And also various viral antagonists to counteract CD317 were identified, such as HIV-1 Vpu, HIV-2 Env and SIV Nef (Neil, Eastman et al. 2006) (Hauser, Lopez et al. 2010) (Gupta, Mlcochova et al. 2009) (Jia, Serra-Moreno et al. 2009) (Sauter, Schindler et al. 2009).

CD317/tetherin functions not only as an intrinsic restriction factor to block the release of viral particles but also as a viral sensor to stimulate innate immune responses. The gene encoding CD317/tetherin was early reported as a potential activator of the

transcription factor NF-κB in a cDNA screening before the identification of its restriction activity (Matsuda, Suzuki et al. 2003). The subsequent researches demonstrated the role of CD317 as a pattern-recognition receptor to stimulate the activation of NF-κB via a mechanism independent of its restriction of virion release (Galao, Le Tortorec et al. 2012) (Tokarev, Suarez et al. 2013). NF-κB is the major transcriptional control factor which plays a pivotal role in immune and inflammatory responses. It is sequestered in cell cytoplasm by its inhibitor IƙB as an inactive form in the cell cytosol. Upon various stimulations such as TNFα, bacterial lipopolysaccharide (LPS) and the growth factors, NF-κB is free of IƙB sequester and then transported to the nucleus where it regulates a list of gene expression including immunoregulatory proteins and cytokines (Gilmore 2006) (Oeckinghaus and Ghosh 2009). The motif studies showed that the restriction of virus release and induction of NF-κB by CD317/tetherin are uncoupled activities. Activation of NF-κB by CD317/tetherin is dependent on the YxY motif in the CTD but not the GPI anchor of CD317/tetherin, while the GPI anchor but not the YxY motif is essential for the restriction of virion release (Galao, Le Tortorec et al. 2012) (Tokarev, Suarez et al. 2013). How HIV-1 Vpu counteracts these two antiviral activities of CD317/tetherin and what HIV-1 could benefit from the antagonism of CD317/tetherin are unsolved issues.

2.3.2 Antagonism of CD317 by HIV-1 Vpu

Vpu-mediated Virion Release Enhancement and CD317 Surface Downregulation

HIV-1 utilizes Vpu to antagonize CD317/tetherin and facilitate virion particle release. The molecular mechanism of how CD317/tetherin tethers mature virions at the plasma membrane of infected cells and how Vpu counteracts the restriction are partially determined. It's clear that the membrane anchor GPI and extracellular coiled-coil domain of CD317/tetherin are involved in crosslinking the virions on the cell membrane, and also Vpu TMD domain is essential for facilitating the virion release. The Paul D. Bieniasz group demonstrated that HIV-1 Vpu directly bind with

CD317/tetherin via TM domains of both proteins and displaced CD317/tetherin from the sites of viral assembly, leading to the failure of CD317/tetherin tethering into the viral envelop and the decreased surface level of CD317/tetherin (Venkatesh and Bieniasz 2013) (McNatt, Zang et al. 2013).The required molecule determinants of Vpu were identified. The highly conserved AxxxAxxxA motif embedded in the TM domain, together with the its β-TrCP binding motif DSGxxS and a trafficking motif ExxxLV located in the CTD domain, as well as a tryptophan (W) residue in the end of Vpu CTD are essential for the optimal virion release enhancement (Vigan and Neil 2010) (Kueck and Neil 2012) (Jafari, Guatelli et al. 2014). Apart from its capacity to antagonize the virion restriction activity of CD317/tetherin, Vpu could reduce the level of CD317/tetherin from the cell surface. The proposed mechanism is that Vpu interacts with CD317/tetherin and targets it to TGN for lysosomal degradation, or traps CD317/tetherin molecules in TGN and blocks the anterograde trafficking (Schmidt, Fritz et al. 2011). Ambiguously, surface down-modulation and total intracellular depletion of CD317/tetherin are not essential for neutralizing CD317/tetherin-restricted release of viral particles, which indicates that the antagonism of CD317/tetherin restriction and downregulation of CD317 are separable activities of Vpu (Goffinet, Homann et al. 2010).

Inhibition of NF-ƙB Signalling in the Presence or Absence of CD317/tetherin by Vpu

In addition to counteract the restriction of virion particle release imposed by CD317/tetherin, Vpu could also inhibit CD317/tetherin-induced NF-ƙB signalling. NF-ƙB can be activated by the presence of CD317, whose expression level is increased due to the enhanced type I interferon upon the infection of HIV-1. Vpu has a negative effect on the stimulation of NF-ƙB in this pathway due to its effect on CD317/tetherin. This inhibitory activity requires the β-TrCP binding motif DSGxxS of Vpu (Tokarev, Suarez et al. 2013). A former study also observed Vpu interfered the NF-ƙB signalling independent of CD317/tetherin. And this capacity of Vpu is attributed to its affinity with β-TrCP which is required for the degradation of NF-ƙB inhibitor-IƙB. In the presence of Vpu, the degradation of IƙB is suppressed, because

the phosphorylated Vpu has high affinity with the adaptor protein β-TrCP of $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex which is needed for marking the phosphorylated IƙB to degradation (Perkins 2007), and thus the activation of NF-ƙB is inhibited (Bour, Perrin et al. 2001; Gilmore 2006).

2.4 Surface Down-modulation of Antigen Presenting Molecule: MHC-I

In addition to the enhancement of viral particle release and the inhibition of NF-ƙB signalling, Vpu may disturb the presentation of HIV-1-derived peptides to $CD8^+$ cytotoxic T lymphocytes (CTL) by down-regulating the surface expression of major histocompatibility complex I (MHC-I) (Kerkau, Bacik et al. 1997). One of the primary antiviral functions of MHC-I is to present pathogen-derived peptides to the specific CTLs (Yewdell and Bennink 1992), which consequently induces the killing of pathogen-invaded cells. MHC-I plays a critical role in eradication of virus infected cells and viruses have evolved strategies to interfere the MHC-I mediated immune responses (Yewdell and Hill 2002). One of the strategies is to decrease the surface expression level of mature MHC-I molecules. HIV-1 utilizes three of its proteins Vpu, Nef and Tat to down-modulate MHC-I (Petersen, Morris et al. 2003). The accessory protein Nef blocks the transport of MHC-I and leads to the surface level reduction and by doing so, Nef enables HIV-1 to evade CTL killing and contributes to the pathogenicity of HIV-1 (Collins, Chen et al. 1998) (Swann, Williams et al. 2001) (DeGottardi, Specht et al. 2008). However, the mechanism and functional relevance of Vpu-mediated downregulation of MHC-I has not been investigated since its effect on the surface expression level of MHC-I was observed (Kerkau, Bacik et al. 1997). One group proposed that Nef and/or Vpu played the major role on the surface reduction of MHC-I during HIV-1 infection to evade the CTLs (Schmokel, Sauter et al. 2011). In this scenario, Vpu may have a positive effect on viral survival during the HIV-1 infection if not directly on the HIV-1 replication, as the pathogenic factor Nef. It is worthwhile to mention that Vpu could target MHC-I, but Vpu-derived peptides are very rarely presented to CTLs (Addo, Altfeld et al. 2002) (Addo, Yu et al. 2002)

(Hasan, Carlson et al. 2012).

2.5 Vpu-mediated Interceptions of NK cells Antiviral Activity

Not only the adaptive immune responses dysfunction during HIV-1 infection, but also the innate immune system is perturbed, as increasing evidences have showed (Alter, Teigen et al. 2005) (Fogli, Costa et al. 2004). As a major component of innate immunity, the phenotypes of NK cells was altered by HIV-1 infection (Iannello, Debbeche et al. 2008). The surface levels of membrane markers such as CD56, as well as members from killer-cell immunoglobulin-like receptors (KIR), present on the surface of NK cells, can be dysregualted during the infection of HIV-1 (Mavilio, Benjamin et al. 2003) (Naranbhai, Altfeld et al. 2013) (Milush, Lopez-Verges et al. 2013). All these evidences attract the researchers to decipher the mechanisms how HIV-1 could escape the first line of host antiviral the surveillance-innate immunity. The Vpu-mediated evasion of NK cells immunity is described as follows.

2.5.1 Vpu Impedes NK cells Killing via NTB-A and PVR Downregulation

NK cells express a repertoire of activating and inhibitory receptors which regulate the functions of NK cells by interacting with their corresponding ligands. In normal conditions, a delicate balance is maintained by the interactions of NK cell receptors and their ligands (Lanier 2008). Upon infection or other stimuli, NK cells could initiate the cytolytic activity by sensing the surface level changes of NK cell ligands on target cells such as tumours and virus-infected cells via the interactions with specific NK cell receptors (Alter and Altfeld 2009). HIV-1 could shape these interactions and thus evade the killing by NK cells. One target is NK-T-B antigen (NTB-A) which is a co-activating receptor required for NK cell degranulation. NTB-A can be downregulated from HIV-1 infected cells specifically by Vpu, which leads to the failure of NK cell degranulation and consequently inability of killing HIV-1 infected cells (Shah, Sowrirajan et al. 2010). Another NK cell ligand PVR (CD155) for the activating receptor DNAM-1 (CD226) expressed by all NK cell, is

also modulated in HIV-1 infection and therefore contributes to the evasion of the cytolytic effect imposed by NK cells. The major viral component responsible for this downregulation activity is HIV-1 Nef, while the optimal downregulation needs the presence of HIV-1 Vpu (Matusali, Potesta et al. 2012). Overall, Vpu is not strictly required for the HIV-1 replication, but it modulates the expression level of the ligands for NK cells functioning and thus leads to the inability of innate immune responses, which adds the strength of HIV-1 to persist in the complicated host cell immune surveillance.

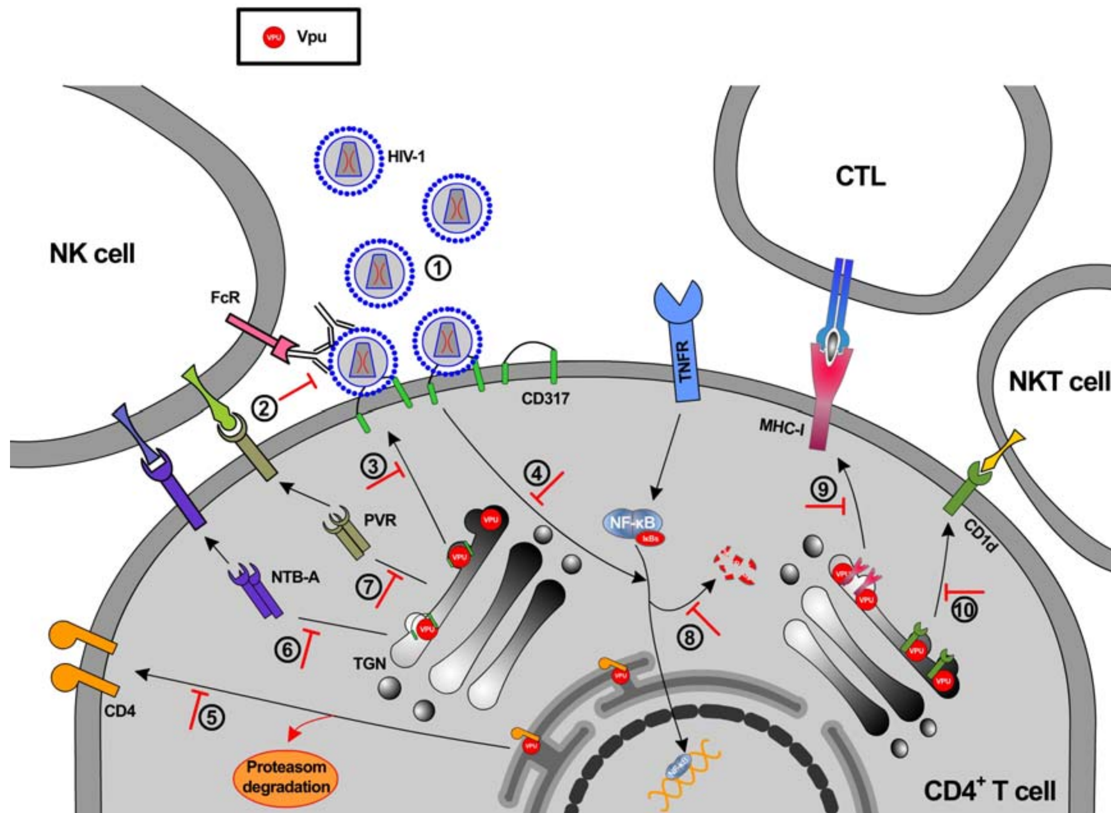

Figure 7: Biological functions of HIV-1 Vpu. ① Vpu enhances HIV-1 particle release HIV-1 particle release by antagonizing CD317. ② Vpu inhibits the NK cell-mediated ADCC response. ③ Vpu traps CD317 at TGN and reduces the surfacel level of CD317. ④ Vpu inhibits CD317-induced NF-ƙB signalling. ⑤ Vpu induces the degradation of newly sythesized CD4. ⑥, ⑦ Vpu disables NK cell killing by

down-regulating NK cell ligands NTB-A and PVR. ⑧ Vpu inhibits NF-ƙB signalling via the inhibition of IƙB. ⑨, ⑩ Vpu decrease the surface expression level of MHC-I or CD1d.

2.5.2 Vpu Modulates NK cell function via its KIR-associated Footprints

In addition to regulate the expression level of NK cells ligands, Vpu was shown to directly adapt to the receptors on NK cells during the co-evolution of HIV-1 and the host. One epidemiology study showed evidences that HIV-1 evolved under the NK cell mediated selection pressure and adapted to KIR receptors present on the surface of NK cells and thus neutralize the lytic effects imposed by NK cells. Two amino acids at the C-terminal of Vpu 71M/74H were found to be associated with one of the inhibitory KIR receptors KIR2DL2. The specific Vpu footprints, together with the corresponding KIR genotype, enable HIV-1 to escape the recognition and killing by NK cells (Alter, Heckerman et al. 2011).

2.5.3 Vpu Protects HIV-1 Infected Cells from NK cell-mediated ADCC

As introduced in **1.4.4**, ADCC is increasingly recognized as a potentially powerful anti-HIV response. Recently, two groups observed that Vpu could circumvent the effects of NK cell-mediated ADCC. The Cohen group showed that Vpu, synergistically with HIV-1 Nef, decreased NK cell-mediated ADCC by antagonizing CD317/ tetherin and preventing CD4 accumulation from the infected cells, which may contribute to the persistence of HIV-1 *in vivo* (Pham, Lukhele et al. 2014). The other study showed that Vpu protected infected cells from ADCC via the antagonism of CD317/tetherin. They proposed that CD317/tetherin retains virions on the infected cells surface and therefore enhances the chances of recognition and binding of HIV-1 particles with HIV-1 specific ADCC antibodies in the absence of Vpu, which makes the infected cells more susceptible to NK cells (Arias, Heyer et al. 2014).

2.6 Functional Discrepancy of Primary Vpu from Different HIV-1 Groups

Only the HIV-1 M group, which is responsible for the global pandemic, has evolved the full functional Vpu to modulate the immune components or pathways described in previous sections. The rare HIV-1 N group Vpu alleles could counteract CD317/tetherin to a very limited extent, but they are inactive to reduce the cell surface level of CD4 due to the lack of determinants in cytoplasmic domain (Sauter, Unterweger et al. 2012). HIV-1 O and P group Vpu alleles are active for CD4 downregulation but deficient to enhance vial particle release (Vigan and Neil 2011). All these evidences suggest that Vpu could be one of the factors to promote the spread in the population level and contribute to the HIV-1 pathogenesis. However, the central issue about the role of Vpu in HIV-1 pathogenicity is not defined yet, which motivates us to give more insights into this issue.

3 Aims of This Study

Vpu is not essential for HIV-1 replication but intensively modulates host immune components including the HIV-1 primary entry receptor CD4, whose cell surface levels are reduced by Vpu. Uniquely, Vpu promotes the release of mature viral particle from infected cells by antagonizing the host restriction factor CD317/tetherin. Moreover, Vpu interferes with NF-ƙB signalling triggered by CD317/tetherin and reduces the cell surface exposure of MHC class I (MHC-I) and natural killer cells ligand NTB-A. While these Vpu activities have been established ex vivo, their relevance for HIV pathogenesis in the infected host remains unclear.

In an attempt to correlate Vpu function with the clinical outcome of HIV-1 infection, we designed to generate the *vpu* alleles derived from two distinct clinical groups of treatment-naïve HIV-1 infected patients. While HIV-1 elite controllers (ECs) naturally control virus replication and keep the viral load below detectable level (<50 copies/ml), chronic progressors (CPs) display viral loads of more than 2 000 copies/ml. Several aspects concerning these two groups of Vpu alleles were to be addressed in the course of this study:

1) Sequence diversity of patient-derived Vpu alleles.
2) If the activities of CD4, CD317, MHC-I, and NTB-A downregulation are conserved among EC and CP Vpus?
3) Whether the Vpu alleles still possess the activities to enhance virion release and inhibit NF-ƙB signalling or not?
4) Is there any systematic difference regarding these functions between EC and CP Vpus? If these functions of Vpu are correlated with each other?
5) Is any activity correlated with disease outcomes?
6) Based on the natural Vpu alleles, define novel functional motifs for the listed activities.

4 Materials and Methods

4.1 Materials

4.1.1 Study Subjects

Fifteen HIV-1 ECs (pVL< 50 RNA copies/ml; median [interquartile range, IQR] CD4 counts 843 [654–955]cells/mm^3) and sixteen CPs (median [IQR] CD4 count 284 [36.75–433] cells/mm^3) were selected from two cohorts described previously (Pereyra, Addo et al. 2008) (Miura, Brockman et al. 2008) (Miura, Brockman et al. 2009) (Brumme, Li et al. 2011) (Mwimanzi, Markle et al. 2013). Briefly, all participants were recruited on the basis of viral load from outpatient clinics at local Boston hospitals and also referred from provides throughout the United States, after institutional review board approval and written informed consent. At the time of plasma sample collection, all patients were treatment naïve. HIV-1 ECs were defined as having plasma HIV RNA levels below the level of detection for the available ultrasensitive assays (< 75 copies/mL by multiple branched DNA (bDNA) or < 50 RNA copies/mL by ultrasensitive PCR) without ART. HIV-1 CPs were defined as having plasma HIV RNA levels above 10,000 copies/mL without ART (Pereyra, Addo et al. 2008).

4.1.2 Viral RNA Isolation and RT-PCR Amplification

Prior to the *vpu* gene amplification, plasma collection, viral RNA isolation and RT-PCR amplification were performed by the researcher from the group of Bruce D. Walker (Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, Boston, MA, USA) (Pereyra, Addo et al. 2008) (Miura, Brockman et al. 2009). The RT-PCR products for our research project were kindly provided by Takamasa Ueno (Center for AIDS Research, Kumamoto University, Japan) who was authorized to have the patients' samples as described below. The plasma collection and viral RNA isolation were described previously (Pereyra, Addo et al. 2008) (Miura, Brockman et al. 2009). Briefly, HIV-1 plasma virus from EC and CP was isolated by ultracentrifugation. Viral RNA was extracted by using the Qiagen viral RNA mini kit with the treatment of RNase-free DNase set

and was eluted in DNase- and RNase-free water and stored at -80°C. HIV-1 gene regions were amplified using nested reverse transcriptase PCR (RT-PCR) followed by the treatment of DNase, as described (Miura, Brockman et al. 2008). Genbank accession numbers for primary *vpu* sequences are EU517721-EU873004 (EC) and FJ469688-FJ469764 (CP).

4.1.3 Oligonucleotide Primers for *Vpu* Sequences Amplification

Based on the primary *vpu* sequences, forward and reverse primers were designed by tracking the nucleotide sequences for editing the first and the last four amino acids respectively. All the primers were listed in Table 2-1 and 2-2.

Table 2-1: List of forward primers for *vpu* gene amplification.

Eco-MQSL	5'-CGCGGAATTCATGCAATCCTTA- 3'
Eco-MQPL	5'-GCCGGAATTCATGCAACCTTTA- 3'
Eco-MQSI	5'-GCCGGAATTCATGCA ATCTATA- 3'
Eco-MQTL	5'-GCCGGAATTCATGCAAACTTTA- 3'
Eco-MQSV	5'-GCCGGAATTCATGCA ATCTGTA- 3'
Eco-MQAL	5'-GCCGGAATTCATGCAAGCTTTA- 3'
Eco-MQTT	5'-GCCGGAATTCATCA AACCA A- 3'
Eco-MIPL	5'-GCCGGAATTCATGATACCTTT A- 3'
Eco-MPSL	5'-GCCGGAATTCATGCCATCTTTA- 3'
Eco-MSPL	5'-GCCGGAATTCATGTCACCTTTA- 3'
Eco-MQLL	5'-GCCGGAATTCATGCAATTGTTA- 3'
Eco-MNSL	5'-GCCGGAATTCATGAACTCTCTA- 3'
Eco-MNAL	5'-GCCGGAATTCATGAATGCCTTA- 3'
Eco-MQYL	5'-GCCGGAATTCATGCAATATTTA- 3'
Eco-MQPI	5'-CGCGGAATTCATGCAACCAATC- 3'
Eco-MQPIQI	5'-CGCGGAATTCATGCAACCTATACAAATA- 3'
Eco-MHVS	5'-GCCGGAATTCATGCATGTATG- 3'
Eco-MYSL	5'-GCCGGAATTCATGTACTCTTTA- 3'
Eco-MLSL	5'-GCCGGAATTCATGCTCTCTTTA- 3'

Table 2-2: List of reverse primers for *vpu* gene amplification.

VDDL BamRev	5'-GCGCGGATCCAGATCATCAAC- 3'
WDVDDL BamRev	5'-GCTCGGATCCAGATCATCAACATCCCA- 3'
WDIDDL BamRev	5'-GCGCGGATCCAGATCATCAATATCCCA- 3'
IDDL BamRev	5'-GCGCGGATCCAGATCATCAAT- 3'
VNDL BamRev	5'-GCGCGGATCCAGAT ATTAAC- 3'
INDL BamRev	5'-GCGCGGAT CAGATCATTAAT- 3'
NDNL BamRev	5'-GCGCGGATC CAGATTATCATT- 3'
INNM BamRev	5'-GCGCGGATCCATATTATTAAT- 3'
GDDIG BamRev	5'-GCGCGGATCCCCTATATCATCACC- 3'
INDM BamRev	5'-GCGCGGATCCATATCATTAAT- 3'
VDNL BamRev	5'-GCGCGGATCCAGATTATCAAC- 3'
TDDL BamRev	5'-GCGCGG ATCCAGATCATCAGT- 3'
IDDQ BamRev	5'-GCGCGGATCCTGATCATCAAT- 3'
DINDM BamRev	5'-GCGCGGATCCATATCATTAATATC- 3'

4.1.4 Specific Primers for Site-directed Mutagenesis

All the primers used for searching novel molecular determinants of Vpu are listed in Table 3. They were designed for site-directed mutagenesis under these conditions: 1) The primer length is in the range of 25-45 bases of nucleotides; 2) The primer contains at least 40% cytosine (C) and guanine (G), starting with a C or G; 3) Melting temperature (Tm) should be above 78°C; 4) The target sequence for mutation should be in the middle of the primer (Laible and Boonrod 2009).

