MECHANISMS OF IMMUNE EVASION

MEDIATED BY HIV-1 VPU

by

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ABSTRACT

Human Immunodeficiency Virus Type 1 (HIV-1), the causative agent of AIDS, encodes four proteins (Nef, Vif, Vpr and Vpu) that have evolved specific roles in promoting efficient viral replication and dissemination. A key attribute of these viral factors is their