Table 3-1: List of forward primers for mutagenesis from NL4.3 Vpu to MEF

NL4.3Vpu I17V	5'-GCAATAGTAGCATTAGTAGTAGCAATAGTA ATAGCAATAGTTGTGTGGTCCATAG- 3'
NL4.3Vpu V26I	5'-GCAATAATAATAGCAATAGTTGTGTGGTCC ATAATAATCATAGAATATAGGAAAATATTAAG- 3'
NL4.3Vpu 49V	5'-GGTTAATTGATAGACTAATAGAAAGAGTAG AAGACAGTGGCAATGAGAGTGAAGG- 3'
NL4.3Vpu 77H	5'-GGCACCATGCTCCTTGGCATGTTGATGATC TG- 3'

Table 3-2: List of reverse primers for mutagenesis from NL4.3 Vpu to MEF

NL4.3 Vpu I17V	5'-CTATGGACCACACAACTATTGCTATTACTATTGCTAC TACTAATGCTACTATTGC- 3'
NL4.3 Vpu V26I	5'-CTTAATATTTTCCTATATTCTATGATTATTATGGACCA CACAACTATTGCTATTATTATTGC- 3'
NL4.3 Vpu A49V	5'-CCTTCACTCTCATTGCCACTGTCTTCTACTCTTTCTA TTAGTCTATCAATTAACC- 3'
NL4.3 Vpu D77H	5'-CAGATCATCAACATGCCAAGGAGCATGGTGCC- 3'

Table 4-1: List of forward primers for mutagenesis MEF to NL4.3 Vpu

MEF V17I	5'-GCAATAGTAGCATTAGTAGTAGCAGCAATACTAGCA ATAGTTGTGTGGTCCATAATAC- 3'
MEF I26V	5'-GCAGCAGTACTAGCAATAGTTGTGTGGTCCATAGTA CTCATAGAATATAGGAAAATATTAAG- 3'
MEF V49A	5'-GATTGATAGAATAGCAGAAAGAGCAGAAGACAGTG GCAATGAGAG- 3'
MEF D77H	5'-GGCATGATGCTCCTTGGGATGTTAATGATCTGGATC C- 3'

Table 4-2: List of reverse primers for mutagenesis from MEF to NL4.3 Vpu

MEF V17I	5'-GTATTATGGACCACACAACTATTGCTAGTATTGCTGC TACTACTAATGCTACTATTGC- 3'
MEF I26V	5'-CTTAATATTTTCCTATATTCTATGAGTACTATGGACCA CACAACTATTGCTAGTACTGCTGC- 3'
MEF V49A	5'-CTCTCATTGCCACTGTCTTCTGCTCTTTCTGCTATTCT ATCAATC- 3'
MEF D77H	5'-GGATCCAGATCATTAACATCCCAAGGAGCATCATGC C- 3'

4.1.5 Reagents

Enzymes and Buffers for Molecular Cloning

Pfu polymerase/10× Pfu buffer	New England Biolabs
Endonuclease / the digest buffer	New England Biolabs
T4 ligase/ligation buffer	New England Biolabs

Kits for Molecular Cloning

NucleoBond AX 100 Kit	Macherey-Nagel

NucleoSpin Extract II Kit	Macherey-Nagel
PCR Purification Kit	Macherey-Nagel
Plasmid Miniprep/Max-prep	Macherey-Nagel

Markers

Protein Standards, pre-stained	New England Biolabs
DNA molecular marker, 1kb DNA ladder	MBI Fermentas, St. Leon-Roth

Inhibitors and Drugs

MG-132	Sigma
Protease Inhibitor Cocktail	Sigma
Recombinant Human TNF-α	Peprotech

Antibodies for Western Blotting, Fluorescence-activated Cell Sorting (FACS) and Immunology Fluorescence Microscopy

Table 5-1: List of primary antibodies

Antibodies	Usage/Dilution	Source
Rabbit-anti-Vpu	WB 1:1000	Vpu-101AP; FabGennix Inc.
Mouse-anti-GFP	WB 1:1000	G6539; Sigma
Rat-anti-GFP	WB 1:1000	3H9 a-GFP; Chromotek
Sheep-anti-HIV-1capsid	WB 1:5000	Babara Müller, Uni. Heidelberg
Mouse-anti-CD317	FACS 1: 500	Clone 26F8; BD Bioscience
CD4-APC	FACS 1: 20	RPA-T4; BD Bioscience
MHC-I-APC	FACS 1: 20	555555; BD Bioscience
Mouse-anti-NTB-A	FACs 3µg/ml	MAB19081; R&D
594 WGA	IF 10 µg/µl	Invitrogen
Mouse-anti-Tfr	WB 1:1000	Zymed Laboratories

Tfr: Transferrin receptor

Table 5-2 List of secondary antibodies

Antibodies	Usage/Dilution	Source
Mouse-APC	FACS 1: 200	Jackson ImmunoResearch
Goat-anti-mouse peroxidase	WB 1:5 000	Dianova
Goat-anti-rat peroxidase	WB 1:5 000	Dianova
Goat-anti-rabbit peroxidase	WB 1:5 000	Dianova
Duck-anti-sheep peroxidase	WB 1:5 000	Dianova

4.1.6 Plasmids

pEGFP N1

Origin: Clontech

Descritption: It is used for the expression of enhanced green fluorescent protein (eGPF) in mammalian cell lines under the Cytomegalovirus (CMV) promoter.

Usage: Construction of the expression plasmids for non-codon optimized natural vpu alleles and reference NL4.3 vpu. The vpu gene with the restriction cites (EcoRI and BamHI) was inserted into upstream of the eGFP sequence. Vpu was expressed as a fusion to the N-terminus of GFP tag.

pIRESGFP

Origin: Clontech

Descritption: It contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the multiple cloning sites (MCS) and the GFP coding region. This permits both the gene of interest (cloned into the MCS) and the *EGFP* gene to be translated simultaneously from a single bicistronic mRNA.

Usage: Construction of the expression plasmids for non-codon optimized natural *vpu* alleles and reference NL4.3 *vpu*. The *vpu* gene with the restriction cites (EcoRI and BamHI) was inserted into upstream of the *eGFP* sequence. *Vpu* was expressed as a fusion to the N-terminus of GFP tag.

pcDNA-SynVpu

Descritption: It contains a codon-optimized HIV-1 NL4.3 *vpu*. It was from Klaus Strebel (Nguyen, llano et al. 2004).

Usage: Served as a positive control for the infectivity assay in the pre-test.

pNL4.3 VpuEGFP, pNL4.3 VpuIRESGFP and pNL4.3 VpuS/A IRESGFP

They were constructed as the same procedure with natural *vpu* alleles described in **4.2**. pNL4.3VpuS/AIRESGFP was generated by site-directed mutagenesis from pNL4.3VpuIRESGFP. pNL4.3VpuS/AIRESGFP encodes for the Vpu protein containing two serine mutations at the amino acids of 52 and 56. They all served as controls for the functional analysis of natural *vpu* alleles.

pHIV-1NL4.3 wt (BH10 Env)

Origin: Valerie Bosch (DKFZ, Germany)

Descritption: provirus plasmids (Bosch and Pawlita 1990)

Usage: Infectivity assay

pHIV-1 NL4.3ΔVpu (BH10 Env)

Origin: Valerie Bosch (DKFZ, Germany)

Descritption: Based on pHIV-1NL4.3 wt (BH10 Env), Vpu expression was abolished by mutating the *vpu* initiation codon to ATT (Pfeiffer, Pisch et al. 2006).

Usage: Infectivity assay

The following plasmids were from Daniel Sauter (Ulm University, Germany). The related experiments were performed by his group.

pCG-WITO *Vpu*

Description: It is derived from a transmitted/founder subtype B HIV-1 group M strain and it is highly active for CD4 downregulation and CD317/tetherin counteraction(Li, Gao et al. 2005) (Doehle, Chang et al. 2012)

Usage: Positive control for the inhibition of NF-κB activation

pCG-human tetherin IRES DsRed2

Description: Human CD317/Tetherin was cloned into the CMV promoter-based pCG expression vector co-expressing DsRed2 as previously described (Sauter, Schindler et al. 2009).

Usage: activation of NF-ƙB signalling.

pNF-ƙB(3x)-Firefly Luciferase

Description: It contains three NF-κB binding sites kindly provided by Bernd Baumann.

Usage: NF-κB reporter plasmid

pTAL-Gaussia Luciferase

Description: It contains the minimal promoter-TATA-like promoter (pTAL) region from the Herpes simplex virus thymidine kinase (HSV-TK) that is not responsive to NF-κB (Sauter, Hotter et al. 2013).

Usage: The internal control for transfection efficiency, that is, used for the normalization of the NF-ƙB-induced expression of firefly luciferase.

pTAL-Firefly Luciferase

Description: The gaussia luciferase in pTAL-Gaussia Luciferase constructwas replaced by firefly luciferase (Sauter, Hotter et al. 2013)

Usage: The negative control for the NF-κB reporter plasmid

IKKβ

Description: constitutively active mutant

Usage: activation of NF-ƙB signalling.

4.1.7 Eukaryotic Cell Lines

HEK293T

Origin: Human Embryonic Kidney cell line expressing the large T antigen of SV40

Cell culture medium: DMEM (high)

Usage: Check the expression of Vpu alleles

A3.01

Origin: Human T cell line, CD4 and CD8 double positive

Cell culture medium: RPMI1640 complete

Usage: CD4/CD317/MHC-I/NTB-A downregulation

TZM-bl

Origin: Hela cell lines introduced separate integrated copies of the luciferase and ß-galactosidase genes under control of the HIV-1 promoter. The TZM-bl cell line is highly sensitive to infection with diverse isolates of HIV-1.

Cell culture medium: DMEM (high)

Usage: Check the expression of Vpu alleles; downregulation of CD4/CD317; infectivity assay

Freezing medium: 90% (v/v) heat inactivated fetal calve serum supplemented with 10% (v/v) DMSO

Trypsin/EDTA: 10% (v/v) trypsin/EDTA (10×, Biochrom), 90% PBS

4.1.8 Bacterial strains

DH5α

Origin: Invitrogin

Genotype: *F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-*

Usage: Amplification of plasmids DNA

Stab II

Origin: Invitrogin

Genotype: F- *mcr*A Δ(*mcr*BC-hsdRMS-*mrr*) *rec*A1 *end*A1*lon* *gyr*A96 *thi* *sup*E44 *rel*A1 λ- Δ(*lac-pro*AB)

Usage: Amplification of HIV-1 proviral plasmids

4.1.9 Buffers and Solutions

DNA loading Buffer

Tris HCl (pH7.5)	50mM
EDTA	50mM
Glycerol	50% (v/v)
Bromphenol blue	0.25%

Paraformaldehyde (PFA) pH7.3

Paraformaldehyde	3% (v/v) in PBS

PBS pH7.4

Sodium Chloride (NaCl)	96mM
Sodium potassium phosphate (Na_2HPO_4)	10mM
Monosodium phosphate (NaH_2PO_4)	2.3mM

PBS-T

Sodium Chloride (NaCl)	96mM
Sodium potassium phosphate (Na_2HPO_4)	10mM
Monosodium phosphate (NaH_2PO_4)	2.3mM
Tween 20	1mM

Luria Bertani (LB) Medium pH7.5

Tryptone	1% (w/v)
Yeast extract	0.5% (w/v)
Sodium chloride (NaCl)	170mM
NaOH	5mM

Autoclaved at 121°C for 20 min

SDS-PAGE Running gel

30% acrylamide (0.8% bisacrylamide)	2.5 ml
1.88 M Tris/HCL, pH 8.8	1.2 ml
0.5% SDS	1.2 ml
Water	1.1 ml
APS 10%	30 µl
TEMED	5 µl

SDS-PAGE Stacking gel

30% acrylamide (0.8% bisacrylamide)	330 µl
0.625 M Tris/HCL, pH 6.8	400 µl
0.5% SDS	400 µl
Water	870 µl
APS 10%	10 µl
TEMED	2 µl

2× SDS Sample Buffer

Tris-HCl (pH6.8)	130mM
β-Mercaptoethanol	10% (v/v)
Glycerol	10% (v/v)
SDS	6% (w/v)
Bromphenol	blue tip of a spatula

6× SDS Sample Buffer

Tris-HCl (pH6.8)	390mM
β-Mercaptoethanol	30% (v/v)
Glycerol	30% (v/v)
SDS	10% (w/v)
Bromphenol blue	blue tip of a spatula

50× TAE Buffer

Tris-acetic acid, pH7.8	2M
Sodium acetate	250mM
EDTA	500µM

Tris-buffered Saline (TBS)

Tris-HCl (pH7.6)	20mM
NaCl	140mM

TBS-T

Tris-HCl (pH7.6)	20mM
NaCl	140mM
Tween 20	1mM

Transfer Blotting Buffer

Tris.HCl, pH8.8	25mM
Glycine	192mM
Methanol	20% (v/v)
SDS	0.05%(w/v)

4.1.10 Lab Equipments, Chemicals and Consumption Items

Table 6-1: List of Lab Equipment.

Equipment	Source
Balance	Ohaus Explorer, Germany
Centrifuge Megafuge 1.0R	Heraeus
Centrifuge Biofuge fresco	Heraeus
Electroporator GenePulser X Cell ™	BioRad, USA
Electrophoresis Power Supply	Amersham Biosciences
Flow cytometer FACScalibur	BD, Germany
Fluorescence Microscopy IX70	Olympus, Japan
Microscopy Olympus CK2	Olympus, Japan
Freezer -80°C	Thermo Scientific, USA
LIEBHERR refrigerator and Freezer	LIEBHERR, UK
Hera cell 150i CO_2 incubator	Thermo Scientific, Germany
Incubator Infors HT	Infors, Bottmingen, Switzerland
LSM510 confocal Microscopy	Zeiss, Germany
Microplate Luminometer	Thermo Scientific, Germany
Multi Channel Pipettes	Thermo Scientific, Germany
Minigel Twin (SDS PAGE)	Biometra, Germany
Nanophotometer	IMPLEN, Germany
Odyssey Infrared Imaging System	Li-cor Biosciences, USA
pH meter	Knick, Germany
Pipettes Labmate	Abimed, Germany
PCR Thermocycler	Biometra, Germany
Power PAC 200	Bio-Rad, USA
Srerile Hood	The Baker Company, USA
UV Transilluminatoren	INTAS, Germany

Table 6-2: List of Chemicals and Consumption Items.

Acrylamide bis aqueous solution 40 (29:1)	Roth, Karlsruhe
Acetone	Zentralbereich INF, Heidelberg
Ammonium chloride (APS)	Bio-Rad; München
Blotting paper (Whatman)	Schleicher&Schuell; Dassel
Dishes/bottles/tube for cell culture	Neolab; Heidelberg
Agar	BD falcon, Heidelberg
Agarose NEEO Ultra quality	Roth, Karlsruhe
Ampicillin	Roth, Karlsruhe

Coverslips (12mm)	Marienfeld
DMEM/OptiMEM/RPMI1640	Invitrogen
Electroporation cuvettes (4 mm gap)	Invitrogen, Karlsruhe
Ethanol 100% (v/v)	JT Baker, Deventer, Netherlands
Ethidium bromide	Merck, Darmstadt
Fetal calf serum (FCS)	Invitrogen
Glycerol	Zentralbereich INF, Heidelberg
Isopropanol	Zentralbereich INF, Heidelberg
Kanamycin	Roth, Karlsruhe
Lipofectamine 2000	Invitrogen, Karlsruhe
Metafectene	Biontex, Martinsried
Milk powder	Roth, Karlsruhe
Mowoil	Calbiochem
β-Mercaptoethanol	Sigma- Aldrich, München
Nitrocellulose, Protran Transfer Membrane	Schleicher&Schuell, Dassel
Penicillin/Streptomycin	Sigma-Aldrich, München
Protease inhibitor Mix	Sigma-Aldrich, München
Sodium acetate ($NaCH_3CO_2$)	Grüssing, Filsum
Sodium chloride (NaCl)	AppliChem; Darmstadt
Sodium dodecyl sulfate (SDS)	Serva; Heidelberg
Sodium hydroxide(NaOH)	Merck; Darmstadt
Sodium pyruvate	Invitrogen; Karlsruhe
Tris (hydroxymethyl aminomethane)	Roth; Karlsruhe
Triton X-100	Pharmacia Biotech; Erlangen
Trypsin-EDTA	Biochrom; Berlin
TEMED	Roth; Karlsruhe
Trypan Blue	Invitrogen; Karlsruhe
Tween-20	Roth; Karlsruhe
Cell Culture Lysis Reagent (5×)	Promega, Wisconsin, USA
Luciferase assay Substrate	Promega, Wisconsin, USA
White polystyrene (Luc. Assay)	Corning, New York, USA
Plastic materials for cell culture	Costar
Penicillin and Streptomycin (50U/ml)	Invitrogen

4.2 Methods

4.2.1 Cloning and Analysis of *vpu* genes from RT-PCR Products

To amply the *vpu* gene sequences, polymerase chain reaction (PCR) was performed to amply the region of *vpu* gene from the RT-PCR samples with specific primers listed in **Table 2-1 and 2-2**. The amplified PCR fragments were digested with EcoRI and BamHI and then ligated into the vector pEGFP-N1 which was digested with the same enzymes. The PCR reaction system, PCR procedure, enzyme restriction digestion of PCR products/vector plasmids were as follows.

PCR Reaction System

Components	Ultimate concentration	Volume (50.0μl)
Templates (RT-PCR)	0.1μg/μl	1.0 μl
Forward primer	0.5 μM	1.5 μl
Reverse primer	0.5 μM	1.5 μl
dNTPs	10mM	1.0 μl
Pfu	0.2 U/μl	1.0 μl
10×Pfu buffer	1×	5.0 μl
H_2O		39.0 μl

PCR procedure

	Cycles	Temperature	Time
Initial denaturation	1	98 °C	30 seconds
Denaturation	30	98 °C	10 seconds
Annealing	30	58 °C	30 seconds
Elongation	30	72 °C	15 seconds
Final elongation	1	72 °C	10 minutes

Restriction Endonuclease Digest of *vpu* PCR products/vector plasimds

The reaction system was as follows and incubated at 37°C for 60-90 minutes. Then it was stopped by addition of 6× DNA loading buffer for further electrophoresis and purification.

PCR products/Plasmids	10.0 µl/3.0 µl
EcoR I	2.0 µl (2.0 units)
BamH I	2.0 µl (2.0 units)
NEB Buffer 4	5.0 µl 10.0×
Add H_2O to total volume	50.0 µl

Gel Electrophoresis and Purification of PCR Products

To purify the restriction-endonucleases-digested PCR fragments and vectors, they were separated by the agarose gels consisting of 1.5% (w/v) agarose and 0.7 µg/ml ethidium bromide in TAE buffer for 20-30 minutes, with the voltage of 85V electricity. The gels were then visualized under UV light. The bands at the indicated size were sliced and purified by NucleoSpin Extract II kit according to the manufacturer's protocol. In the end, the purified products were dissolved in H_2O.

Ligation of pEGFP and *Vpu* Fragments

Vpu gene were introduced into pEGFP N1 vector by the ligation of *vpu* PCR products and pEGFP N1 plasmid via their complementary base pairs in the ends which were generated from the endonuclease (EcoR I/BamH I) restriction digest as described above. The ligation mixture contained a molar ratio of 1:3 pEGFP N1 to *vpu* and catalysed by 2 units of T4 ligase (1.0 unit/µl). As a control, the vector fragments were incubated with no *vpu* insertion to estimate the percentage of ligated vector fragments. The ligation mixture was incubated at room temperature for 2 hours or 4°C for overnight. Then 10.0 µl of the ligation mixture was further performed for transformation described as below.

Heat Shock Transformation

To amplify the ligated pVpuEGFP products or other plasmids, they were transformed into bacterial competent cells DH5α or StabII. The experimental procedure was as follows:

① Took competent cells DH5α 50.0 μl or StabII 50.0 μl (for provirus plasmids production) from -80°C freezer, incubated on ice for thaw the competent cells

② Added ligation reaction mixture 10.0 μl or plasmids 1.0 μl (1.0 μl/μg)

③ Incubated the mixture of competent cells and DNA for 20-30 minutes on ice

④ Heat shock the reaction in water bath at 42°C for 90 seconds to

⑤ Incubate the reaction on ice for 2 minutes to reduce damage to cells

⑥ Added 1.0 ml of LB (with no antibiotic) to the mixture and then incubated the for 1 hour at 37°C

⑦ Evenly plated 100 μl of the resulting culture on LB plates (with appropriate antibiotic added – usually ampicillin or kanamycin). Incubated at 37°C overnight.

⑧ Picked colonies about 12-16 hours post transformation

Plasmid DNA Isolation (Mini-prep)

To identify the resulted natural *vpu* clones from the former steps or other DNA plasmids from the single clone, DNA plasmids need to be isolated. The DNA plasmids preparation was based on the manufactory's protocols.

① Grew bacterial (DH5α or StabII) culture in LB medium with appropriate antibiotics at 37 °C overnight with shaking in 4.0 ml medium.

② Harvest the bacterial culture into 1.5ml tube, spinning at maximum speed for 20 seconds, then discarded the supernatant.

③ Suspended the bacterial cells pellets with 300 μl of ice cold solution 1 (50 mM Tris-HCl, 10 mM EDTA, 100 μg/mL RNase A, pH 8.0).

④ Lysed the bacterial cells with 300 μl of solution 2 (200 mM NaOH, 1%SDS) at room temperature (RT). Mixed the solutions by inverting them a few times gently, leaving the mixture at RT for less than 5 minutes.

⑤ For neutralization, 300 μl of ice-cold solution 3 (2.8 M potassium acetate, pH 5.1), was added, leaving the mixture on ice for 5 minutes.

⑥ After spinning the mixture at maximum speed for 10 minutes, the supernatant was transferred to a new 1.5 ml tube, around 900 µl.

⑦ After another spinning, 600µl isopropanol was added to the 900 µl supernatant to precipitate the DNA nucleic acids.

⑧ Spin at maximum speed for 10 minutes in cold centrifuge, harvest the plasmids DNA pellets and discarded the supernatant.

⑨ Plasmids DNA pellet was washed with 75% ethanol. Dry the plasmid DNA pellet at room temperature.

⑩ Plasmid DNA was dissolved with 50 µl brown water.

Quality Control of Plasmid DNA

Before sending the isolated plasmids to sequence or performing further experiments, the plasmid DNA was first evaluated by the endonuclease restriction digest agarose gel (1.5%) electrophoresis (as described above) to see whether the plasmid showed the right bands and whether the target gene was in the right size.

Sequencing

Five *vpu* single clones derived from one patient were picked up for mini-prep. The isolated DNA plasmids were fist restricted by endonuclease digest (EcoR I/BamH I) as described above. The clones showing the right insertion were sent to GATC biotech for sequencing via the primer for pEGFP N1 promoter CMV provided by the company. The homepage is referred to *http://www.gatc-biotech.com/en/home.html*.

Plasmid DNA Isolation (Max-prep)

To extract DNA plasmids in a large scale, the NucleoBond A× 500 kit, which empolys a modified alkaline /SDS lysis procedure to prepare the bacterial cell pellet for plasmid purification, was used according to the manufacture's protocol. The NucleoBond PC 500 column was used for the purification of plasmid DNA, the basic principle is The experimental procedure was as follows:

① Grew bacterial (DH5α or StabII) culture in LB medium with appropriate antibiotics at 37 °C overnight with shaking in 400 ml medium.

② Harvest of the bacterial cells and successional treatments with solution 1/2/3 (12 ml of each solution per one extraction) were identical with the mini-prep.

③ Resulted bacterial lysate was centrifuged 4000 rpm for 10 minutes for clearing the lysate. At this period, equilibrated DNA binding column (NucleoBond PC 500) with the equilibration buffer N2 (100 mM Tris, 15% ethanol, 900 mM KCl, 0.15% Triton X-100, adjusted to pH6.3 with H_3PO_4) 6 ml.

④ The cleared lysate was applied to the equilibrated NucleoBond Column PC500 through a wet folded filter in order to remove remaining particles before loading the column. The plasmid DNA is bound to the anion-exchange resin.

⑤ The column was washed with buffer N3 (100 mM Tris, 15 % ethanol, 1.15 M KCl, adjusted to pH 6.3 with H_3PO_4)

⑥ Plasmid DNA was eluted from the column into a new 50 ml tube with 15ml elution buffer (100 mM Tris, 15 % ethanol, 1 M KCl, adjusted to pH 8.5 with H_3PO_4).

⑦ Plasmid DNA was precipitated by the adding 15 ml of room temperature isopropanol to to avoid spontaneous co-precipitation of salt.

⑧ After centrifugation at 4000rpm for 45 minutes at 4°C, the plasmid DNA was pelleted. It was washed with 75% ethanol and dried at RT.

⑨ Plasmid DNA was dissolved within 300 µl Braun water.

Measurement of DNA Concentration and Purity

The concentration and purity of plasmid DNA solution were determined by measuring absorbance (optical density (OD)) at the absorbance at 260 nm and 280 nm respectively using NanoDrop™ spectrophotometer. The ration of OD_{260}/OD_{280} within the range of 1.8-2.0 was considered as quality plasmid for further application.

4.2.2 Home-made Site-directed Mutagenesis

Primer Phosphorylation

5' end of the primers need to be phosphorylated. The reaction system is as follows:

Primer	5.0 µl
MgSO4 100mM	0.5 µl
ATP 10mM	10.0 µl
T4-PNK (5U)	0.5 µl
10× Kinase Buffer A	5.0 µl
Add water to	50.0 µl

The mixture was incubated at 37°C for 60 min, then heat inactivated and stored in -20°C. The PCR reaction system was the same principle described in 4.2.1. The thermo cycling procedure is different with normal PCR amplification. The annealing temperature was 55°C, and elongation time at 72°C was 10 min. The cycling was shortened to 18 (Laible and Boonrod 2009).

DpnI digestion

To get rid of the background, DpnI.restriction endonuclease was used to digest the methylated template plasmid before tansformation. For each reaction, 2U of DpnI was incubated with the PCR products at 37°C for 2 hours. Transformation was described in **4.2.1**.

4.2.3 Analysis of Vpu sequences

Validation of *Vpu* Sequences

More than one *vpu* allele is circulating in HIV-1 infected patient, and the contamination was a major concern for the cloning procedure. To exclude the contamination and pick up the predominant *vpu* allele circulation in each patient, the phylogenetic tree was generated based on the primary sequences in the databse and clonal *vpu* sequences (GATC sequencing results of pVpuEGFP plasmids) by using of the maximum-likelihood method (DNAml, PHYLIP), which was performed by our colleagues Takamasa Ueno group (Center for AIDS Research, Kumamoto University, Japan). The clonal *vpu* alleles, which assembled the primary sequences, were selected for functional analysis. And also, the intact enzyme restriction sites, open reading frame and the GFP tag for each construct were checked by using the software Clone Manager.

Multiple Sequence Alignment

All the validated *vpu* alleles, the sequences of their encoding amino acid were aligned against NL4.3 Vpu by using ClustalW2 from EMBL-EBI. The home page is referred to *http://www.ebi.ac.uk/Tools/msa/clustalw2/.*

Mutation Frequency of Candidate Amino Acids

The candidate amino acids, which were suspected to be critical for Vpu functions, were determined by calculating the mutation frequencies out of all HIV-1 clade B subtype Vpu proteins extracted from Los Alamos National Laboratories (http://www.hiv.lanl.gov). The amino acids with mutation frequency lower than 0.1% were arbitrarily selected for further site-mutagenesis and functional analysis.

4.2.4 Delivery of Plasmid DNA to Mammalian Cells

Distinct methods were applied to deliver plasmid DNA into mammalian cells depending on cell lines and size of plasmid DNA. A3.01 cells, derived from human T lymphoblast, were transfected with *vpu* allele plasmids by electroporation. The delivery of provirus plasmids were mediated by lipofectamine 2000. Polyethylenimine (PEI) and metafectene were also used for the transfection of TZM-bl and 293T cells respectively. The methods and protocols used in this study were described as follows.

Transfection of HEK 293T Cells

The expression of natural *vpu* alleles was first evaluated in HEK 293T cells. Metafectene was used to transfect HEK 293T cells with pVpuEGFP plasmids.

① One day prior to transfection, cells were seeded in 6 well-plate, 5×10^5 cells/well.

② To remove the cell debris in old culture medium, fresh DMEM complete medium were added to the cells.

③ The following solutions were prepared in EP tubes: Solution A: 3 µg of expression plasmid in 100 µl Opti-MEM medium. Solution B: 6 µl metafectene100 µl Opti-MEM medium. The solutions were gently mixed.

④ The solutions were then combined with no mixture procedure, and then incubated the complex at room temperature for 25 minutes.

⑤ The complex was then drop gently to the cells with gently swirling afterwards. Incubate at 37°C in a CO_2 incubator.

⑥ 24 hours post transfection, the cells were harvested for evaluating the expression of Vpu alleles.

Electroporation of A3.01 Cells

① One day prior to electroporation, A3.01 cells were cultured in fresh medium.

② 8×10^6 cells per one electroporation, the cells were harvested by centrifugation and dissolved in 500 µl pre-warmed RPMI1640 medium with no supplements of FCS and antibiotics.

③ The suspended cells were transferred to the 4.0 mm cuvelte and added 30µg pVpuEGFP or pVpuIRESGFP plasmid.

④ Electroporation was performed by using the Bio-Rad Genepulser under the procedure: voltage: 250 volts, capacitance: 950 microfarads (µF).

⑤ Electroporated A3.01 cells were cultured in 4.0 ml RPMI1640 complete medium. Incubate at 37°C in a CO_2 incubator.

⑥ 24 or 48 hours post electroporation, cells were harvested for further analysis.

Transfection of TZM-bl Cells by PEI

① One day prior to transfection, TZM-bl cells were seeded in 12 well-plate, 7×10^4 cells/well.

② To remove the cell debris in old culture medium, fresh DMEM complete medium were added to the cells.

③ 3 µg of expression plasmids were first added to a 1.5 ml EP tube with 100 µl Opti-MEM medium, briefly vortex the mixture, then 9 µl PEI was added, gently mixing and leaving the mixture at room temperature for 35-50 minutes

④ DNA/PEI mixture was then transferred to cells, with gently swirl afterwards. Incubate at 37°C in a CO_2 incubator.

⑤ 24 hours post transfection, harvest the cells were harvested for further analysis.

Transfection of TZM-bl Cells with Provirus Plasmids by Lipofectamine 2000

PEI is widely used for the transfection of mammalian cells. However, it did not mediate the delivery of provirus plasmids in my hands (data not show). Then lipofectamine 2000 was used to co-transfect the TZM-bl cells with provirus and vpu expression plasmids for the infectivity assay, which was described in **4.2.7**.

4.2.5 Confocal Microscopy

A3.01 T cells adhered to poly-lysine coated coverslips were fixed with 3% PFA. Coverslips were mounted with mowiol medium and analysed with a Zeiss LSM510 confocal microscope with a 100x PLAN-APO objective lens. Images were recorded with the Zeiss proprietary software LSM5 and processed with Adobe Photoshop 4.0.

4.2.6 Biochemical Assays

Preparation of Cell lysates

For the separation and detection of target proteins, the transfected cells were lysed using SDS sample buffer or KEB lysis buffer in this study. Briefly, cells were washed and subjected to ice-cold KEB buffer (100 µl for 8×10^6 cells) followed by swirling at 4°C. After centrifugation (10 min at 12,000 rpm at 4°C), supernatants were transferred into a new tube, stored at -20°C or immediately subjected to the applications such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Discontinuous SDS-PAGE

Cell lysates were denatured by treating with 2× SDS sample buffer and heating (95°C for 5 min). The proteins were separated by discontinuous SDS-PAGE. Equal amounts of cell lysates were loaded into the polyacrylamide gel and first run in the stacking gel (4.0% polyacrylamide), and then the stacked proteins were separated in the resolving gel (12.5% polyacrylamide) according to their relative molecular mass. The electrophoresis ran at 25 milliamps (mA), with the indication of a pre-stained protein marker ranging from 6 to175 kDa. Gels were subsequently applied to western blotting.

Western Blotting

After electrophoretic separation of proteins, gels were performed semi-dry proteins blotting to transfer the proteins to a nitrocellulose membrane according to the standard blotting flow. The nitrocellulose membrane, filter paper (Whatman blotting paper) and gel were soaked in the transfer blotting buffer and then assembled in a strict order to set the gel was close to cathode (-) and the membrane was close to anode (+). The gel

and membrane were sandwiched by the soaked blotting paper, then the whole component ran for electronic transfer under the current of 100 mA per Gel for 45-60 minutes.

The nitrocellulose membrane, with blotted proteins, was then detected for the expression of proteins by using antibodies and the reporter enzyme, horseradish peroxidase (HRP) in this study. The experimental procedure was described below.

① Membranes were incubated with 5% milk to block unspecific binding sites.

② Membrane were incubated with primary antibody overnight at 4°C after washing with PBST.

③ To remove the unbinding primary antibody, membranes were washed for three times wash with PBST (15 min per time).

④ The respective secondary antibody conjugated with HRP (diluted in 5% milk) was then added to membranes and incubated for 1 hour at RT. For the detection of proteins by LiCor Odyssey, the secondary antibody coupled with Alexa 700 (dilution of 1:10,000 in 2% BSA) was added to the membrane and incubated in dark for 1 hour. Afterwards, the membrane was washed to remove the remaining secondary antibody and milk.

⑤ The expression of target proteins was evaluated by enhanced chemiluminescent substrate (ECL) (Pierce), which was reflected on a photosensitive film. Membranes incubated with Alexa 700 conjugated antibody were scanned on Licor Odyssey with a wavelength of 700 nm. The quantification was assessed using Licor Odyssey software.

4.2.7 Fluorescence-Activated Cell Sorting (FACS)

To evaluate the surface level of CD4, CD317, MHC-1, NTB-A molecules in cells expressing Vpu alleles, FACS analysis was performed as the following steps:

① A3.01 or TZM-bl cells were transfected with plasmid DNA in a 6-well plate or 12-well plate scale as described in **4.2.4**. Untransfected cells from the same batch

were served as control for FACS settings. They were harvest and suspended at 48 hours or 24 hours post transfection.

② All samples were divided to 4 aliquots, each aliquot of cells were stained with 100 µl of diluted CD4-APC, CD317-APC, MHC-I-APC or NTB-A –APC respectively listed in Table 6, then were incubated in dark and on ice for 45 min.

③ After staining, the cells were washed with PBS (3000rpm, 5 minutes, 4°C), and the pellets were suspended with 150 µl-300 µl PBS in each tube for performing FACS analysis.

④The FACS analysis procedure was: firstly, untransfected and unstained sample was used to define the population of living cells; then, transfected but unstained control sample, together with transfected and stained sample, was used to adjust the detectors; finally, run samples after setting gates for GFP positive and negative cells to determine the CD4, CD317, MHC-I or NTB-A surface level in those two population of cells.

4.2.8 Infectivity Assay

Vpu has no effect on HIV-1 infectivity in cell culture system. Vpu-mediated virus release enhancement was evaluated by infectivity assay in TZM-bl cells.

Transfection of TZM-bl Cells by Lipofectamine 2000

① One day prior to transfection, TZM-bl cells were seeded in 12 well-plate, 7×10^4 cells/well.

② To remove the cell debris in old culture medium, fresh DMEM medium with no serum and antibiotics were added to the cells.

③ The following solutions were prepared in EP tubes: Solution A: 2.7 µg of provirus and pVpuIRESGFP or pIRESGFP plasmids in 50 µl Opti-MEM medium. The amount of plasmids was shown below. Solution B: 2.7 µl lipofectamine 2000 in 50 µl Opti-MEM medium. The solutions were gently mixed. Triplicates were performed for each transfection.

Table 7: Co-transfection of TZM-bl cells with proviral plasmid and Vpu construct or empty vector.

HIV-NL4.3 wt 1.2 μg	pIRESGFP 1.5 μg
HIV-1 NL4.3 ΔVpu 1.2 μg	pIRESGFP 1.5 μg
HIV-1 NL4.3 ΔVpu 1.2 μg	pVpuS/AIRESGFP 1.5 μg
HIV-1 NL4.3 ΔVpu 1.2 μg	pVpuIRESGFP 1.5 μg
HIV-1 NL4.3 ΔVpu 1.2 μg	EC and CP pVpuIRESGFP alleles 1.5 μg

④ The solutions were then combined with no mixture procedure, and then incubated the complex at room temperature for 25 minutes.

⑤ The complex was then drop gently to the cells with gently swirling afterwards. Incubate at 37°C in a CO_2 incubator.

⑥ The plates were then transferred to P3 lab and was changed to the fresh DMEM complete medium.

⑦ 48 hours post transfection, the cell culture supernatant was harvested for the infection of newly-seeded TZM-bl cells which were then used for infectivity assay. The transfected cells were lysed and subjected to Western blotting for the evaluating the expression of Vpu or GFP, viral capsid p24 and p55. To detect the real expression level of those proteins in one single transfection, we avoided to detach the TZM-bl cells by trypsin/EDTA, which could break the syncytia cells or fragile cells then consequently loss proteins. The 2× SDS sample buffer was directly added into two of the triplicates for each sample and the cells were harvested by scrapping. TZM-bl cells in one of the triplicates were detached by by trypsin/EDTA, washed with PBS and then fixed with 3% PFA for 1 hour at room temperature, and the fixed cells were performed FACS analysis for determining the surface level of CD317. Infection of new TZM-bl cells, harvest and fixation of transfected cells were done in P3 lab. Western blotting and FACS analysis were done in P2 lab, and the procedures were the same as described in **4.2.7** and **4.2.8** respectively.

Infectivity Assay

① Prior one day of infection, TZM-bl cells were seeded in 96-well plates, 5×10^3 cells/well.

② 50 µl the cell culture supernatant was added to the fresh TZM-bl cells, and incubated the infected cells for 72 hours.

③ The infected cells were harvested with 40 µl cell lysis buffer (1:5 diluted in H_2O) per well.

④ The cell lysate was transferred to a new 96-well plates and brought to P2 lab.

⑤ Transfer 10µl cell lysis to white plate, add 50 µl luciferin substrate per 10 µl cell lysis sample

⑥ Measure the luciferase units in luminoskan ascent.

4.2.9 Software

Table 8: List of software used in this study.

Software	Version	Source
Adobe Illustrator	CS4	Adobe System
Clone Manager	Professional suite 8	Sci-Ed Software
CellQuestPro	4.0.2	BD Biosciences
Cyflogic	1.2.1	http://www.cyflogic.com/
Endnote	X5.0	Thomson Reuters
GraphPad Prism	5.0	GraphPad Software
Image J	–	http://imagej.nih.gov/ij/
LSM Image Browser	LSM 5	http://www.zeiss.com/

4.2.10 Statistical Evaluation

All statistical analysis were evaluated using GraphPad Prism 5 and statistical significance was determined using the Mann-Whitney U test (* $p \leq 0.05$, ** $p \leq 0.01$). Correlations between data sets were evaluated by applying Spearman's or Pearson coefficient analysis (* $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$).

5 Results

5.1 Validation of Patient-derived *Vpu* Genes

All *vpu* alleles were amplified from the RT-PCR products provided by Takamasa Ueno (Center for AIDS Research, Kumamoto University, Japan). The acquisition of the RT-PCR products from the plasma HIV-1 isolates were previously described (Fellay, Shianna et al. 2007) (Miura, Brockman et al. 2008. Patient-derived Gag, Env, and Nef from the same EC and CP cohorts were characterized {Miura, 2009 #25) (Brumme, Li et al. 2011) (Mwimanzi, Markle et al. 2013). To evaluate the properties of the *vpu* alleles, the amplicons were cloned into pEGFP N1 expression vectors respectively. Five Vpu clones per patient were sequenced and then validated by maximum-likelihood phylogenetic tree to exclude the contamination. A single colone that closely resembled the original primary sequence in the database was picked up for further functional analysis. Genbank accession numbers for primary Vpu sequences are EU517721- EU873004 (EC) and FJ469688-FJ469764 (CP).

Altogether 15 EC and 16 CP Vpu alleles were selected based on the validated sequences and comparable expression levels. All the sequences were assembled into a maximum likelihood phylogenetic tree (Figure 8). As shown, each representative patient Vpu allele has unique sequence, and all EC and CP Vpu sequences distributed equally to the reference NL4.3 Vpu, which indicated that there is no correlation between Vpu sequence diversity and disease progression. Clonal Vpu sequences from EC showed no evidence of gross defects or recent shared ancestry, which is consistent with previous analyses of bulk plasma HIV RNA sequences from our EC cohort (Miura, Brockman et al. 2008).

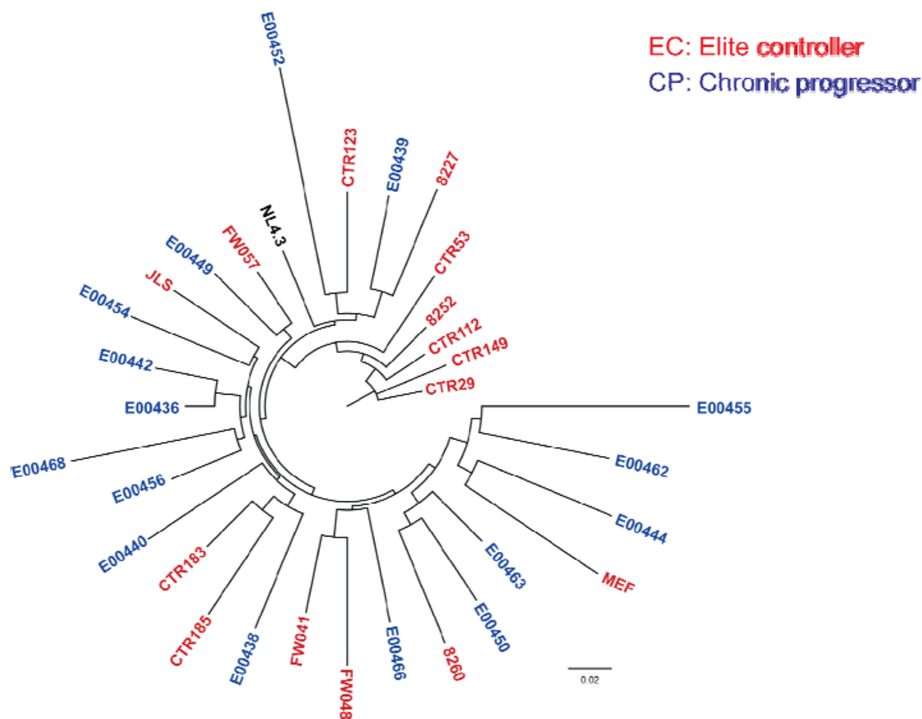

Figure 8: Maximum-likelihood phylogenetic tree of plasma HIV RNA-derived *vpu* clonal sequences. EC-derived Vpus are red, CP-derived Vpus are blue, and control strain NL4.3 Vpu is black. (This figure was performed by Takamasa Ueno, Center for AIDS Research, Kumamoto University, Japan)

5.2 Alignment and Sequence analysis of Vpu Alleles

With the validated Vpu alleles, we aligned and analyzed their amino acid sequences. Sequence variations for both groups were in the range of 16% to 24% (calculated by the software Clone Manager), which is similar to another study showing that the diversity of intra-patient Vpu alleles was around 20% (Pickering, Hue et al. 2014). Even though the representative Vpu sequences were highly variable, the documented critical motifs for CD4 downregulation and CD317 antagonism were principally conserved (Figure 9). Strikingly, no natural mutation occurred in the DSxxES motif, which is the target for CK-II phosphorylation and essential for CD4 degradation and CD317/tetherin antagonism (Margottin, Bour et al. 1998) (Goffinet, Allespach et al. 2009) (Mangeat, Gers-Huber et al. 2009). In the transmembrane domain, the indicated determinants for CD317/tetherin counteraction were well conserved, especially the

central ones A10/A14/A18/W22 for CD317/tetherin contact (Vigan and Neil 2010), in addition, I4/A7 showing conservation to a slightly less extent. Two positively charged residues 30R and 31K critical for trans-Golgi network localization, located in the hinge region between the TM domain and the cytoplasmic domain, together with the residues V25/Y29 crucial for lipid raft association (Fritz, Tibroni et al. 2012) (Dube, Roy et al. 2009), were highly conserved. In the C-terminal domain, the trafficking motif ExxxLV displayed mild diversity. For example, MEF and E00452 showed insertion or deletion at the last amino acid, and the mutations at the variable xxx sites also gave rise to relatively higher diversity in the C-terminal domain. The recently identified 76W residue, which was shown unique importance for virion release enhancement (Jafari, Guatelli et al. 2014), was conserved among both group Vpus, except for E00452 with 76R. Enrichment of KIR2DL2-adapted polymorphism at 71M/74H in EC Vpus was a striking property. Only 3 of 15 EC Vpus, including CTR183, MEF and FW048, displayed mutation of 74R/D/L, whereas, 11 of 16 CP Vpus showed 74R/D/L. The CTL targeting epitope EYRKILRQR was highly conserved. However, the other one HAPWDVNDL showed high variations in the first amino acid and flanking amino acids, such as E00449 and E00452. Last but not least, 61S for Vpu stability (Estrabaud, Le Rouzic et al. 2007), was principally conserved, except for FW048 and E00468 with the mutation of K and A respectively. Altogether, the key functional motifs were generally preserved, the sequence variations mainly come from the N-terminal and C-terminal (Figure 9).

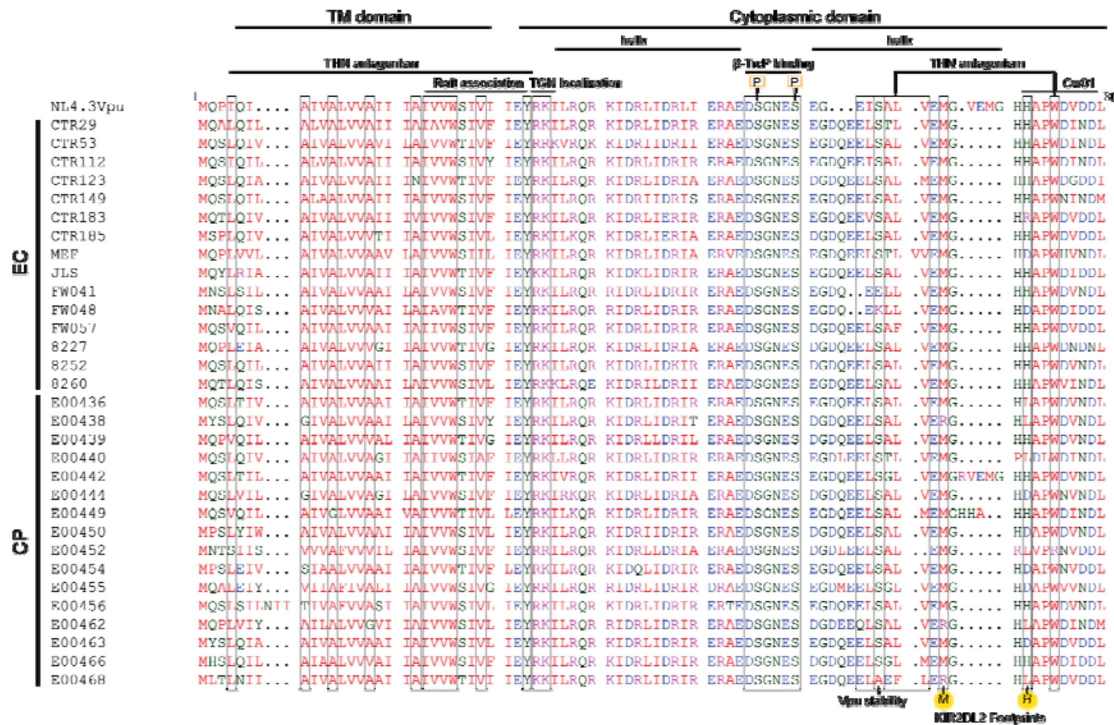

Figure 9: Sequence alignment and analysis of patient-derived Vpu alleles. Amino acid sequence alignment of the Vpu proteins analyzed generated using Clustal Omega (EMBL-EBI). HIV-1 NL4.3 Vpu on the top serves as reference sequence. Boxes indicate the position of functionally relevant residues and motifs. Different colours present the properties of the amino acid: red colours for small side chains and aromatic Y, blue for acidic residues, green for hydroxyl, sulfhydryl, amine groups and G, magenta for basic-H bonding or positive charged groups.

5.3 Expression and Detection of Natural Vpu Alleles

Besides the representative Vpu sequence for each patient, the following limited steps for further functional analysis are whether the expression of the non-codon-optimized Vpu clonal constructs can be detected and whether they express on the comparable level. We first checked the expression and detection of all generated pVpu.GFP constructs in 293T cells (data not shown), together with the sequence information, a set of 31 constructs is confirmed for further analysis. Then we evaluated the expression of the 15 EC and 16 CP *vpu* alleles in more relevant cell lines. Briefly, A301 cells transfected with pVpu.GFP or TZM-bl cells transfected with pVpuIRESGFP were collected and western blot analysis was performed. The

transferin receptor showed equal loading (Figure 10A, B; lower panel). As non-fusion proteins, the similar intensity of GFP bands reflected comparable transfection efficiency and comparable Vpu expression level for each allele (Figure 10B; middle panel). Whereas, Vpu probe showed more variant bands in fusion and non-fusion version of Vpu alleles by using the rabbit polyclonal Vpu antibody (Figure 10A, B; upper panel). This antibody was generated against a peptide encompassing amino acids 58-80 (EGD QEE LSA LME MGH HAP WNV ND). The failure of detection by Vpu antibody mainly resulted from the mutation from H to R/D/L at the position 74 which is one of KIR2DL2 footprints. CP Vpus showed more sequence diversity at this position compared with EC Vpus, which was reflected by the more frequent detection

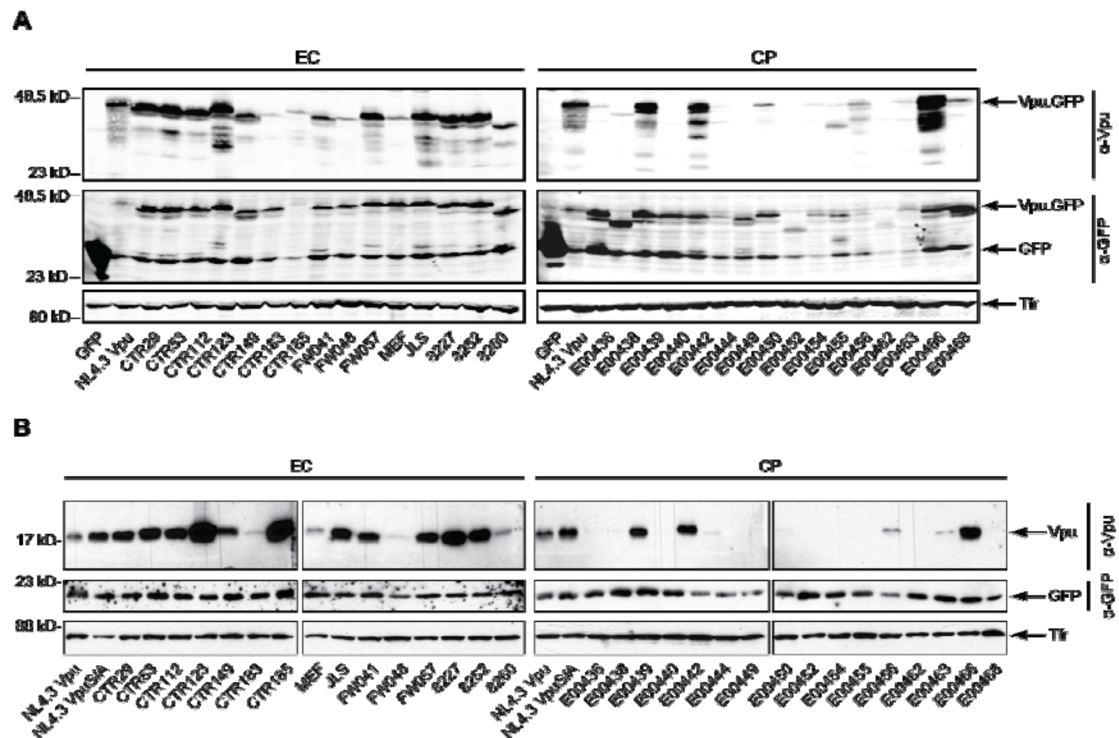

Figure 10: Expression and detection of non-codon-optimized *vpu* alleles. A: A301 cells were electroporated with Vpu.GFP plasmids (30 μg). **B**: TZM-bl cells were transfected with VpuIRESGFP plasmids (3 μg). 24 hours post-transfection cells were harvested and the cell lysates were analyzed by immunoblotting for Vpu, GFP, and transferin receptor.

of EC Vpus (Figure 10A, B; Upper panel). In addition, the insertion of HHA at C-terminal for E00449 and insertion of NII at N-terminal for E00456 could be the reasons for the poor detection or expression (Figure 2, 3). CTR183, E00452, E00462 and E00466 showed less pronounced bands as fusion proteins. Nevertheless, they displayed similar bands as non-fusion proteins (Figure 2A). Overall, the expression levels of all alleles as fusion protein were comparable, which made us move on the further analysis.

5.4 Subcellular Localization Patterns of Natural Vpu Alleles

The subcellular distribution of Vpu in ER and TGN are essential for Vpu-induced CD4 degradation and virion release enhancement (Dube, Roy et al. 2009) (Vigan and Neil 2011). We analyzed the subcellular localization in A301 cells expressed with Vpu.GFPs. The plasma membrane was defined by WGA594, localization of Vpu alleles was indicated by GFP. The control NL4.3 Vpu.GFP was accumulated in the cellular compartment(s) (Figure 11). The majority of the EC and CP Vpus shown the similar cellular accumulation pattern as the reference NL4.3 Vpu.GFP, but CTR112 from EC group displayed plasma membrane patches (Figure 11). Based on the comparable expression levels and localization in relevant cell lines, we investigated the functional analysis of the patient-derived Vpu alleles.

5.5 Conservation of CD4 and CD317 Modulation Activities

5.5.1 Surface Modulation of CD4 and CD317 by Vpu.GFPs

We first analyzed the downregulation of CD4 and CD317 molecules from the surface of A301 cells. Vpu.GFPs were transiently expressed in A301 cells, and cell surface levels of CD4 and CD317 were examined by FACS 48 hours post transfection. eGFP and NL4.3 Vpu.GFP were set as negative and positive controls respectively. The y axis showed the mean fluorescence intensity (MFI) of CD4-APC or CD317-APC, and x axis represented GFP expression level (Figure 12A, 13A). MFI ratio of GFP positive

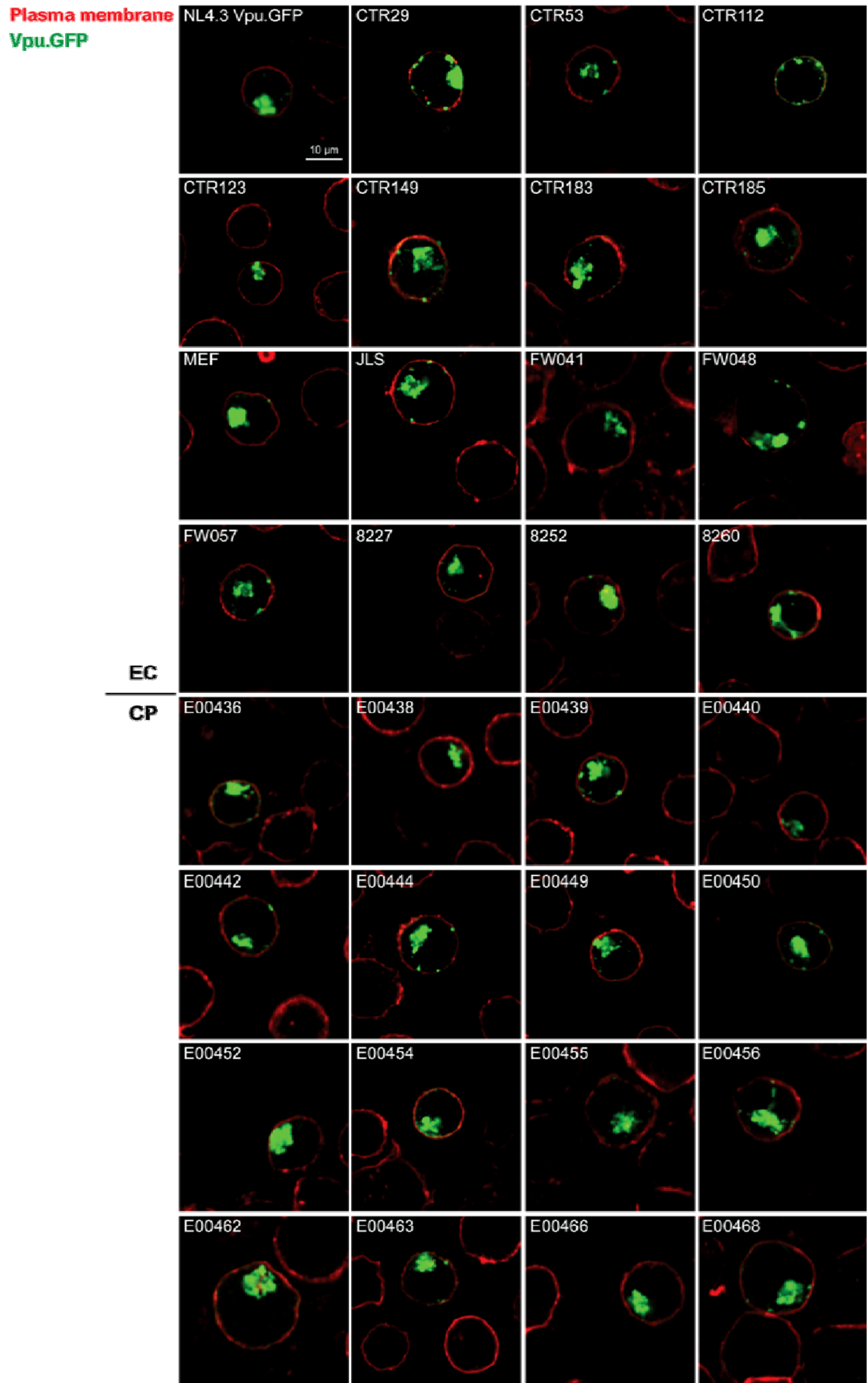

Figure 11: Subcellular localization of Vpu alleles. A3.01 cells were electroporated with Vpu.GFP expression constructs, harvested and fixed on coverslips after 24 hours. The plasma membrane was defined by WGA-594. Coverslips were analyzed with a Zeiss LSM510 confocal microscope with a 100x PLAN-APO objective lens. Images

were recorded with the Zeiss proprietary software LSM5 and processed with Adobe Photoshop 4.0. Scale bar: 10 μm.

cells/MFI of GFP negative cells was first subtracted from the background of eGFP and the resulted value for each Vpu was further normalized to NL4.3 Vpu that was then arbitrarily set as 100% (Figure 12B, 13B). The ability to reduce surface levels of CD4 was conserved among the majority of analysed Vpus, except CTR183 and MEF from EC group, which showed obvious impairment, less than 50% of the NL4.3 Vpu (Figure 12B). Median with interquartile range (IQR) was evaluated to compare the downregulation potency of both groups. EC Vpus displayed lower capacity (median 116.0 [IQR 77.0–129.0]%) to decrease the surface expression level of HIV-1 receptor CD4 molecules than CP Vpus (median 135.0 [IQR 119.0–149.8]%) ($p< 0.01$; Figure 12 C).

Downregulation of CD317 was analysed as the same procedure described before with the same batch of transfected cells. The positive control NL4.3 Vpu removed around 80% of CD317 from the cell surface (Figure 13A). CP Vpus showed comparable activity with NL4.3 Vpu. However, EC Vpus had more impaired alleles, such as CTR183 and 8227 with lower than 50% of NL4.3 Vpu ability (Figure 13 B). Statistically, EC Vpus displayed lower capacity (median 76.7 [IQR 65.2–82.4]%) to decrease the surface expression level of HIV-1 receptor CD317 molecules than CP Vpus (median 97.4 [IQR 84.8–97.1]%) ($p< 0.01$; Figure 13C). It indicated that CD4 and CD317 downregulation activities of patient-derived Vpu alleles are principally conserved with few impaired ones in EC group. And the statistical differences resulted from the higher frequencies of alleles with reduced ability in EC Vpus.

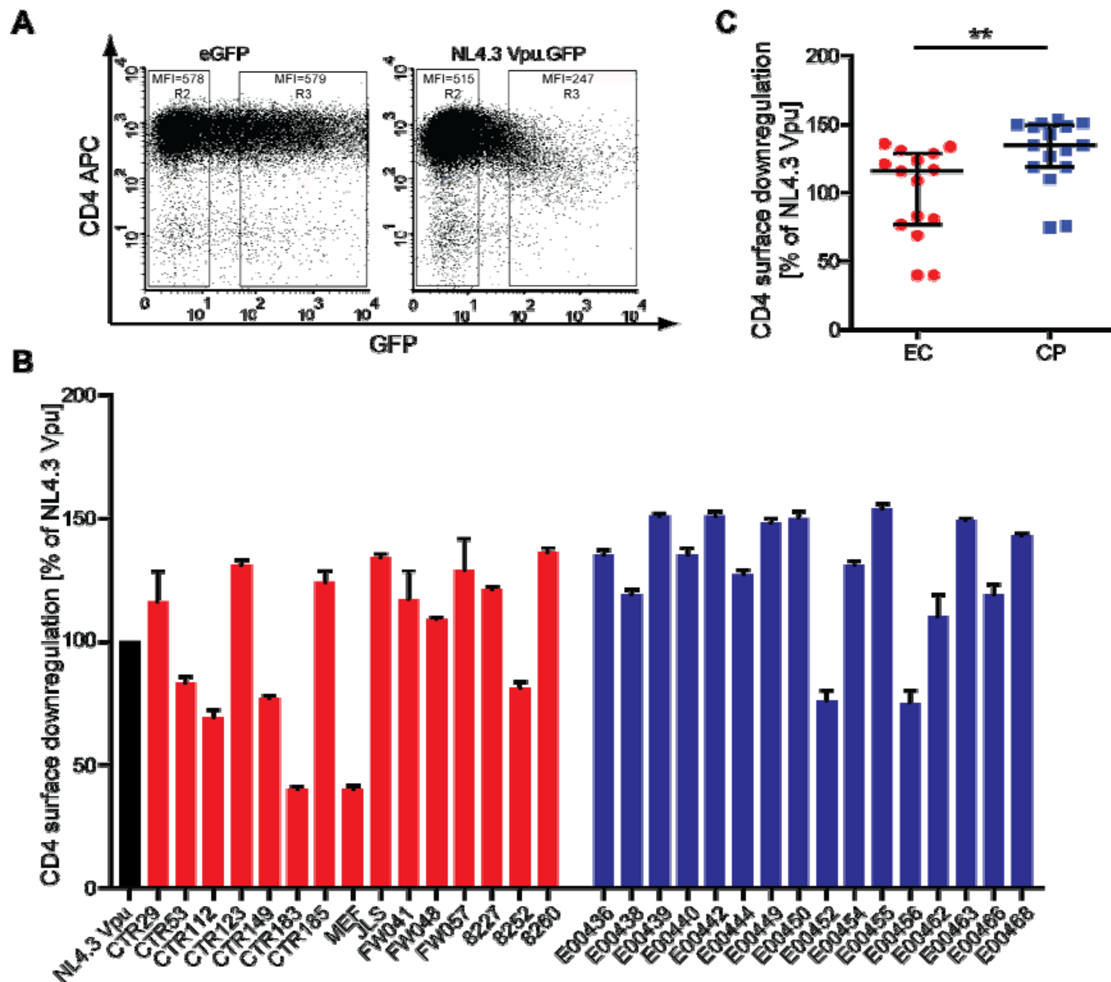

Figure 12: CD4 downregulation activity of Vpu.GFP alleles. A3.01 cells were electroporated with Vpu.GFP expression constructs, harvested after 48 hrs, and stained for cell surface CD4. **A**: Flow cytometry plots of eGFP and NL4.3 Vpu.GFP: CD4-APC (y-axis) vs. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to NL4.3 Vpu that was arbitrarily set to 100%. **B**: Graph showing the CD4 downregulation activity of patient derived Vpu alleles relative to NL4.3 Vpu. Shown are mean values of triplicate transfections with the indicated standard deviation. The result is a representative of three independent experiments. **C**: Comparison of CD4 downregulation activity of EC Vpus and CP Vpus.

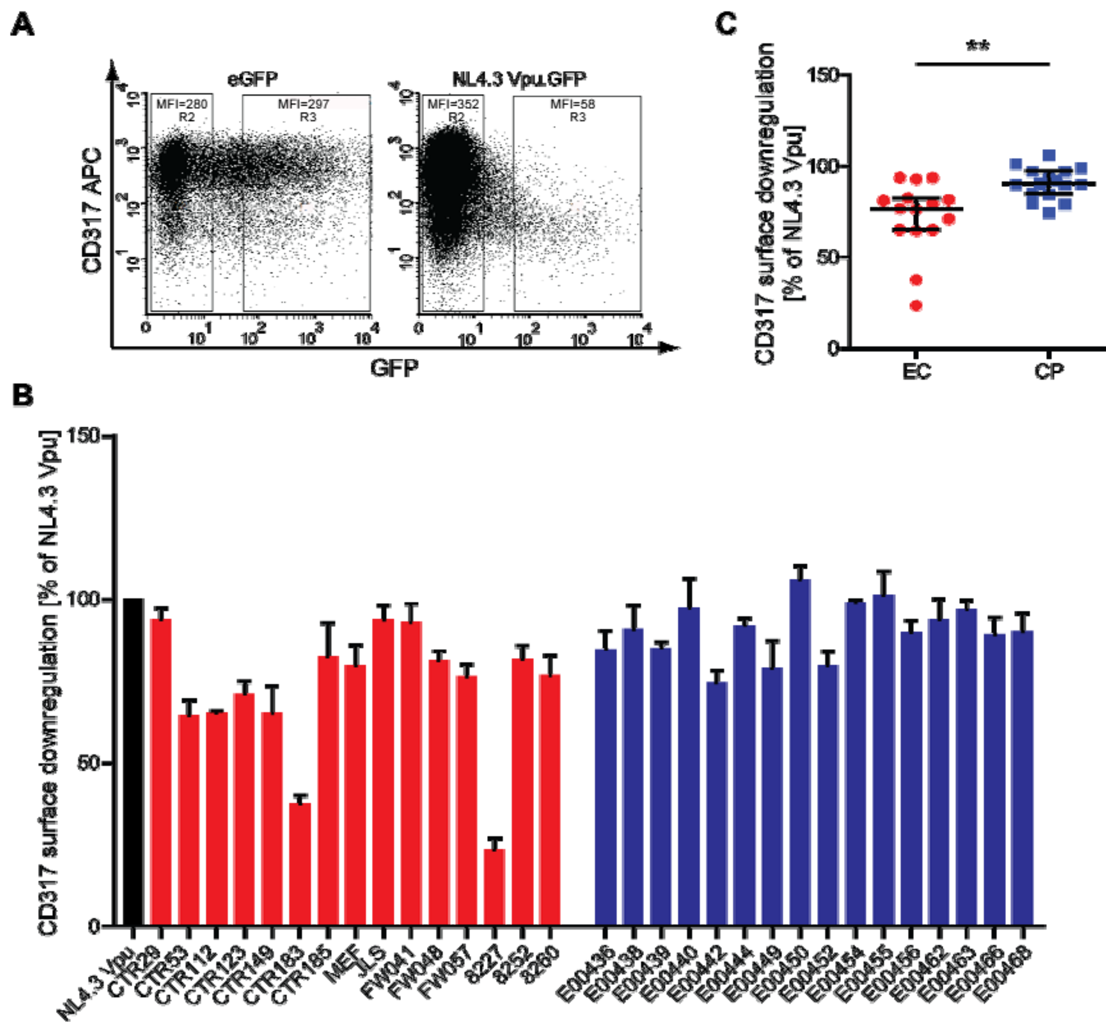

Figure 13: CD317 downregulation activity of Vpu.GFP alleles. A3.01 cells were electroporated with Vpu.GFP expression constructs, harvested after 48 hrs, and stained for cell surface CD317. **A**: Flow cytometry plots of eGFP and NL4.3VpuGFP: CD317-APC (y-axis) vs. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to NL4.3 Vpu that was arbitrarily set to 100%. **B**: Graph showing the CD317 downregulation activity of patient derived Vpu alleles relative to NL4.3Vpu. Shown are mean values of triplicate transfections with the indicated standard deviation. The result is a representative of three independent experiments. **C**: Comparison of CD317 downregulation activity of EC Vpus and CP Vpus.

5.5.2 Surface Modulation of CD4 and CD317 by VpuIRESGFPs

We further evaluated the capacity of Vpu to modulate CD4 and CD317 in the non-fusion context. EC and CP VpuIRESGFPs were transiently expressed in TZM-bl cells. Surface CD4 and CD317 remaining levels were determined by FACS

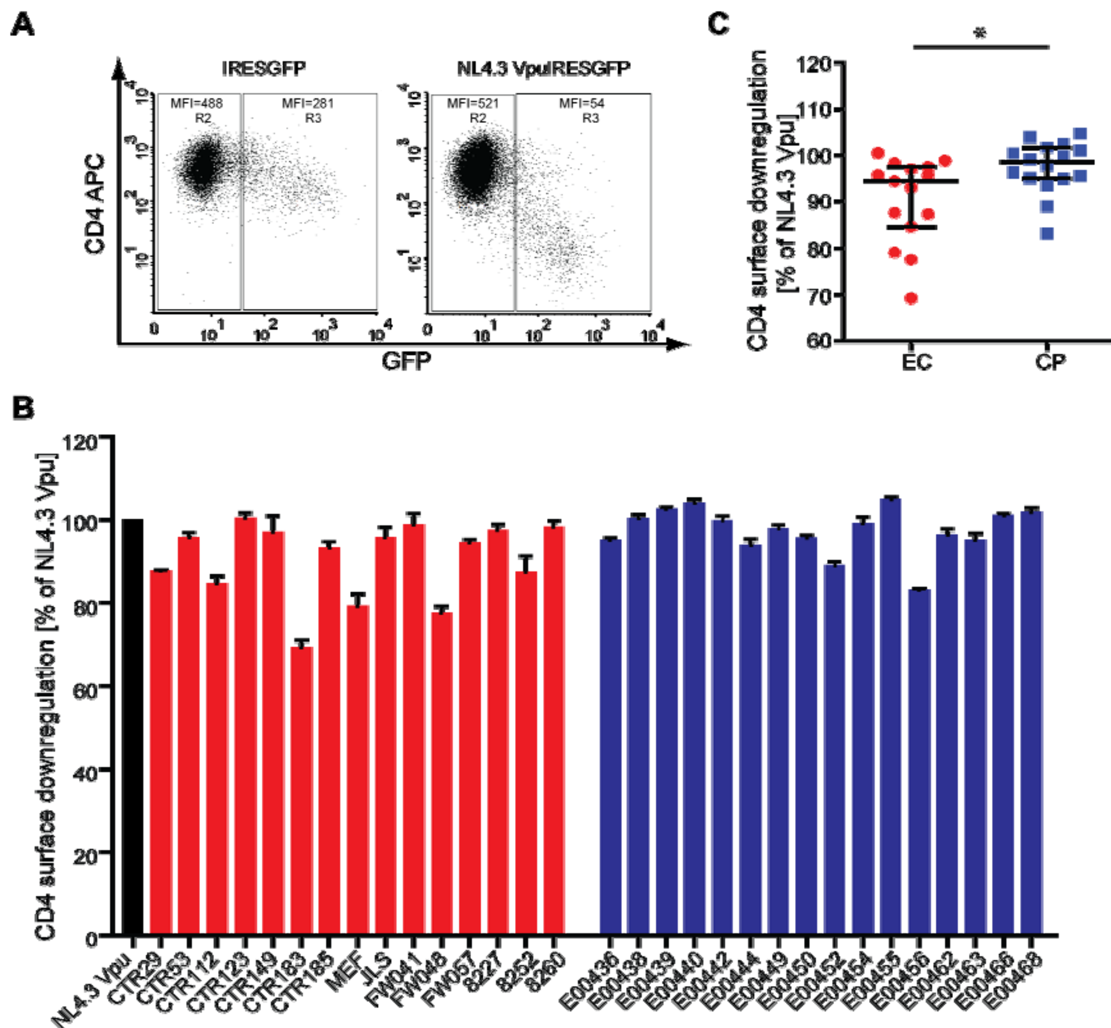

Figure 14: CD4 downregulation activity of VpuIRESGFP alleles. TZM-bl cells were transfected with VpuIRESGFP constructs, harvested after 24 hours, and stained for cell surface CD4. **A**: Flow cytometry plots of IRESGFP and NL4.3 VpuIRESGFP: CD4-APC (y-axis) vs. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to NL4.3 Vpu that was arbitrarily set to 100%. **B**: Graph showing the CD4 downregulation activity of patient derived Vpu alleles relative to NL4.3 Vpu. Shown are mean values of triplicate transfections with the indicated standard deviation. The result is representative of three independent experiments. **C**: Comparison of CD4 downregulation activity of EC Vpus and CP derived Vpus.

as described in **5.3.1**. As shown in the primary data for controls, the reference NL4.3 Vpu removed 80% of CD4 or CD317 from the surface (Figure 14A, 15A). The following evaluation showed that majority of patient-derived Vpus showed comparable potency to reduce CD4 surface levels as the reference strain NL4.3 Vpu

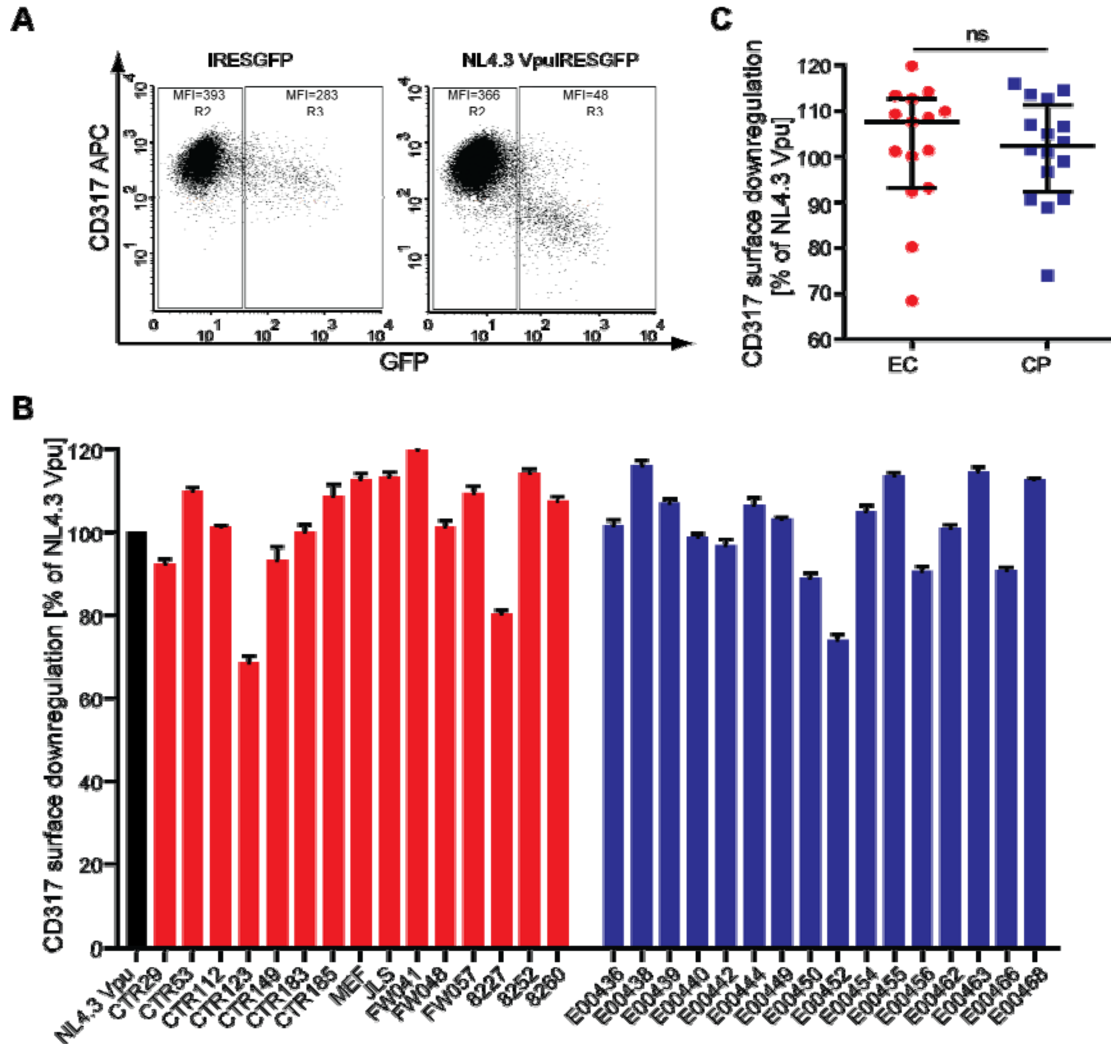

Figure 15: CD317 downregulation activity of VpuIRESGFPs alleles. TZM-bl cells were transfected with VpuIRESGFP constructs, harvested after 24 hours, and stained for cell surface CD317. **A**: Flow cytometry plots of IRESGFP and NL4.3 VpuIRESGFP: CD317-APC (y-axis) VS. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to NL4.3 Vpu that was arbitrarily set to 100%. **B**: Graph showing the CD317 down-regulation activity of patient derived Vpu alleles relative to NL4.3 Vpu. Shown are mean values of triplicate transfections with the indicated standard deviation. The result is representative of three independent experiments. **C**: Comparison of and CD317 downregulation activity of Vpus and CP derived Vpus.

(Figure 14B). Few alleles from EC group, such as CTR183, MEF and FW048, displayed lower than 80% of NL4.3 Vpu activity (Figure 14B). EC Vpus maintained significantly lower activity (median 94.5 [IQR 84.7–97.5]%) than CP Vpus (median

98.6 [IQR 95.2–101.6]%) ($p < 0.05$; Figure 14C). Similarly, majority of primary Vpus displayed potent abilities as reference NL4.3 Vpu to interfere with CD317 surface expression (Figure 15B). While unlike in the fusion version, EC Vpus showed similar CD317 downregulation activities (median 107.6 [IQR 93.2–112.7]%) as CP Vpus (median 102.4 [IQR 92.3–11.3]%). There was no statistically significant difference between these two groups ($p > 0.05$; Figure 15 C). The data generated in both fusion and non-fusion contexts showed the basic CD4 and CD317 downregulation activities were well conserved among all Vpus.

5.6 EC Vpus Displayed Lower MHC-I and NTB-A Downregulation Activities

5.6.1 Downregulation of MHC-I by Vpu.GFP and VpuIRESGFPs

Vpu is one of the proteins that HIV-1 exploits to modulate the surface level of antigen presenting molecules MHC-I (Petersen, Morris et al. 2003). Downregulation of MHC-I by HIV-1 Nef was one of the strategies that HIV-1 escapes CTL killing (Collins, Chen et al. 1998) (Kirchhoff, Schindler et al. 2008). EC Nefs showed attenuated MHC-I downregulation activities compared with CP Nefs (Mwimanzi, Markle et al. 2013). Up-to-data, MHC-I reduction mediated by patient-derived Vpu alleles has not been investigated yet. We evaluated surface modulation of MHC-I in A301 cells transiently expressed fusion or non-fusion Vpu proteins. The experimental procedure and data analysis were the same with **5.3.1**. Since the expression level of MHC-I and NTB-A on TZM-bl cells were low (data not shown). The data in this section were generated in A301 cells. NL4.3 Vpu removed 30%～40% of MHC-I from the cell surface level as fusion or non-fusion protein (Figure 16A, 17A). The majority of CP Vpus alleles showed comparable abilities as NL4.3 Vpu to reduce MHC-I molecules (median 98.2 [IQR 88.2–106.3]%) or (median 87.4 [IQR 77.9–110.0]%) as fusion or non-fusion proteins (Figure 16A, 17A). However, EC

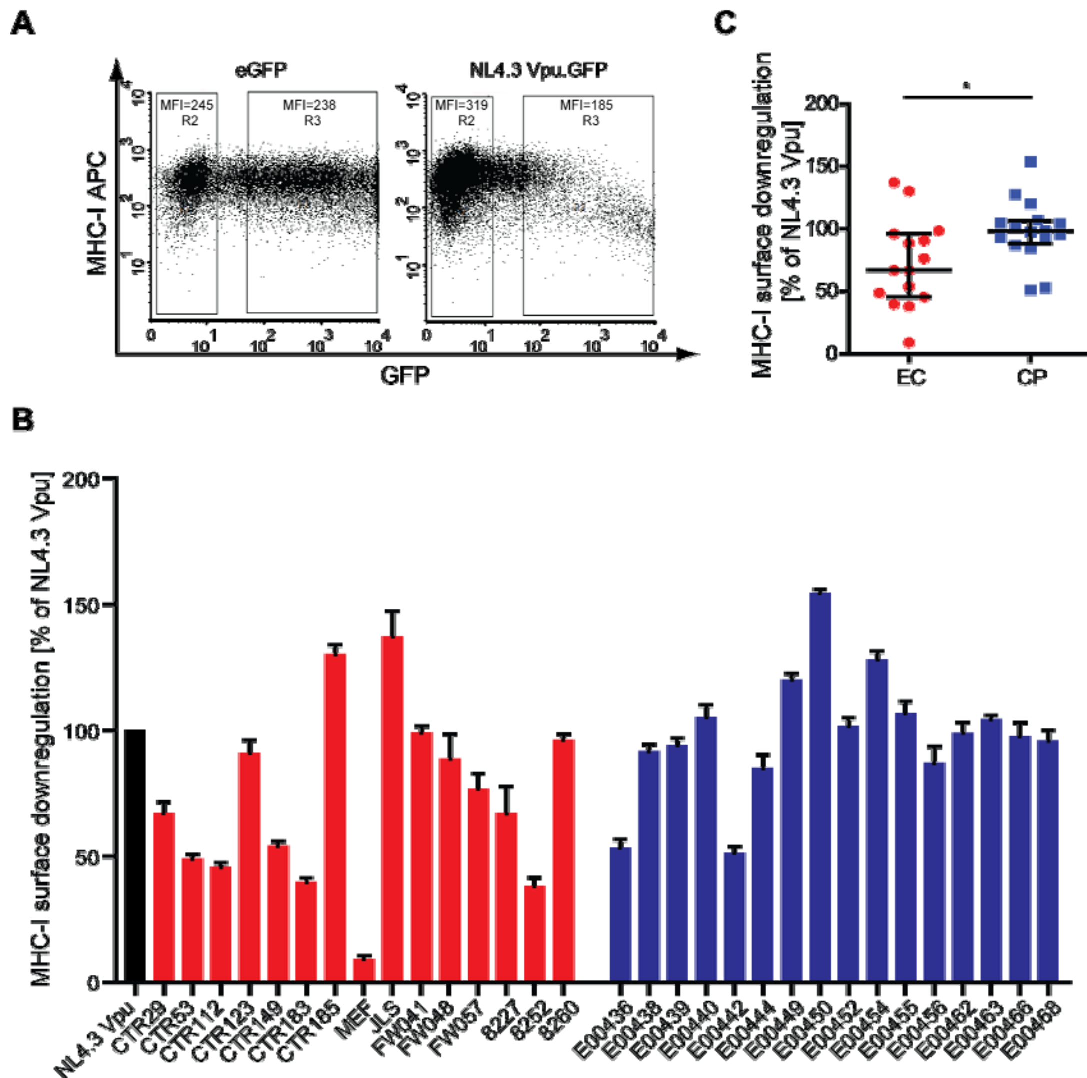

Figure 16: MHC-I downregulation activity of Vpu.GFP alleles. A301 cells were electroporated with Vpu.GFP expression constructs, harvested after 48 hours, and stained for cell surface MHC-I. **A**: Flow cytometry plots of GFP and NL4.3Vpu.GFP: MHC-I-APC (y-axis) vs. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to NL4.3Vpu that was arbitrarily set to 100%. **B**: Graph showing the MHC-I downregulation activity of patient derived Vpu alleles relative to NL4.3Vpu. Shown are mean values of triplicate transfections with the indicated standard deviation. The result is representative of two independent experiments. **C**: Comparison of MHC-I activity of EC Vpus and CP Vpus.

Vpus displayed more variable and attenuated MHC-I reduction activity (median 67.1[IQR 45.5–96.2]%) or (median 65.4 [IQR 52.7–90.4]%) (Figure 16B, 17B). The different patterns for EC and CP groups were reflected in the *t* test. There was

statistically significant difference between EC and CP Vpu alleles as fusion or non-fusion proteins ($p < 0.05$; Figure 16C, 17C).

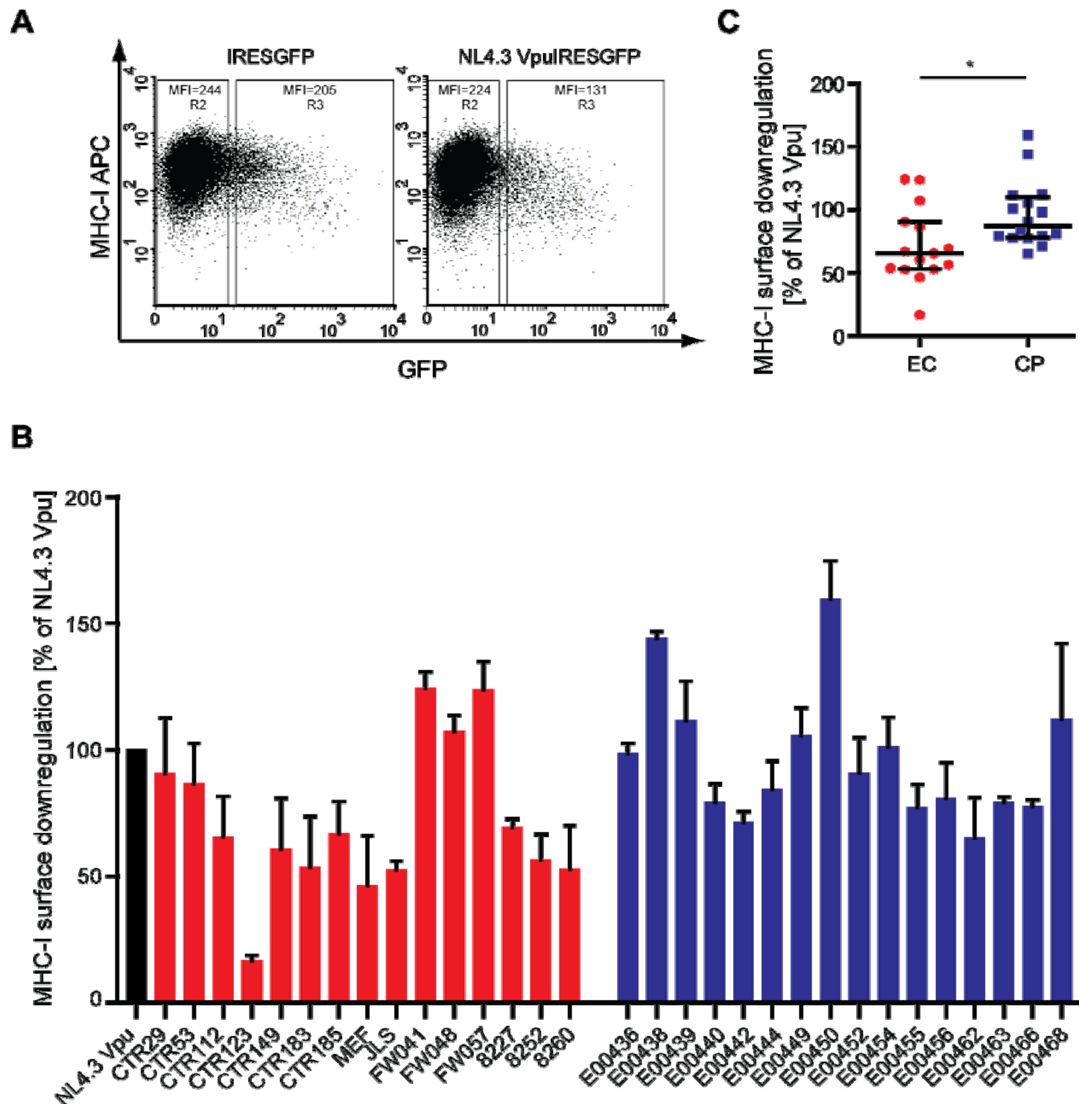

Figure 17: MHC-I downregulation activity of VpuIRESGFP alleles. A301 cells were electroporated with VpuIRESGFP expression constructs, harvested after 48 hours, and stained for cell surface MHC-I. **A**: Flow cytometry plots of GFP and NL4.3Vpu.GFP: MHC-I-APC (y-axis) vs. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to NL4.3Vpu that was arbitrarily set to 100%. **B**: Graph showing the MHC-I downregulation activity of patient derived Vpu alleles relative to NL4.3Vpu. Shown are mean values of triplicate transfections with the indicated standard deviation. The result is representative of two independent experiments. **C**: Comparison of MHC-I activity of EC Vpus and CP Vpus.

5.6.2 Downregulation of NTB-A by Vpu.GFP alleles

NTB-A is a co-activating ligand for NK cell activation and degranulation. Vpu is the only protein that HIV-1 utilizes to decrease NTB-A cell surface levels and consequently avoid the recognition of NK cells (Shah, Sowrirajan et al. 2010). It is unknown whether this activity of Vpu is conserved among natural Vpu alleles and whether there is any systematic difference between EC and CP Vpus. We analysed the NTB-A surface modulation activity mediated by patient-derived-Vpu alleles in A301 cells as fusion and non-fusion proteins. Our positive control NL4.3Vpu decreased NTB-A surface expression by about 40% (Figure18A), which confirmed the previous result (Shah, Sowrirajan et al. 2010) (Bolduan, Hubel et al. 2013). Among both EC and CP Vpu alleles, NTB-A downregulation activity showed high degree of variation compared with CD4 and CD317 downregulation activity. In EC groups, there were more impaired ones again, such as 53, CTR112, CTR149, CTR183 and MEF showing around 25% of NL4.3 activity (Figure18A). Overall, the EC Vpus showed more lower NTB-A downregulation activity (median 60.7 [IQR 28.4–78.8]%) than CP Vpus (median 98.6 [IQR 57.5–109.4]%) ($p< 0.01$; Figure 18C). All non-fusion constructs, including our positive control NL4.3 Vpu, could only remove up to 10% of NTB-A from the A301 cell (data not shown).

5.7 Vpu-mediated Enhancement of HIV-1 Virion Release

5.7.1 Defect of GFP fused Vpu Proteins to Enhance HIV-1 Virion Release

Vpu was shown to promote HIV-1 virion release by antagonizing the restriction of CD317/tetherin (Neil, Eastman et al. 2006). We evaluated the virion release enhancement mediated by Vpu alleles in TZM-bl cells which are introduced in integrated copies of luciferase genes under control of the HIV-1 promoter and sensitive to HIV-1 infection. Briefly, TZM-bl cells were co-transfected with HIV-1 NL4.3 ΔVpu proviral plasmids and indicated Vpu alleles, empty vector or negative control VpuS/A mutant. The resulting supernatant was used to infect newly-seeded TZM-bl cells. The transfected cells were harvested for GFP, p24 and p55 immunology

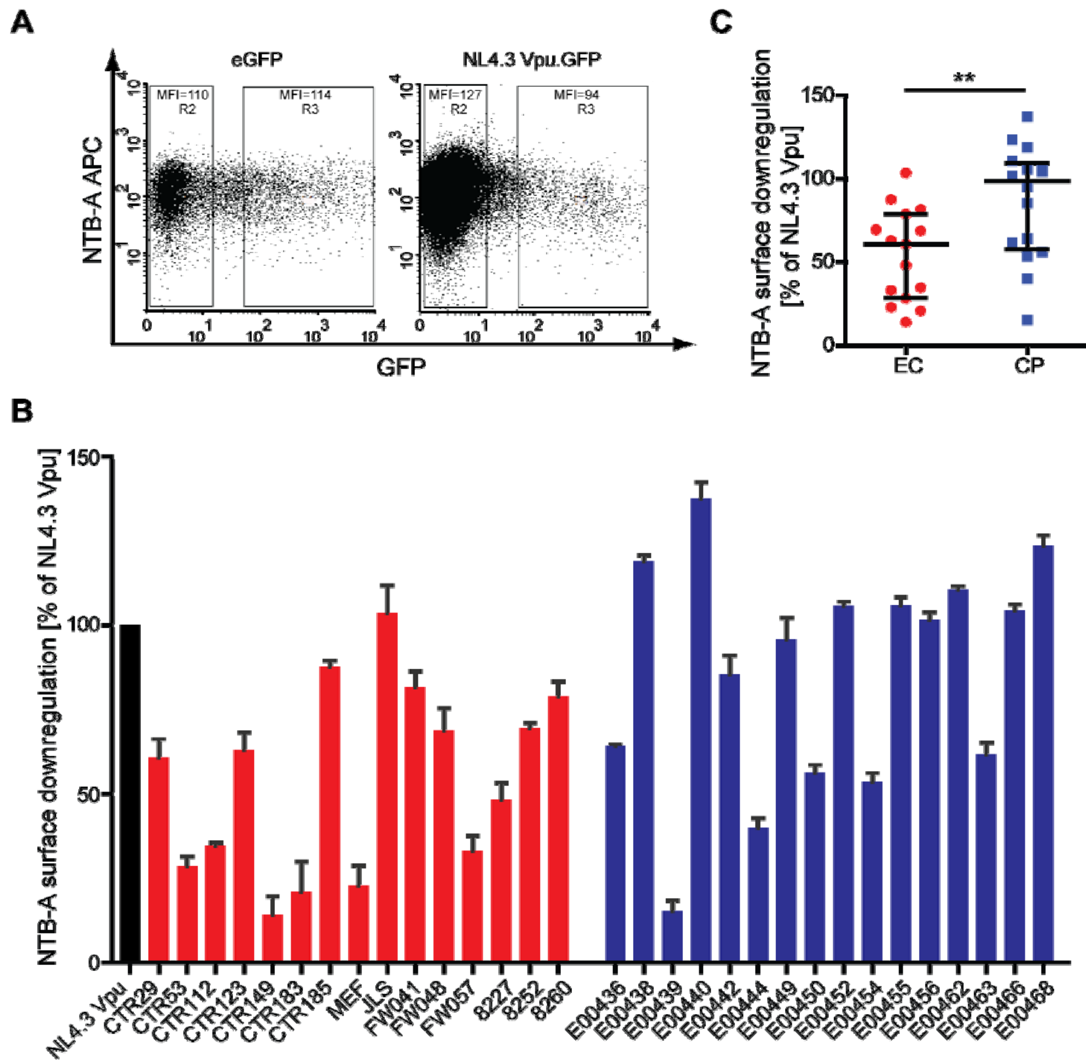

Figure 18: NTB-A downregulation activity of Vpu.GFP alleles. A301 cells were electroporated with Vpu.GFP expression constructs, harvested after 48 hours, and stained for cell surface NTB-A. **A**: Flow cytometry plots of GFP and NL4.3Vpu.GFP: NTB-A-APC (y-axis) vs. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to NL4.3Vpu that was arbitrarily set to 100%. **B**: Graph showing the NTB-A downregulation activity of patient derived Vpu alleles relative to NL4.3Vpu. Shown are mean values of triplicate transfections with the indicated standard deviation. The result is representative of two independent experiments. **C**: Comparison of NTB-A activity of EC Vpus and CP Vpus.

blotting. The infected cells lysate was assayed for luciferase units which was the readout of the quantity of the infectious virions in the supernatant. Vpu was shown no effect on the infectivity of released HIV-1 virions, so quantity of the infectious virions in the supernatant indicated the quantity of the released virions. We first tested this activity of NL4.3 Vpu.GFP at a series of concentrations. The 0.8 μg non-codon optimized NL4.3 Vpu.GFP was expressed at the comparable level of the positive control 0.1μg of codon-optimized SynVpu. NL4.3 Vpu.GFP could not promote virion release but the non-fusion Vpu protein was able to enhance the virion release at proper concentration (Figure 19). This directed us to go for non-fusion Vpu proteins for the full panel of virion release evaluation.

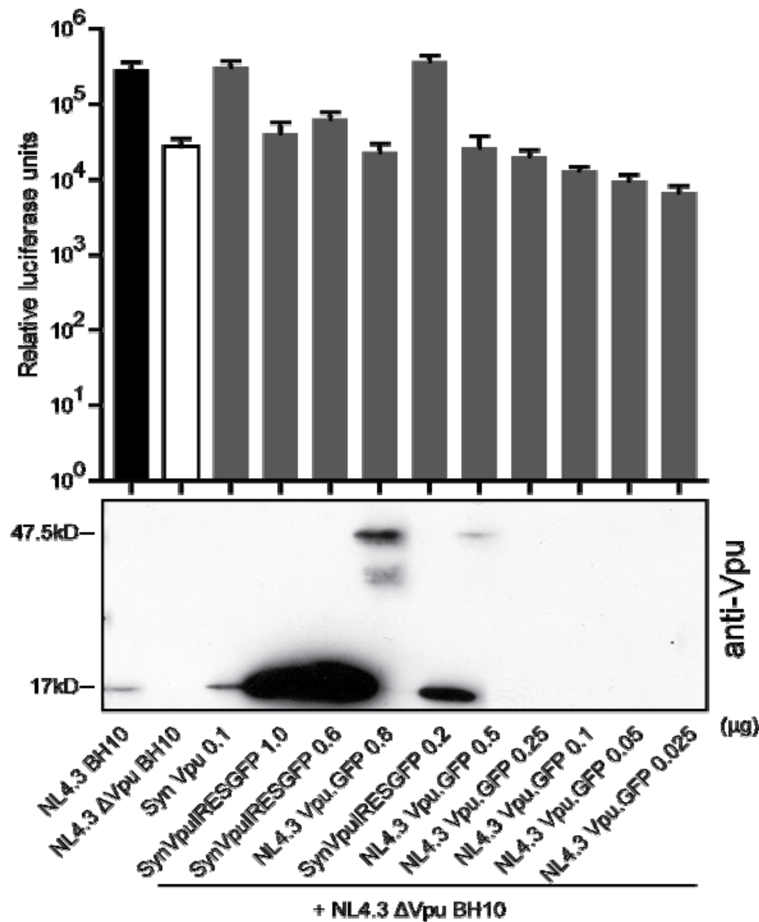

Figure 19: Virion release enhancement mediated by Vpu as fusion and non-fusion protein. TZM-bl cells were transiently transfected with HIV-1NL4.3 ΔVpu provirus plasmid and the indicated Vpu allele. NL4.3 BH10 served as one

positive control. 48 hours post transfection, the cell supernatants containing viral particles were assayed for infectivity on TZM-bl reporter cells to determine the amount of infectious virions produced. TZM-bl cell lysates were subjected to Western blot detection.

5.7.2 Virion Release Enhancement is Conserved among Patient-derived Vpus

To examine whether patient-derived Vpus are able to enhance HIV-1 particle release or not, all indicated individual VpuIRESGFPs, together with HIV-1 NL4.3 ΔVpu proviral plasmids, were co-transfected TZM-bl cells and then processed for infectivity assay, western blotting and downregulation of CD317/tetherin as described in **5.7.1.** Most of EC and CP Vpus displayed well-conserved abilities to enhance virion particle release similar to NL4.3 Vpu, with the exception of impaired EC 8227 and CP E00456. (Figure 20a, upper bars). To preclude the possibility of different levels of virion production and Vpu expression among different transfections, we analysed GFP, p24 and p55 levels in the transfected cells. The p24 and p55 antigen in the lysate from transfected cells from each Vpu allele were identical, which reflected similar virion production for each transfection of HIV-1 NL4.3 ΔVpu proviral plasmids (Figure 20a, lower panel); the expression levels of GFP were similar to each other (Figure 20a, upper panel), and this was used to define expression levels of Vpus as the Vpu antibody displayed different degree of affinity towards the Vpus (Figure 10). EC Vpus displayed a lower trend of virion release enhancement activity (median 4.6 [IQR [3.2–6.6] fold of HIV-1 NL4.3 ΔVpu) than CP Vpus (median 6.7 [IQR [5.2–13.3] fold of HIV-1 NL4.3 ΔVpu), but the statistic evaluation showed no significant different between these two groups (Figure 20a B; $p=0.07$). The activity to enhance HIV-1 release was well conserved in both EC and CP Vpus and showed no systematic difference.

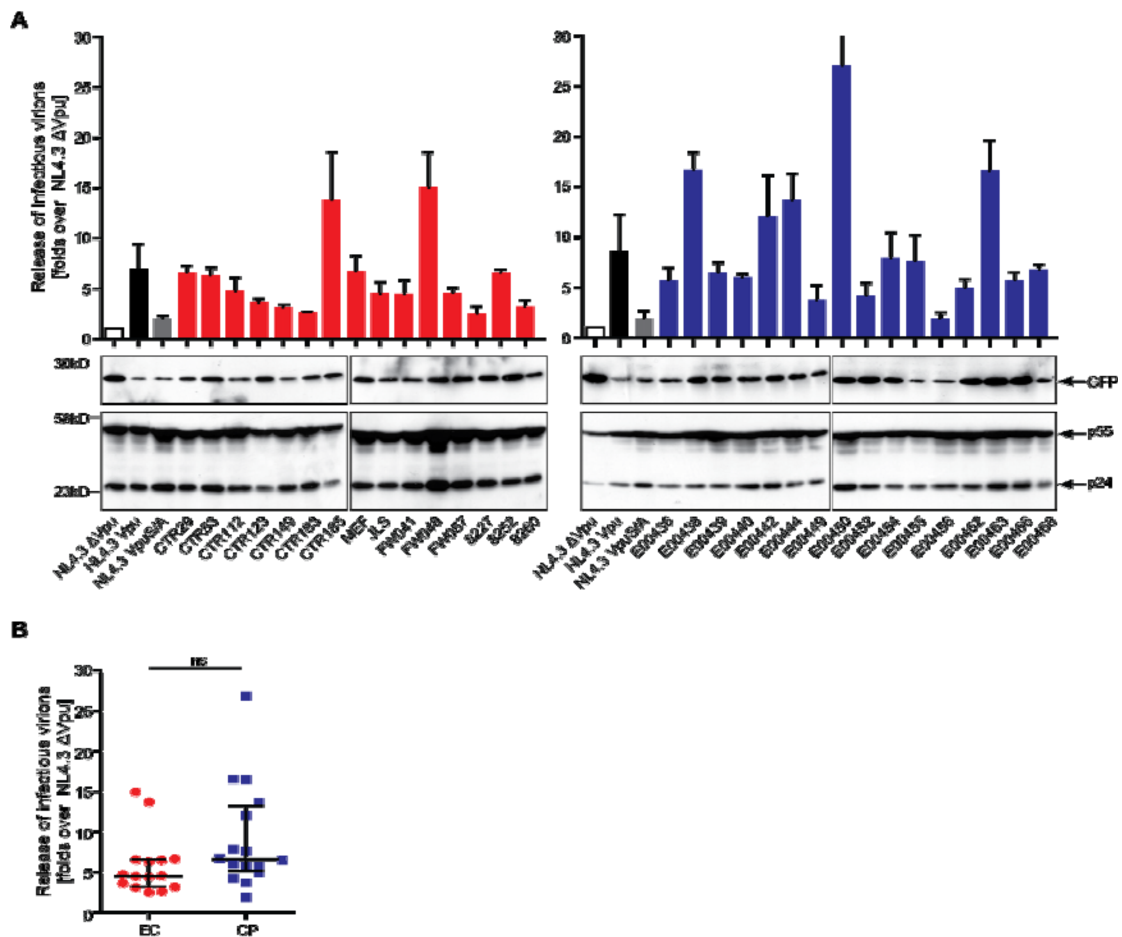

Figure 20a: Rescue of HIV-1 particle release by patient-derived Vpu alleles. TZM-bl cells were transiently transfected with HIV-1NL4.3 ΔVpu provirus plasmid and the indicated Vpu allele or control. 48 hours post transfection the cell supernatants containing viral particles were assayed for infectivity on TZM-bl indicator cells to determine the amount of infectious virions produced. TZM-bl cell lysates from one replicate of the assay were subjected to Western blot analysis. TZM-bl cells from the same replicate were harvested and assayed for cell-surface level of CD317. **A**: The yield of infectious HIV-1 in the supernatant and cell-associated levels of GFP, p24CA, and p55Gag were analyzed. HIV-1 particle release in the supernatant was assessed by measuring the induction of Luciferase units (top) in infected TZM-bl cells. Values (y axis) are normalized to that of control NL4.3ΔVpu, which was set to 1, error bars are standard error of the mean (SEM) for three independent experiments. Western blot results show the expression level of GFP, p24, and p55 in one representative experiment. **B**: Comparison of enhancement of virion release mediated by EC and CP Vpu alleles. Statistical significance was assessed using two-tailed Mann–Whitney U-Test ($p = 0.07$), bars represent median and inter-quartile ranges.

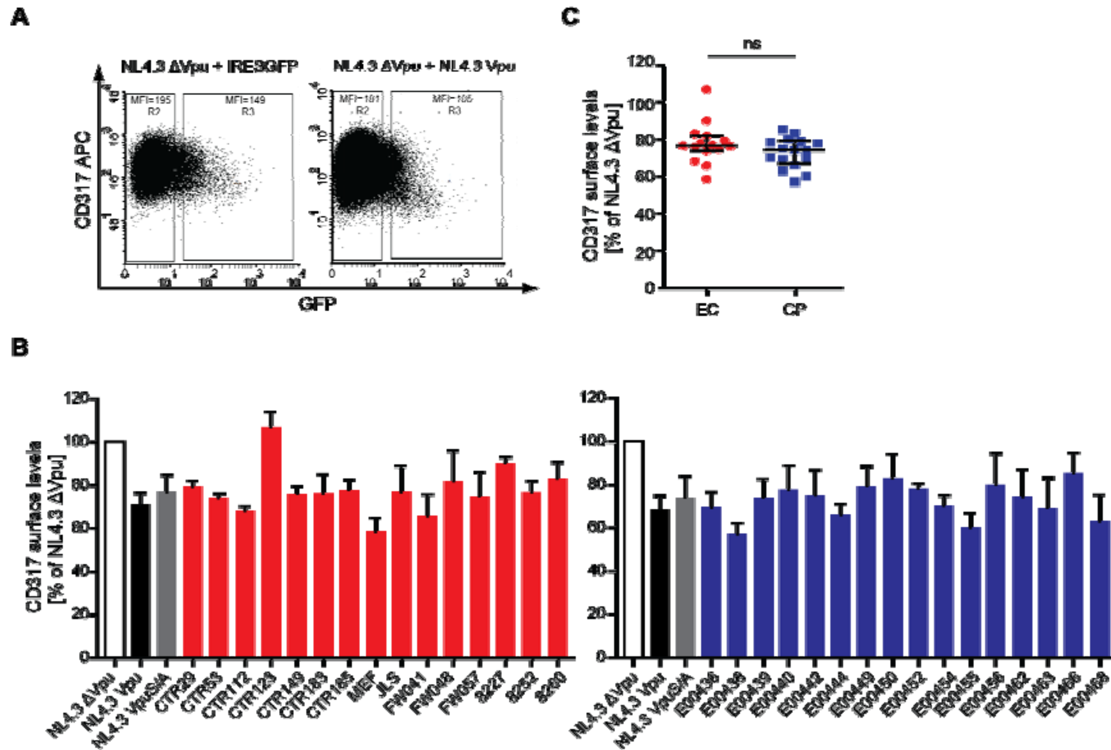

Figure 20b: CD317 cell surface downregulation by patient Vpu in the context of infection. TZM-bl cells transfected with HIV-1 NL4.3 ΔVpu and the indicated VpuIRESGFP plasmids were harvested 48 hrs post-transfection and CD317 cell surface levels quantified by flow cytometry. **A**: Flow cytometry plots of IRESGFP and NL4.3 VpuIRESGFP: CD317-APC (y-axis) VS. GFP (x-axis). Downregulation activity (R3/R2 ratio) was normalized to HIV-1 NL4.3 ΔVpu that was arbitrarily set to 100%. **B**: The y-axis represents the relative CD317 cell surface levels remaining normalized to control cells transfected with NL4.3ΔVpu and IRESGFP (set to 100%). Shown are mean values of triplicate transfections with the indicated standard deviation. The result is representative of three independent experiments. **C**: Comparison of CD317 cell surface levels in TZM-bl cells producing viral particles and expressing EC or CP Vpu alleles. Statistical significance was assessed using two-tailed Mann–Whitney U-Test (p= 0.29), bars represent median and inter-quartile ranges.

5.7.3 Correlation of Vpu-mediated Virion release Enhancement and CD317/tetherin Surface Downregulation

Vpu expression ultimately leads to the surface reduction of CD317/tetherin, while whether the removal of CD317/tetherin from an infected cell surface is required for overcoming the barrier of the virion release is contradictory from the reports of different groups (Strebel 2014). We investigated the surface level of CD317 in the presence of HIV-1 proviral constructs and individual Vpu alleles. In our system, reference NL4.3 Vpu reduced about 35% of CD317/tetherin from the cell surface in the presence of proviral HIV-1 plasmids (Figure 20b, A), the negative control NL4.3 VpuS/A, with two serine mutations in DSGxxS motif, was shown activity to downregulate surface CD317/tetherin molecules but inactive to enhance virion release (Figure 20a, 20b). Analysis for patient-derived Vpus showed that Vpu-mediated surface reduction of CD317/tetherin was not strongly correlated with Vpu-mediated enhancement of virion release in our system (Figure 20a, 20b). First of all, E00450, CTR185 and FW048 showed the evidence high level of virion release did not require stronger CD317/tetherin downregulation activities. CTR123 and 8227, which showed very slight removal of CD317/tetherin, promoted virion release to a moderate extent. Overall, majority of EC and CP Vpus could reduce the cell surface level of CD317/tetherin in the presence of HIV-1 proviral constructs with no systematic difference (Figure 20b C; $p=0.29$), and the trend was similar in the absence of HIV-1 proviral constructs (Figure 13, 14).

5.8 Vpu Antagonizes NF-ĸB Activation

5.8.1 Equal Efficiency to Inhibit CD317/tetherin-induced NF-ĸB Activation

Prior to the identification of a host restriction factor to retain viral particles at the plasma membrane, CD317/tetherin was shown to activate the central transcriptional factor NF-ĸB in a cDNA screening (Matsuda, Suzuki et al. 2003). Recently the role of CD317/tetherin sensing virions and stimulating NF-ĸB activation was described. Vpu,

as the specific antagonist, could counteract the induction of NF-ƙB by CD317/tetherin (Galao, Le Tortorec et al. 2012) (Tokarev, Suarez et al. 2013).

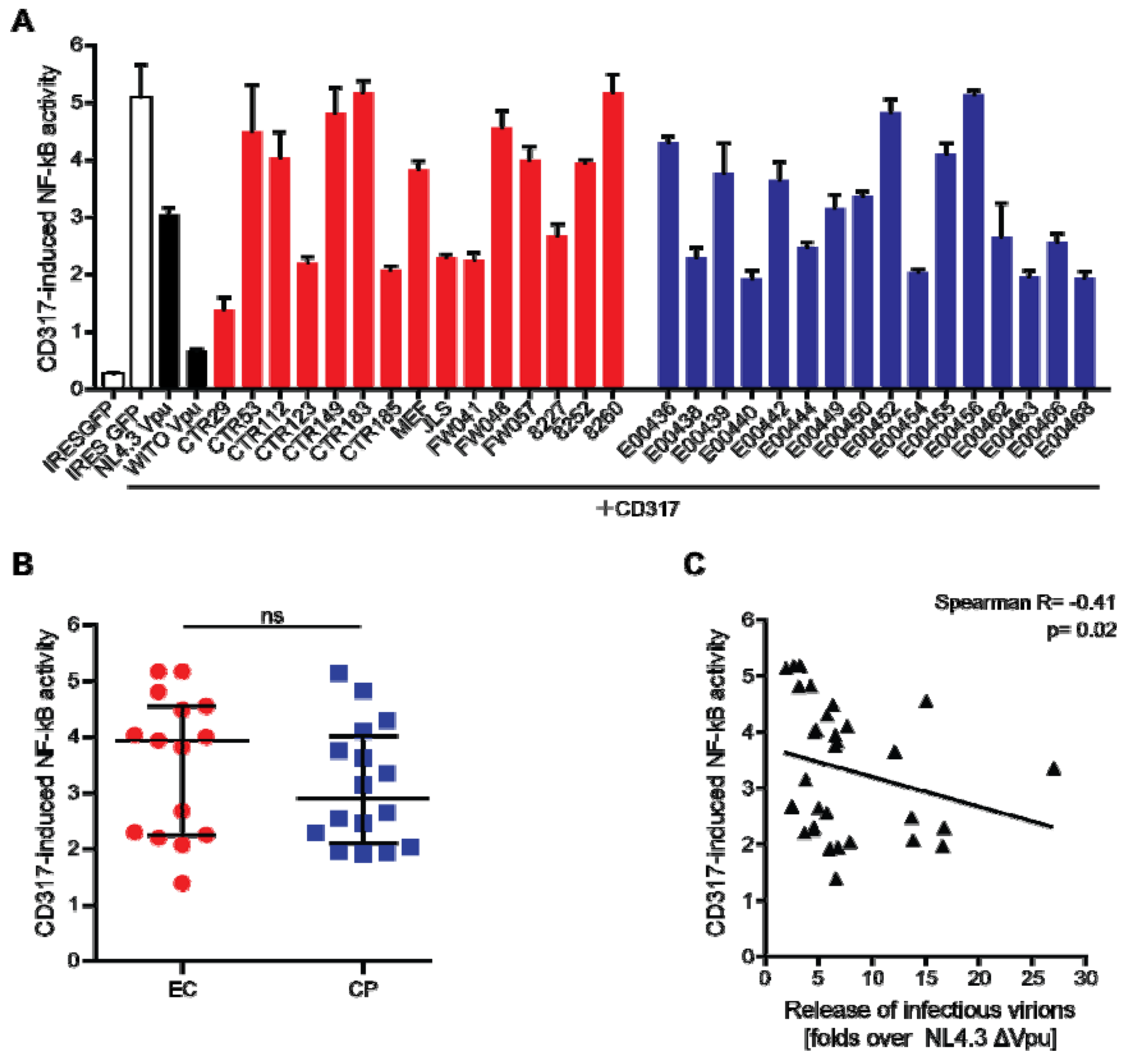

Figure 21: Antagonism of CD317-induced NF-kB signaling. A: Inhibition of CD317-induced NF-ƙB activation by Vpu alleles. Luciferase activity of the NF-ƙB reporter was determined 40 hours post transfection of HEK293T cells with expression plasmids for CD317 or filler, the indicated VpuIRESGFP expression plasmids and luciferase reporter plasmids. HIV-1 NL4.3 Vpu and HIV-1 M WITO Vpu served as controls. Mean values of 6-9 transfections are shown with the indicated standard deviation. **B**: Effect of EC and CP Vpu alleles on CD317-induced NF-ƙB activation. Statistical significance was assessed using two-tailed Mann–Whitney U-Test (p= 0.29), bars represent median and interquartile ranges. **C**: Correlation of Vpu-mediated block of CD317-induced NF-ƙB activity and Vpu-mediated release of infectious

virions. Statistical analyses were done using Spearman's correlation. (Data generated by Daniel Sauter, Ulm University)

Inhibition of CD317/tetherin-induced NF-ƙB activation by Vpu was assessed in a reporter system in which the expression of firefly luciferase is driven by a promoter response to NF-κB. The transient expression of CD317 in HEK293T cells, which lack endogenous CD317, induced NF-κB activity up to 6 fold compared to the negative control in the absence of CD317 as shown by the white bars (Figure 21A). Expression of NL4.3 Vpu and also another more active WITO Vpu from founder HIV-1 virus could inhibit the CD317/tetherin-induced NF-ƙB activation. This inhibition activity does not differ between the EC and CP Vpus, with around 50% of them in each group able to suppress the CD317/tetherin-induced NF-ƙB activation in both groups (Figure 21B). There was very mild correlation between Vpu-mediated virion release enhancement and NF-ƙB activation measuring the Spearman R (Figure 21C; R= -0.41, p=0.02). We suspect that other host factors may be involved in Vpu-mediated inhibition of CD317-induced NF-ƙB activation and Vpu-mediated recovery of virion release.

5.8.2 Majority of Vpus Failed to Inhibit IKKβ-induced Activation of NF-kB

NF-ƙB activation could also be initiated by the stimulation of catalytic unit IKKβ, which is critical for the phosphorylation and subsequent degradation of the inhibitor of NF-ƙB (IkB) by β-TrCP E3 ubiquitin ligases (Perkins 2007). Vpu could interfere with this NF-ƙB signalling pathway via competing the binding with β-TrCP (Bour, Perrin et al. 2001). We investigated whether the Vpus were able to inhibit IKKβ-induced activation of NF-ƙB in the similar reporter system used in **5.8.1**. NF-ƙB was activated in the presence of IKKβ, as shown in white bars (Figure 22A). The pCG-WITO Vpu was set as a further positive control, in contrast to WITO Vpu, reference NL4-3 Vpu was inactive in this experiment and even enhanced NF-ƙB activation. This is in agreement with previous experiments using expression vectors

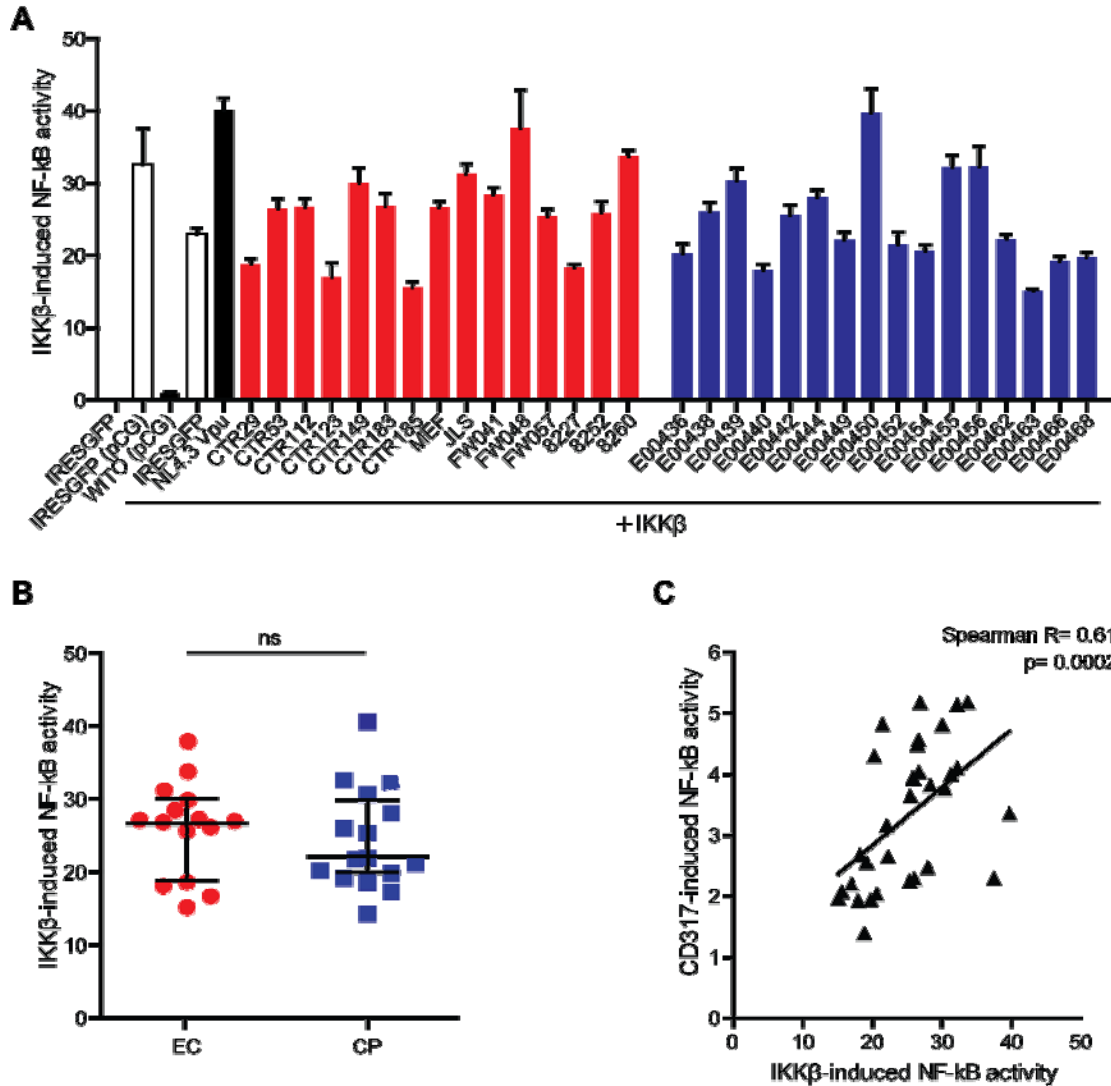

Figure 22: Antagonism of IKKβ-induced NF-kB signaling. **A**: Effect of EC and CP Vpu alleles on IKKβ-induced NF-ƙB activation. Luciferase activity of the NF-ƙB reporter was determined 40 hours post transfection of 293T cells with expression plasmids for IKKβ or filler, the indicated VpuIRESGFP expression plasmids and luciferase reporter plasmids. HIV-1 M WITO Vpu served as positive control. **B**: Comparison of inhibition IKKβ-induced NF-ƙB activation mediated by EC and CP Vpu alleles. Statistical significance was assessed using two-tailed Mann–Whitney U-Test ($p = 0.27$), bars represent median and interquartile ranges. **C**: Correlation of the Vpu-mediated block of CD317-induced NF-ƙB activity and IKK-ß-induced NF-ƙB activity among all Vpu alleles analysed. Statistical analyses were done using Spearman's correlation. (Data generated by Daniel Sauter, Ulm University)

driven CMV promoter (Jafari, Guatelli et al. 2014). Only few of the alleles, including CTR29, CTR123, CTR185 and 8227 from EC group, as well as E00436, E00440, E00454, E00463, E00466, E00468, displayed slight inhibitory activities (Fig. 15 A). There was no significant difference between EC and CP Vpus (Figure 22B). Vpu-mediated inhibition of CD317/tetherin-induced NF-ƙB and IKKβ-induced NF-ƙB activation was not conserved among primary Vpus, while these two inhibitory functions of Vpu were well correlated (Figure 22C; R= 0.61, p=0.02), which suggest the two pathways may be integrated at some point.

5.9 Scanning of Novel Functional Determinants of Vpu

Loss of function for some alleles can be explained by lack of motifs, for some alleles. The assignment was not possible, indicating the existence of important yet un-characterized motifs of Vpu. Based on the results of functional analyses and natural amino acid variants, MEF allele from EC group interested us as its ability to modulate CD4, MHC-I, and NTB-A cell surface levels was significantly impaired, while MEF displayed full activity for CD317 downregulation and virion release enhancement. We first compared its expression level with NL4.3 Vpu to preclude the possibility that impaired activities did not result from the expression level. The expression of NL4.3 Vpu and MEF were comparable (Figure 23A), so the functional differences come from the inherent properties existing in the amino acid sequence. Since all described motifs critical for Vpu functions are conserved in MEF (Figure 9), we first aligned MEF and NL4.3 Vpu sequences. The variable regions or single amino acid were marked in red, then we referred to all the HIV-1 M group Vpu sequences in Los alamos national laboratory database (http://www.hiv.lanl.gov/content/index) and checked the mutation frequencies for these variable regions. The amino acids marked in the green box showed rare mutation frequencies, and were collected for further analysis (Figure 23B).

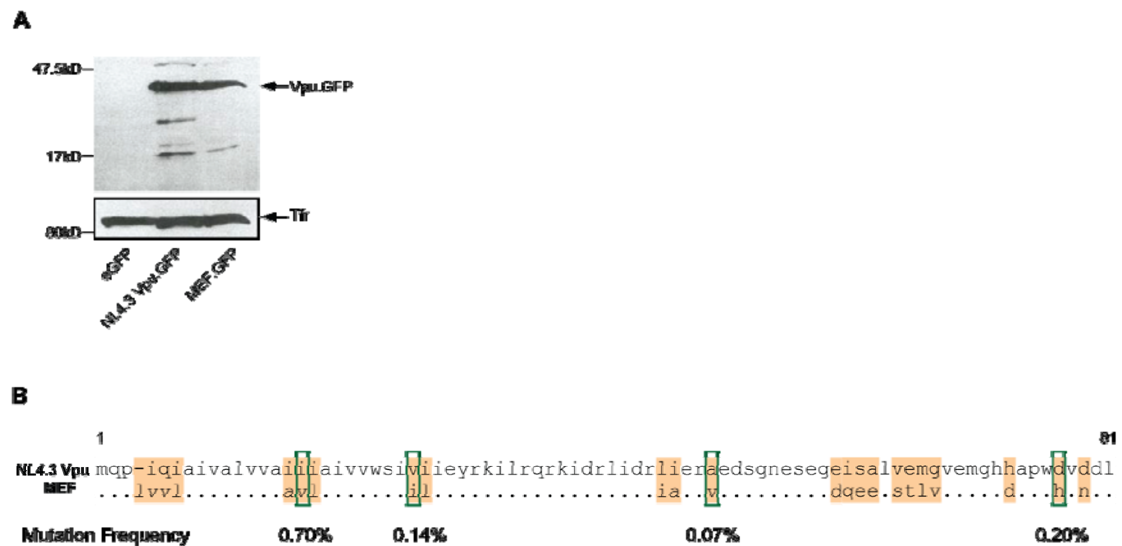

Figure 23: Identification of critical amino acids for full functionality. A: Validation of comparable expression level of NL4.3 Vpu and MEF. A301 cells were transfected with non-code optimized NL4.3 Vpu.GFP and MEF.GFP plasmids (30 μg). 24 hours post-transfection cells were harvested and the cell lysates were analyzed by immunoblotting for Vpu and transferin. **B**: Sequence alignment and mutation frequency calculation. The potential amino acids in red were compared with all HIV-1 clade B sequences in HIV-1 database (http://www.hiv.lanl.gov/content/index).

We constructed a set of MEF alleles with single or grouped amino acid mutations, as well as a corresponding set of NL4.3 Vpu mutants by site-directed mutagenesis. Downregulation of CD4 and MHC-I was assessed by FACs. For NL4.3 Vpu, V25I and A49V impaired the CD4 downregulation activity, but I16V and D77H had no effect (Figure 24A). Single substitute of V17I, I26V and V50A increased rescued CD4 downregulation activity of MEF, especially V50A almost making MEF as active as NL4.3 Vpu (Figure 24A). Interestingly, All the single mutants displayed impaired MHC-I downmodulation, whereas, the corresponding mutants V17I, I26V and V50A MEF did not rescue this ability, except H78D showing mild effect (Figure 24B). Within the grouped mutations, all listed single amino acids are required for optimal CD4 downregulation activity in the context of NL4.3 and MEF (Figure 24B). The grouped NL4.3 Vpu mutant showed almost comparable potency as a wild type to reduce MHC-I cell surface level. However, the MEF IVA rescued its ability while the

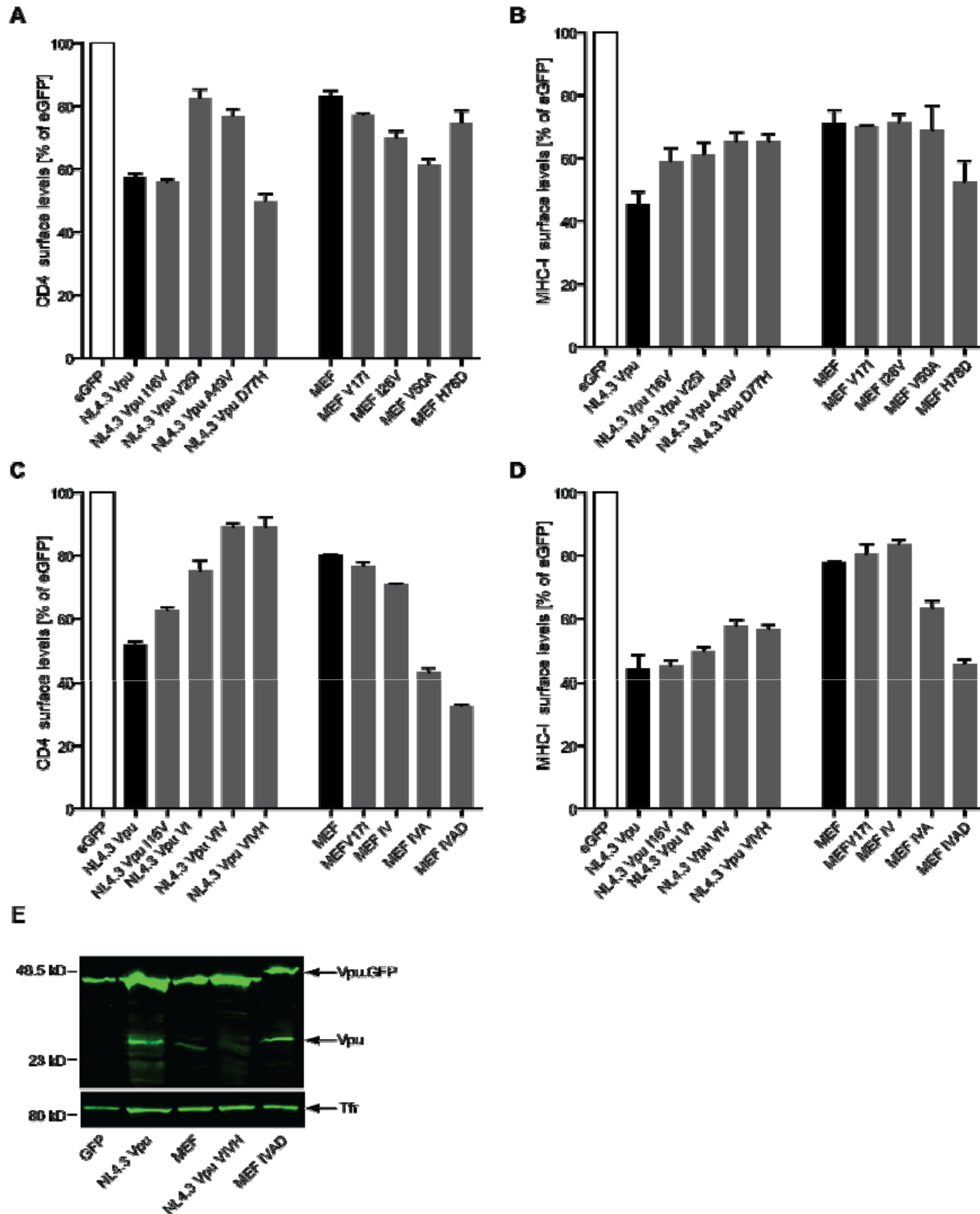

Figure 24: CD4 and MHC-I downregulation activity of mutated NL4.3 Vpu and MEF. A3.01 cells were electroporated with the indicated Vpu.GFP expression constructs, harvested after 48hrs, and stained for cell surface CD4 and MHC-I. Downregulation activity was normalized to eGFP that was set to 100%. **A, C**: Graphs showing the CD4 downregulation activity of Vpu alleles relative to eGFP. Shown are mean values of triplicate transfections with the indicated standard deviation. **B, D**: Graphs showing the MHC-I downregulation activity of Vpu alleles relative to eGFP. Shown are mean values of triplicate transfections with the indicated standard

deviation. **E**: A301 cells were transfected the indicated Vpu.GFP alleles (30 μg). 24 hours post-transfection, The GFP positive cells were isolated, and the cell lysates were analyzed by immunoblotting for Vpu, and transferin.

single mutant MEF V50A did not. Moreover, the full grouped mutant MEF IVAD completely recovered this activity (Figure 24D). To summarize, all listed single amino acids are required for optimal CD4 downregualtion, especially, 25V and 49A. While for MHC-I downmodulation, the grouped mutant MEF IVAD totally recovered for its activity, but the corresponding NL4.3 Vpu VIVH only showed little effect. The preliminary western blotting data showed the grouped mutants expressed at similar levels (Figure 24E). While the mechanism by which the listed amino acids affect Vpu's activity in different alleles need to be further elucidated.

5.10 Summary of the Results

The results were summarized in the following Table. Downregulation of CD4 and antagonism of CD317/tetherin were well conserved among EC and CP Vpus. IN contrast, inhibition of NF-ƙB signalling was not conserved in both patients. More EC Vpus showed impaired activity to modulate MHCI and NTB-A.

EC	CD4 ↓	CD317↓	MHC-1↓	NTB-A↓	Release assay	NF-ƙB CD317	NF-ƙB IKKβ	HLA C		KIR-associated polymorphism		KIR2DL2
								C1	C2	71M	74H	
CTR29	+	+	+/−	+/−	+	+	+	C0401	C1701			−
CTR53	+	+	−	−	+	−	−	C0202	C0701			u.d.
CTR112	+	+	−	−	+	−	−	C0701	C0602			−
CTR123	+	+/−	+	+/−	+	+	+	C0304	C1202			+
CTR149	+	+	+/−	−	+	−	−	C0304	C0602			−
CTR183	+/−	+	−	−	+	−	−	C0401	C1402	71M	74R	u.d.
CTR185	+	+	+	+	++	+	+	C0202	C1203			−
MEF	+/−	+	−	−	+	−	−	C0205	C0303	71M	74D	u.d.
JLS	+	+	+	+	+	+	−	C04	C12			+
FW041	+	+	+	+	+	+	−	C1601	C1601			u.d.
FW048	+/−	+	+	+/−	++	−	−	C0702	C0401	71M	74D	+
FW057	+	+	+	−	+	−	−	C0102	C0602			u.d.
8227	+	+/−	−	−	−	+	−	C1402	C1801			u.d.
8252	+	+	+/−	+/−	+	−	−	C0401	C0701			u.d.
8260	+	+	+	+	+	−	−	C0802	C1601			u.d.
CP												
E00436	+	+	+/−	+/−	+	−	+	C03	C08	71M	74L	+
E00438	+	+	+	+	++	+	−	C04	C07	71R	74L	u.d.
E00439	+	+	+	−	+	−	−	C05	C15			u.d.
E00440	+	+	+	+	+	+	+	C07	C07	71M	74L	u.d.
E00442	+	+	+/−	+	+	−	−	C03	C04			−
E00444	+	+	+	−	+	+	−	C0202	C06	71M	74D	u.d.
E00449	+	+	+	+	+	+	−	C04	C05			−
E00450	+	+	+	+/−	++	+	−	C01	C07	71M	74D	u.d.
E00452	+	+/−	+	+	+	−	−	C04	C07	71M	74L	+
E00454	+	+	+	+/−	+	+	−	C02	C12	71M	74D	−
E00455	+	+	+	+	+	−	−	C07	C08	71M	74D	−
E00456	+	+	+	+	−	−	−	C02	C04			−
E00462	+	+	+	+	+	+	−	C02	C04	71R	74L	u.d.
E00463	+	+	+	+/−	++	+	+	C04	C07	71M	74D	u.d.
E00466	+	+	+	+	+	+	+	C06	C07			u.d.
E00468	+	+	+	+	+	+	+	C07	C07	71R	74L	u.d.

Table 10: Summary of Vpu alleles function, HLA background and HLA or KIR associated amino acid polymorphisms. The activity of patient derived *vpu* alleles was rated relative to that of NL4.3 Vpu and the following activity classes were defined: CD4 and CD317: ＋: more than 80% of NL4.3 Vpu activity, +/－: 50% - 80% of NL4.3 Vpu activity, －: lower than 50% activity of NL4.3 Vpu. MHC-I and NTB-A downregulation: ＋: more than 75% activity of NL4.3 Vpu, ＋/－: 75% - 50% of NL4.3 Vpu activity, －: lower than 50% activity of NL4.3 Vpu. Virion release: ＋＋: fold increase over NL4.3 vpu > 12, ＋: fold increase over NL4.3 vpu > 3, －: fold increase over NL4.3 vpu < 3. Interference with NF-kB activation by CD317: ＋: equally or more active than NL4.3 Vpu (significant difference to IRESGFP), p > 0.001, －: inactive (not different from IRESGFP, p> 0.01. Interference with NF-kB activation by IKKβ: ＋: significant reduction relative to IRESGFP, p< 0.01, －: no significant reduction relative to IRESGFP, p> 0.01. n.d., not determined.

6 Discussion

The aim of this study was to assess whether there are systematic differences between Vpu alleles derived from EC and CP HIV-1 infected individuals, and further evaluate whether variation of Vpu functions could contribute to host control of virus replication and thus disease progression in EC HIV patients (see summary of all results in Table 10).

We assessed the surface downmodulation of CD4, CD317, MHC-I and NTB-A, as well as enhancement of virion particle release and antagonism of NF-κB signalling by using clonal plasma HIV RNA sequences from 15 EC and 16 CP in the *in vitro* system. We observed that all Vpu alleles were generally functional for the most characteristic activities of CD4/CD317 downregulation and virion particle promotion. In contrast, inhibition of NF-κB signalling was not conserved and showed no significant difference between EC and and CP Vpus. It was the first time that downregulation of MHC-I and NTB-A by patient-derived Vpus was investigated. We showed that EC Vpus displayed higher frequencies of attenuated ones regarding these activities, resulting in significantly lower median activities of EC Vpus than those of CP Vpus. The enrichment of KIR2DL2 footprints in EC Vpus was the most significant sequence difference compared with CP Vpus. Whether the different frequencies of adaption to the KIR2DL2 receptor had an impact on the individual analysed functions or could have other possible but not-yet-studied effects will be discussed. Moreover, natural mutations in Vpus and the effect on functions, correlation of individual activities, and implications of conserved and un-conserved functions will be discussed in the following sections.

6.1 Patient-derived Vpu alleles Displayed Comparable Activities as GFP fusion and non-fusion Proteins

In the beginning, we constructed *vpu* gene to the expression vector pEGFP N1 as fusion aiming to facilitate detection of the expression and the subcellular localization of Vpus. As shown in Figure 19, Vpu.GFP protein was not functional for the virion release enhancement. As a commonly used tag, GFP-associated defects were observed

in actin-myosin interaction, NF-ĸB and JNK signalling (Agbulut, Coirault et al. 2006) (Baens, Noels et al. 2006). Very interestingly, one early report showed that actin-myosin interaction was involved in HIV-1 release (Sasaki, Nakamura et al. 1995), which then could explain how the GFP tag disabled the Vpu-mediated virion particle release. From another point of view, GFP tag may affect this activity of Vpu by stimulating miss-folding or blocking the functional residues of its C-terminus. Indeed GFP tag is very close to the 76W residue which is required for the promotion of virion release.

To evaluate the Vpu-mediated virion release enhancement, we constructed the *vpu* gene to the pIRESGFP expression vector which allows the simultaneous and separate expression of Vpu and GFP. With all pVpuIRESGFP constructs, we performed the analysis of all downregulation activities of Vpu as well. To assess whether the modulation abilities of Vpu alleles were disturbed by the GFP tag, we analysed the correlation for CD4, CD317 and MHC-I downregulation activities of our alleles as Vpu.GFP and VpuIRESGFP proteins. Pearson's correlation (r) was measured for the two data sets of each activity. Both CD4 and CD317 downregulation by all primary Vpu alleles were highly correlated as fusion or non-fusion proteins. MHC-I downregulation showed moderate correlation efficiency ($r=0.38$, $p< 0.05$; Figure 25). Overall, the general trend of CD4, CD317 and MHC-I downregulation mediated by Vpus was similar as fusion or non-fusion proteins, which indicated that the different levels of a specific downregulation ability among Vpus resulted from the inherited property of Vpus. Moreover, this validation analysis supported that the functional comparison of EC and CP Vpus based on Vpu.GFP proteins was valid.

6.2 The Effects of Natural Amino Acid Variations on Functions of Vpus

As shown in Figure 9, sequence of patient-derived Vpus displayed high diversity, especially in the N- and C-terminus, which is identical with former reports

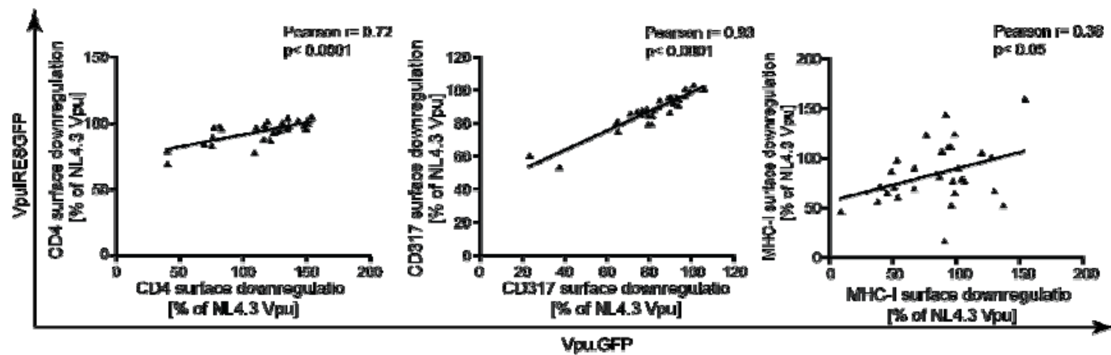

Figure 25: Comparison of CD4, CD317 and MHC-I downmodulation activities by Vpu.GFP and VpuIRESGFP. Pearson correlation coefficient between data sets of CD4, CD317, and MHC-I downregulation by Vpu.GFP (x-axis) and by VpuIRESGFP (y-axis) was calculated by GraphPad prism.

(Pickering, Hue et al. 2014) (Jafari, Guatelli et al. 2014). The natural changes, occurring at the amino acids critical for its functions defined based on NL4.3 Vpu, had some effects on the loss or gain of functions. CTR123, with a mutation at the amino acid 18 from alanine (A) to asparagine (N), was impaired in reducing CD317 surface levels and in enhancing virion particle release. This finding is consistent with former studies showing A18 was required for antagonism of CD317 (Vigan and Neil 2010). An A14L mutation previously displayed impaired ability to antagonize CD317 (Vigan and Neil 2010), while the substitution of A14V or A14G did not show negative effect for any of the functions, such as CTR185 and FW048. The mutations at the residue of A7 only occurred in CP Vpus including E00438, E00444, E00452, E00454, E00455 and E00456. The Neil group showed A7 was one of the residues to interact directly with CD317 (McNatt, Zang et al. 2013). The substitutions of A7 in E00438, E00444, E00454 and E00455 did not result in the loss of function in enhancing viral particle release (Figure 20a), and this may be because other variations in the Vpu protein could compensate the adverse effect. The impairment of E00452 in the promotion of virion release is very likely a result from the mutation of W76R which was identified recently (Jafari, Guatelli et al. 2014). For E00456, the reason is very

likely due to the insertion of three amino acids in N-terminus adjacent to the CD317 interacting domain.

Our results confirmed the role of recently-defined V25 for CD4 downregulation activity (Magadan and Bonifacino 2012). MEF, with a substitution of Isoleucine (I) at position 25, showed suboptimal activity to induce CD4 degradation (Figure 12). However, the replacement of Val by Ala had no effect on E00440. These two observations are consistent with a former study based on NL4.3 Vpu, which demonstrated that the mutation of Val to Iso impair the Vpu-mediated CD4 downregulation by reducing the extent of Vpu-induced CD4 polyubiquitination which is essential for CD4 degradation (Magadan and Bonifacino 2012). We contributed one new residue A48 that is required for optimal CD4 and MHC-I downregulation but not for CD317 downregulation. A48 is located in the end of the first cytoplasmic α-helix responsible for the physical binding with CD4 and adjacent to the β-TrCP binding motif DSGxxS (Margottin, Bour et al. 1998) (Magadan, Perez-Victoria et al. 2010). The mutation at this residue could have an impact on the binding of CD4 or β-TrCP and therefore lead to the impairment of CD4 downregulation activity.

Principally, our sequences and functional analysis confirmed the key residues for each of the Vpu activities. The compensation from other amino acids in a specific allele should be taken account into the overall effect.

6.3 Enrichment of KIR2DL2 Footprints 71M/74H and the Implications

Adaption to KIR2DL2 receptor on NK cells was shown in several regions of HIV-1 genome, especially in the overlapped segment of *vpu* and *env*. The adaption of HIV-1 to the inhibitory receptor KIR2DL2 could enhance the inhibitory ability of KIR2DL2$^+$ NK cells and consequently escape the NK cell killing. In contrast, the viruses containing variations from these adaptions were strongly inhibited by KIR2DL2$^+$ NK cells (Alter, Heckerman et al. 2011). The different frequencies of the adaption to NK cell receptors, together with the specific KIR genotype, are thus very likely to have different impact on the viral replication. However, the sequences and genetic

information in our hands do not support the specific association of KIR2DL2 and 71M/74H as reported before. For example, the alleles derived from KIR2DL2 negative patients, such as CTR29, CTR112, CTR149, E00449 and E00456, have the 71M/74H polymorphisms. In contrast, FW048 and E00436, which were derived from KIR2DL2 positive patients, do not have the adapted 71M/74H footprints.

The most striking difference of amino acid sequences was the higher frequency of KIR-associated polymorphisms at 71M/74H in EC Vpus (12 out of 15) than CP Vpus (5 out of 16) (Figure 9). To test whether the composition of the M/H determines the functional differences, all alleles were grouped according to their genotype. No significant difference was observed for any of the downregulation activities or the inhibition of NF-κB among the Vpus ($p > 0.05$; Figure 26A-E). However, Vpu 71M/74H (+) alleles showed slightly lower activity to reduce CD317 cell surface levels and promote virion particle release than Vpu 71M/74H (-) alleles ($p < 0.05$; Figure 26F-G). Taken together, the 71M/74H polymorphisms generally did not cause loss of functions and do not explain the functional differences observed between EC and CP Vpus. With the genetic background information and data sets, we are not able to define the driving force responsible for the differences between EC and Vpus.

In addition to the KIR-associated polymorphisms in Vpu sequence, they were also extensively found throughout the HIV-1 genome, such as the KIR2DL2-associated footprints in Nef 9S and Gag 138L (Alter, Heckerman et al. 2011). Similar with the effect of Vpu 71M/74H on NK cell recognition, the genotype of Nef 9S or Gag 138L, together with the presence of the KIR2DL2 receptor, could inhibit the killing of HIV-1 by NK cells (Alter, Heckerman et al. 2011). We analysed the available sequences of Nef and Gag proteins from the same two cohorts herein studied. The KIR2DL2 footprints Nef 9S and Gag 138L displayed higher frequencies in EC than in CP (Table 11). It seemed the accumulation of KIR2DL2-associated amino acids is a general property for HIV-1 isolates form EC cohort. Whether the different frequencies have an impact on different viral replication potency in more physiologic conditions need to be further elucidated.

Clinical cohorts	Frequencies of KIR2DL2-associated footprints		
	Vpu 71M/74H	Nef 9S	Gag 138L
Elite controllers	61%	78%	75%
Chronic progressors	31%	57%	63%

Table 11: Frequency of KIR2DL2-associated footprints in ECs and CPs proteins
The ratio was calculated from the sequences with KIR2DL2-associated amino acid(s) out of all available sequences. (All sequences were provided by Takamasa Ueno (Center for AIDS Research, Kumamoto University, Japan)

Even though the observed differences in the Vpu function is not a result from the 71M/74H polymorphism, the different levels of 71M/74H conservation may have an impact on the efficiency of immune responses in EC and CP patients due to different levels of adaption to immune pressure(s). Among the Vpu alleles studied, the majority of the differences concerned H74, which is the first residue of the HLA-Cw1 restricted epitope HL9 of Vpu (HAPWDVDDL) (Liu, McNevin et al. 2007) (Liu, McNevin et al. 2011). As known, HLA-Cw1 is the ligand of KIT2DL2 receptor of NK cells (Wagtmann, Rajagopalan et al. 1995) (Symons and Fuchs 2008) (Moesta, Norman et al. 2008) and HLA-C-presented peptides have an impact on the binding and stablization with the KIR2DL2 (Snyder, Brooks et al. 1999) (Blais, Dong et al. 2011). In this scenario, the alteration at 74H may influence the interaction of HLA-Cw1 and KIR2DL2 and thus lead to the inhibition or the activation of NK cells, which needs to be further characterized.

From another point of view, higher mutation frequency of CP 74H could just facilitate the escape of HLA-Cw1-mediated CTL killing, and this may contribute to the establishment of server HIV-1 infection in CP patients. Interestingly, H74 is highly enriched in transmission-founder viruses (present in 100% of founder virus sequences vs. 53% of subtype B sequences), indicating that the underlying selection pressures may be particularly relevant during the acute phase of HIV infection.

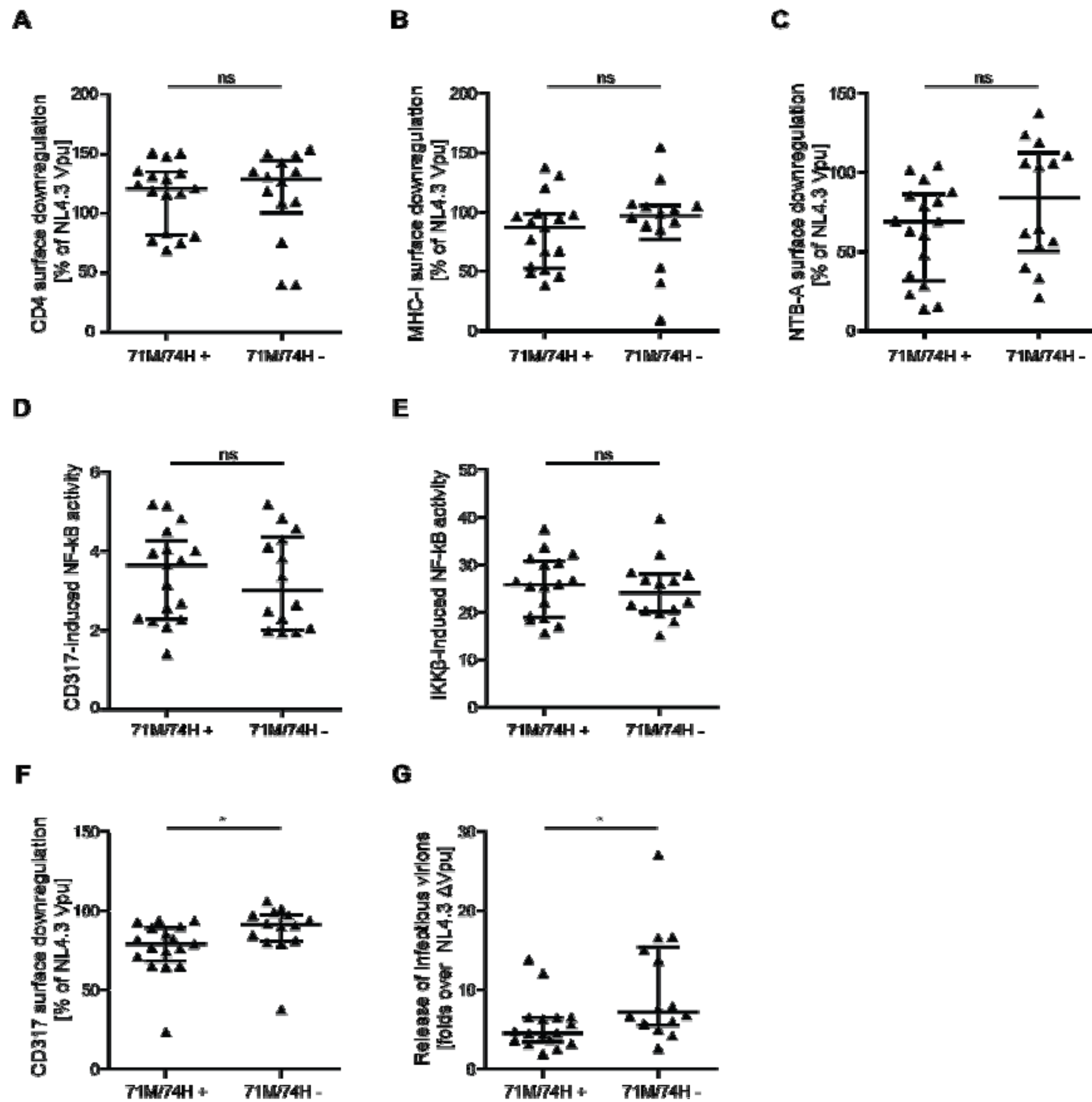

Figure 26: Comparison of activities mediated by Vpu 71M/74H positive and negative alleles. A-C, F: Comparison of CD4, MHC-I, NTB-A and CD317downregulation activities between Vpu 71M/74H positive and negative alleles. D, E, G: Comparison of inhibition of NF-κB signalling and enhancement of virion particle release mediated by Vpu 71M/74H positive and negative alleles. Statistical significance was assessed using two-tailed Mann–Whitney U-test, bars represent median and interquartile ranges.

6.4 Moderate Association between Downregulation Activities of Aatient-derived Vpus

For all the activities imposed by natural Vpus, we evaluated their correlation with each other by measuring the Spearman R. The correlation between activities of

CD317 and MHC-I downregulation was significant (R= 0.69, p< 0.0001; Figure 27E), which suggests that Vpu may use similar mechanisms to downmodulate these two molecules. Vpu reduces the cell surface levels of CD317 by perturbing anterograde transport of newly synthesized CD317 and retaining CD317 molecules in Golgi-apparatus(Schmidt, Fritz et al. 2011). The mechanism of Vpu-mediated MHC-I downregulation has not been elucidated yet, but it was hypothesized that Vpu acts on newly-synthesized MHC-I molecules in ER and directs them for proteasomal degradation as how Vpu induces CD4 surface downregulaton (Kerkau, Bacik et al. 1997). While the correlation between CD4 and MHC-I downregulation was intermediate (R= 0.52, p=0.003; Figure 27B). Taken together, our data suggests that Vpu may interfere with the trafficking of MHC-I similarly with CD317 in Golgi-apparatus.

There was mild correlation between CD4 and CD317 downregulation (R= 0.41, p=0.02; Figure 27A), which is reasonable and not surprising from the knowledge based on the studies of the lab-adapted NL4.3 Vpu. The mechanisms of Vpu-mediated CD4 and CD317 surface level reduction are different as described above, but some of the amino acids or motifs are required for both of the activities. The determinants located in Vpu TMD, such as W22, A14 and A18 are essential for the downregulation of CD4 and CD317. Our data generated from natural Vpus principally supports that these two activities of Vpu are separable.

The correlation between NTB-A and MHC-I downregulation was significant (R= 0.53, p=0.002; Figure 27F), which may reflect Vpu over-layered mechanisms for these two activities. Vpu was shown to slow down the anterograde transport of the NTB-A and retain it in the Golgi-apparatus, distinct from the block of anterograde transport of CD317 (Bolduan, Hubel et al. 2013). Our two sets of correlation analyses suggest Vpu

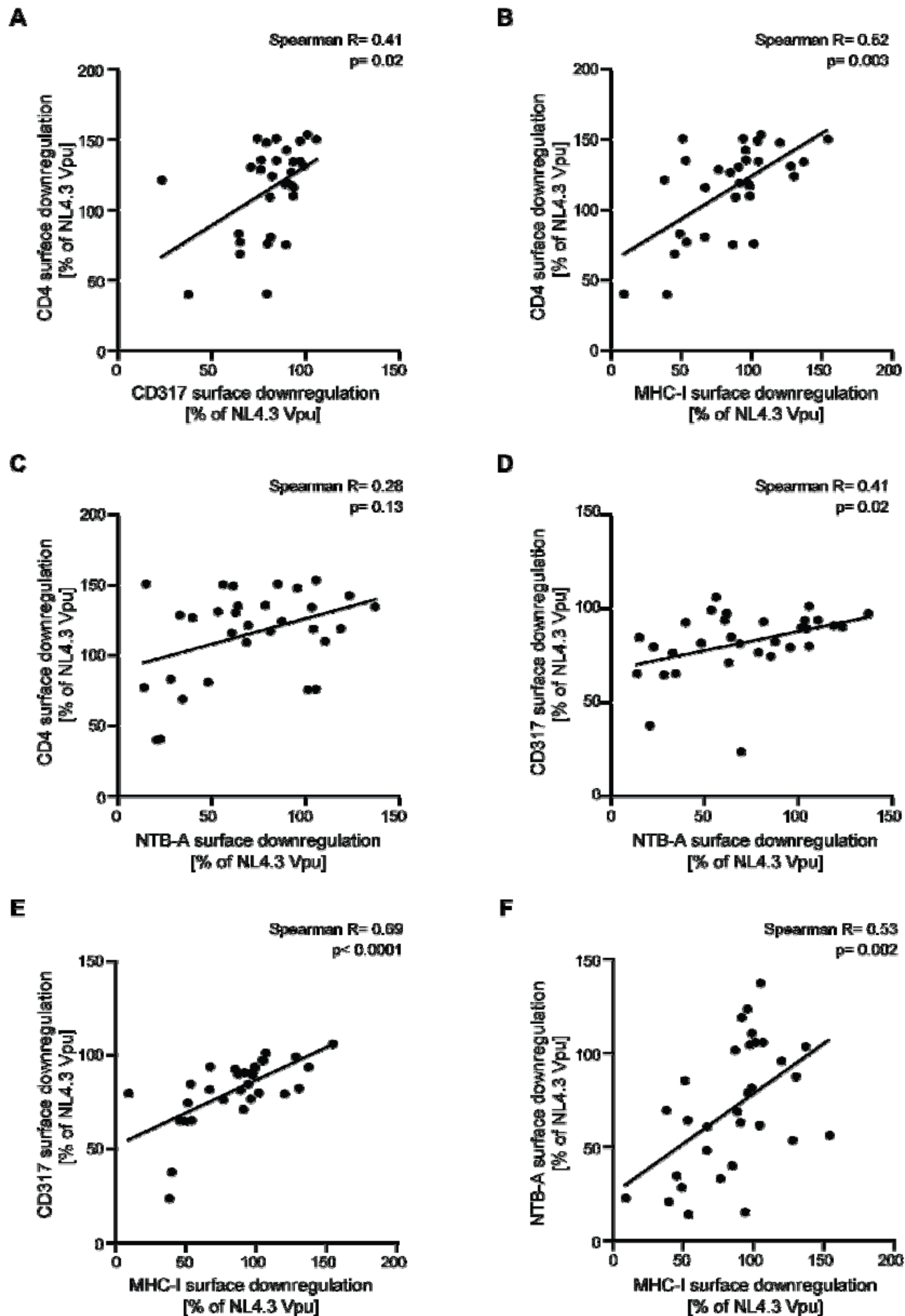

Figure 27 Correlation between different functions of patient-derived Vpus. Correlation of Vpu functions were assessed by the strength of the spearman R coefficient. **A-C**: Correlation of CD4 with CD317, MHC-I or NTB-A. **D, E**: Correlation of CD317with MHC-I or NTB-A. **F**: Correlation of MHC-I with NTB-A.

may also affect the trafficking of MHC-I molecules as it does for CD317 or NTB-A. While since Vpu-mediated MHC-I and NTB-A downregulation are not as potent as the other activities, the correlation between them could be simply from the narrow dynamic range. Interestingly, there was no or very mild correlation between NTB-A and CD4 or CD317 downregulation (Figure 27C, D), which reflects the distinct mechanisms as described above. All together, the varying degrees of correlation reflect that Vpu uses different structural and functional domains and mechanisms to modulate the surface levels of these molecules.

6.5 Conservation and Variation of Functions Mediate by EC and CP Vpus

6.5.1 Implications from Conservation of Vpu-mediated CD4 Degradation and Difference between EC and CP Vpus

Both EC and CP Vpus had conserved abilities to down-regulate CD4 despite high sequences diversity (Figure 12, 14). The Neil group also showed the conservation of CD4 downreguation activities of Vpu alleles derived from different clinical stages of 15 HIV-1 infected individuals including rapid progressors, normal progressors and long-term non-progressors at different stages (Pickering, Hue et al. 2014). Similar observations were made in a cohort of acutely infected patients (Jafari, Guatelli et al. 2014). This conservation may reflect that surface removal of HIV-1 CD4 receptor is strictly required for the preservation and persistence of HIV-1 in the infected individuals and even in EC patients who can naturally control HIV-1 replication but not elimination. The importance of CD4 down-modulation for HIV-1 fitness in the *in vivo* environment is also reflected by the fact that HIV-1 utilizes a combination of three proteins Nef, Vpu and Env to diminish its primary receptor CD4 molecules, and each activity implies different relevance for HIV-1 survival according to their respective expression patterns (Chen, Gandhi et al. 1996) (Wildum, Schindler et al. 2006). Vpu acts on newly-synthesized CD4 molecules in the relatively late stage of HIV-1 life cycle, which was proposed to be critical for liberating Env from the Env-CD4 complex in the ER and thus facilitating the Env processing and viral

production and infectivity (Willey, Maldarelli et al. 1992) (Lama, Mangasarian et al. 1999) (Levesque, Zhao et al. 2003) (Gautam and Bhattacharya 2013). In contrast, Nef plays the major role on reducing CD4 surface level by enhancing CD4 internalization from the plasma membrane and subsequent degradation in the early phase of HIV-1 infection (Piguet, Chen et al. 1998), which is important for effective viral replication by preventing superinfection (Wildum, Schindler et al. 2006).

The evidences from cohorts studies showed that HIV-1 Nef alleles from long term non-progressors were attenuated in down-regulating CD4 molecules (Tobiume, Takahoko et al. 2002) (Mwimanzi, Markle et al. 2013). Moreover, the study performed on pig-tailed macaque showed that Vpu-mediated CD4 degradation is associated with pathogenesis of HIV-1 (Stephens, McCormick et al. 2002). Our EC Vpu alleles maintained lower ability to decay CD4 surface expression levels than CP Vpu alleles. With all these evidences, we could deduce that full CD4 down-regulation by Vpu is very likely to add the strength of HIV-1 to combat with the host, and the different potency to remove CD4 from the cell surface may cause different clinical outcomes.

6.5.2 Benefits from the Conservation of CD317 Antagonism by Vpus

Vpu is the only protein that HIV-1 exploits to antagonize the host restriction factor CD317 by reducing its surface expression or displacing it from nascent virion particles at the plasma membrane (Neil, Zang et al. 2008). Surface downregulation of CD317 and promotion of virion particles were well conserved among EC and CP alleles, which is again identical with other cohorts studies (Pickering, Hue et al. 2014) (Jafari, Guatelli et al. 2014). Our data suggest surface removal of CD317 was not strictly required for enhancing virion release (Figure 20a, 20b), which indicates that Vpu-mediated virion particle release and CD317 downregulation are separable activities. How HIV-1 could benefit from these two Vpu-mediated functions is unknown. Recent reports provide new interpretations for the functional relevance and importance of CD317 antagonism during HIV-1 infection. CD317 molecules could

provide a platform for host NK cells to recognize the retained HIV-1 virions from the infected cell surface and facilitate the recognition and killing by NK cells via ADCC (Arias, Heyer et al. 2014) (Pham, Lukhele et al. 2014).

The *in vivo* relevance of virion release was puzzling in the beginning because cell-free virions are not the only way for HIV-1 spreading. It is even more efficient for HIV-1 spreading from cell to cell via virological synapses (Chen 2012). However, it was elucidated later that the quantity of cell free virions were associated with the efficiency of virion distribution and expansion in a humanized mice model, by this way the virion propagation was boosted especially in the early phase (Sato, Misawa et al. 2012). All together, the conserved abilities of CD317 surface downregulation and virion release enhancement among Vpus derived from different clinical outcomes just implies the important role of these conserved activities in the establishment and persistence of HIV-1 infection.

6.5.3 Vpu-mediated MHC-I Down-modulation and its Functional Consequences

MHC-I is responsible for loading pathogen-derived peptides and presenting them to Cytotoxic T lymphocytes (CTLs). Interference with MHC-I antigen-presentation is a general strategy used by many viruses to escape the CTL killing, such as HIV-1, HCV and HSV (Yewdell and Bennink 1999). HIV-1 Nef-mediated MHC-I downregulation and its consequences were well studied. In the presence of HIV-1 Nef, CTL killing is inefficient due to reduced MHC-I surface expression levels (Collins, Chen et al. 1998) (Petersen, Morris et al. 2003). Furthermore, CP Nef alleles from the same cohort studied herein displayed significantly higher capacities to down-regulate MHC-I surface expression level than EC Nef alleles (Mwimanzi, Markle et al. 2013). No further studies were followed to elucidate the functional relevance and the mechanism since the first report about HIV-1 Vpu-mediated MHC-I downregulation (Kerkau, Bacik et al. 1997). Our data showed that most EC Vpus was attenuated in MHC-I downregulation compared with our reference NL4.3 Vpu or majority of CP Vpus (Figure 16, 17). The relatively stable and higher MHC-I level may be advantageous to

the control of HIV-1 because more viral peptides can be loaded and presented on the infected cells surface via MHC-I and should consequently lead to more efficient CTL responses. And indeed the antigen loading is associated with disease progression. From this point of view, the potent downregulation of MHC-I by CP Vpus may help HIV-1 infected cells to escape CTL surveillance and therefore allow disease progression. Whether the attenuated MHC-1 downregulation by EC Vpus is the consequence or one of the reasons for natural HIV-1 suppression or due to the interaction is complicated to be investigated.

6.5.4 Modulation of NTB-A: Selection Pressure from Innate Immunity

A listing of increasing evidences support that the accessory protein Vpu is widely involved in the interference with NK cells functioning during HIV-1 infection (Shah, Sowrirajan et al. 2010) (Matusali, Potesta et al. 2012) (Arias, Heyer et al. 2014) (Pham, Lukhele et al. 2014). Apart from the adaption to specific KIR receptors to avoid the recognition of NK cells, Vpu has the capacity to modulate NK cell ligands including NTB-A and PVR on the surface of HIV-1 infected cells and thus disturbs the recognition and killing by NK cells (Shah, Sowrirajan et al. 2010) (Matusali, Potesta et al. 2012). However, whether these two activities of Vpu are conserved among natural Vpu alleles has not been investigated before. In this study we assessed NTB-A modulation by patient-derived Vpu alleles. Our data displayed that down-modulation of NTB-A was not conserved among natural Vpu alleles. The majority of EC Vpu alleles were attenuated but most of the CP Vpu alleles still were shown optimal or suboptimal activity in modulating NTB-A molecules (Figure 18). It is unknown how the autologous NK cells will react to decreased surface levels of NTB-A ligands in HIV-1 infected cells. Very likely, CP Vpus may have stronger potency to inhibit the activation of NK cells than EC Vpus by suppressing the activating factor-NTB-A, which is logically right at the time point of sample collection when all patients had established infection status.

The variations observed in NTB-A downregulation activity may also indicate that the

patient-derived Vpu alleles were undergone different degrees of immune pressure(s) imposed by NK cells. And indeed it was proposed that NK cells are involved in the immune control of HIV-1 infection (Iannello, Debbeche et al. 2008). NK cells may play a central role in the acute stage of the HIV-1 infection because the drop in the VL of patients occurs before the CTL response is fully activated (Alter, Teigen et al. 2007), and HIV-1 has undergone selection pressure imposed NK cell at this stage prior to entering to the chronic phage when our samples were collected. The variations of Vpu-mediated NTB-A may just reflect the different potency of NK cells selection of each patient. Even though the phenotypic background of the NK cells in our cohorts is unknown, the attenuated NTB-A downregulation activities by EC Vpu alleles may be a result from strong NK cells pressures, and the impairment of Vpu in turn contributes to the suppression of HIV-1 infection.

6.5.5 Vpu-mediated inhibition NF-ƙB activation: An agonist or antagonist?

As a central transcriptional factor for regulation of a specific gene expression, NF-ƙB plays a pivotal and dynamic role in innate and adaptive immunity upon virus infection (Gilmore 2006) (Ghosh and Hayden 2012). Induction of NF-ƙB is a double-edged sword for it can enhance HIV-1 replication and also induce gene expression of antiviral factors (Hiscott, Kwon et al. 2001) (Chan and Greene 2012). The HIV-1 infection can be sensed by a diversity array of pattern recognition receptors (PPRs) in the immune system, which leads to the activation of NF-ƙB (Hiscott, Kwon et al. 2001). The virion release restriction factor CD317 can also serve as a PPR to stimulate NF-ƙB signalling and consequently induces the downstream proinflammatory responses (Galao, Le Tortorec et al. 2012) (Tokarev, Suarez et al. 2013). Vpu-mediated surface downregulation and viral antagonism of CD317 were well conserved among natural alleles, while half of our Vpu alleles, no matter derived from ECs or CPs, lost the inhibitory effect on CD317-induced NF-ƙB activation (Figure 21). The variations of Vpu-mediated inhibition of CD317-induced NF-ƙB signalling were also observed from Vpu alleles collected from CPs and long-term

non-progressors (LNTP) from the point of initial infection to five years during HIV-1 infection, which was reported by the Neil group (Pickering, Hue et al. 2014). Among the patients they studied, Vpus, derived from 3 out of 4 CPs and 3 out of 5 LTNPs, displayed significant decreasing capacities to inhibit NF-ƙB signalling. But there were also Vpu alleles derived from two patients showing increased activities to suppress NF-ƙB signalling. All these cohort studies indicate that inhibition of NF-ƙB signalling can be fine-turned depending on the stage of the infection and the micro-environment in a specific patient.

Our Vpu alleles were inactivated to intercept IKKβ stimulated NF-ƙB activation. This might be due to the CMV promoter in our constructs, which was reported that the expression of constructs with CMV promoter was disturbed in the presence of interferon (Harms and Splitter 1995). In our system, the production of interferon can be triggered by the over-presence of IKK-β.

Regarding the mechanisms, it was proposed that CD317-mediated restriction of virion release and stimulation of NF-ƙB signalling are separable activities because they require different structural domains of CD317 (Galao, Le Tortorec et al. 2012) (Tokarev, Suarez et al. 2013) (Sauter, Hotter et al. 2013). This fact adds the complexity of the mechanism of how Vpu antagonize CD317-induced NF-ƙB activation, which is not defined yet. The study from scanning the molecular determinants revealed that these two activities of Vpu are uncoupled (Pickering, Hue et al. 2014). The Neil group showed that the motif such as E29 required for the promotion of virion particle release was not needed for suppression of NF-ƙB activation, but the mutations at 59G and E62 were defect for inhibition of NF-ƙB activation but not enhancement of virion particle release. While both activities involved residue A18 located in TMD of Vpu. CTR123, with a substitution of asparagine (N) at 18, was inactive for the antagonism of CD317. For the rest of alleles, the molecular determinants rendering the loss of ability to suppress NF-ƙB activation need to be further identified.

Overall, our result suggests that Vpu-mediated inhibition of NK-ƙB signalling is not a must for the persistence of the HIV-1 infection at the time point of sample collection.

However, this does not exclude the importance of Vpu-mediated inhibition of NF-ƘB signalling in the early stage of the HIV-1 infection. Since NF-ƘB activation could benefit both HIV-1 replication and host antiviral immune responses, it is not surprising that the inhibitory effect of Vpu alleles on NF-ƘB is quite various, which is probably dependent on the stage of the infection and the immune environment of the patient. The loss or gain of this function may be associated with the kinetics of HIV-1 replication or host antiviral responses.

6.6 EC Vpu alleles may be more conserved under CTL pressure

Unlike HIV-1 accessory protein Nef, which has partially overlapped functions with Vpu and is intensively targeted by CTLs (Schmokel, Sauter et al. 2011) (Price, Goulder et al. 1997) (Leslie, Kavanagh et al. 2005). HIV-1 Vpu was rarely targeted by CTLs and the sequence diversity does not majorly result from the CTL-associated selective pressures (Addo, Yu et al. 2002) (Addo, Altfeld et al. 2002) (Altfeld, Addo et al. 2001) (Hasan, Carlson et al. 2012). However, at least four Vpu-derived epitopes, such as LAIVALVVA, EYRKILRQR, ALVEMGHHV and HAPWDVNDL, which are spanning from the transmembrane domain and the end of cytoplasmic tail (HIV lanl database), can be recognized by HIV-1 specific CD8+ T cells and elicit immune responses. Among them, the HLA-Cw0102 restricted CTL epitope HAPWNVND showed a significantly higher variation frequency in CP Vpus than EC Vpus (Figure 9). Our data showed the variation in this epitope did not cause the loss of Vpu functions but rather slightly higher capacity to antagonize CD317 than the Vpu alleles with founder epitope, which is consistent with previous study that the epitope of HAPWNVND was undergone positive selection which is no cost for viral fitness and beneficial to HIV-1itself (Liu, McNevin et al. 2007) (Liu, McNevin et al. 2011).

The dynamics of CTL responses and the evolution of this epitope were assessed over the first four years of HIV-1 infection in an ART-naïve patient. This founder epitope was undergone positive selections, and the CTL responses declined dramatically around 400 days post onset of the primary infection due to the epitope evolution (Liu,

McNevin et al. 2011). So the maintenance of this epitope keeps the consistent effect of initially developed CTLs which is critical for the control of HIV-1 infection. Under this scenario, frequent variations in this CTL recognition epitope may reduce the recognition of HIV-1 infected cells in CP patients. While the conservation of EC Vpus CTL epitope may facilitate the CTL responses and thus contribute to the undetectable viral loads status.

In summary, functions of Vpu were generally conserved among all patient-derived alleles except for the antagonism of NF-ĸB activation. All of the experiment data sets and analysis showed slight systematic differences between EC and CP Vpu alleles regarding CD4, MHC-1 and NTB-A downregulation based on our rather small sample size. The driving force for these differences is undefined but not due to the KIR associated polymorphisms. The functional relevance of these differences needs to be elucidated in more relevant or physiological conditions.

7 Perspectives

To give more insight into the unsolved issue whether Vpu is a pathogenic factor of HIV-1 or not, we compared the sequences and functions of Vpu alleles derived from two distinct and treatment naïve HIV-1 clinical groups. Our results, together with the studies performed in the cell culture system, support that Vpu enhances the persistence and pathogenesis of HIV-1, even though it is not strictly required for the viral replication in vitro. Based on my knowledge, several aspects are proposed for further investigations into Vpu.

7.1 Implications from the Studies of Animal Models

Recently, the Bieniase group showed HIV-1 could establish AIDS like phenotype by successively passaging CCR5 strain HIV-1 in the pigtailed macaque and with the treatment of CD8 antibody during the acute infection, which has never been achieved previously. The adaption of Vpu to the macaque CD317/tetherin is one of the key factors to establish that phenotype (Hatziioannou, Del Prete et al. 2014). Their model

provides direct evidences that the viral adaption or genotype, as well as the immunological events during early infection, are crucial for the disease outcomes. In line with this, the role of HIV-1 genetics is proposed as important as host factors in the pathogenesis and virulence of HIV-1 (Fraser, Lythgoe et al. 2014).

The TMD of Vpu is critical for the acquired ability to antagonize CD317/tetherin, as the Bieniase group observed. However, the DSGxxSG motif, which is essential for its functions, is not studied. It could be worthwhile to introduce the Vpu S/A mutant into the macaque-adapted HIV-1 strain to assess the significance of the DSGxxSG-related functions using their model.

7.2 Biological and Clinical Relevance of KIR-associated Footprints in Vpu alleles

The Vpu 71M/74H polymorphism has an effect on the NK cell killing through the interaction with specific type of KIRs. Based on our sequence information and data sets from all functional analysis, the 71M/74H footprints do not explain the impaired or optimal functions of Vpu alleles. To further look into the relevance of biological and clinical relevance of KIR-associated footprints in Vpus, we are trying to obtain the genetic information of each patient. First, it's necessary to check if the adaption to KIR2DL2 is also true for our cohorts. In our EC cohort, not only for Vpu, but also in Gag and Nef, the frequency of adaption to a specific KIR receptor is generally lower compared with those from CP cohort (Table 8). If there is a strict adaption in our cohorts, then we could evaluate the possible consequences of the adaption in each patient. If the adaption to KIR2DL2 does not fit our cohorts, the higher frequency of Vpu with 71M/74H in EC might come from other immune selection pressures, which can only be elucidated by further epidemiological and experimental data.

7.3 Critical Motifs of Vpu-mediated Inhibition of virion sensing

Vpu is the only protein that HIV-1 exploits to antagonism CD317 which functions as both the restriction factor and virion sensor. These two activities of Vpu is not well correlated, and we can get some hints from our alleles. E00456, which is active for

CD317 surface downregulation but inactive for recovery of virion release enhancement and inhibition of NF-ĸB, has around three amino acid insertion in its N-terminal compared with clade B HIV-1 isolates. This insertion might be responsible for its inability for these two activities. Fw048 is a good example for the discrepancy of these two activities, which was active for recovery of virion release enhancement but inactive for inhibition of NF-ĸB signalling, the 61K could be one of the reasons. The two mentioned candidate motif, together with the relatively variable C-terminal, are potential determinants for inhibit NF-ĸB signalling in my opinion. Further study can be done to verify these motifs and the mechanism.

7.4 Disability of NK cell Degranulation by EC and CP Vpus

NK cell degranulation is triggered via the interactions of activation receptors and co-activation receptors with their specific ligands .This process releases cytotoxic granule contents towards the bounded target cells and is of central importance in NK cell-mediated killing. However, NK cell degranulation is compromised in HIV-1 infection. The reason behind this is that Vpu blocks the transport of the co-activator NTB-A to surface of infected cells. Since the Vpu-mediated inhibition of NK cell degranulation and lysis is a prominent phenotype from lab-adapted Vpu, and there is a significant difference between EC and CP Vpu alles regarding this activity, it might be worthwhile to perform further functional assays to check: 1) Could all patient-derived Vpu alleles inhibit the NK cell killing? 2) If the Vpu-mediated inhibition of NK cell killing is associated with viral loads or other clinical parameter or patient genetic information or not? 3) Is the NTB-A down-modulation between EC and CP correlated with differentiated NK cell cytolytic ability?

7.5 Interference of ADCC by Vpu: a Potential Candidate for Vaccine Design?

As mentioned in the introduction, ADCC showed protection in macaques and in the Thai trail (Gomez-Roman, Patterson et al. 2005) (Florese, Demberg et al. 2009) (Haynes, Gilbert et al. 2012). The higher ADCC antibody titres and broader ADCC responses may partially contribute to the viral suppression in EC (Baum, Cassutt et al.

1996) (Lambotte, Ferrari et al. 2009). Vpu derived peptides were shown to induce IFN-γ expression of NK cells as well as Env-derived peptides (Stratov, Chung et al. 2008) (Johansson, Rollman et al. 2011). And this property of Vpu may put it as a candidate for vaccine design. It would be very interesting to uncover the following issues: 1) Define the peptides eliciting ADCC antibodies in each individual Vpu alleles and compare whether there are amino acids different among the known peptides, especially in C-terminal 19mer one. It was shown even one single amino acid change in C-terminal of Vpu could totally abort NK cell-mediated ADCC responses (Stratov, Chung et al. 2008). 2) Evaluating the magnitude and breadth of ADCC response elicited by potential patient-derived Vpu peptides. 3) Systematically compare the difference between EC and CP groups regarding the peptides that can be recognized and evaluated the contribution of Vpu-elicited ADCC for viral control.

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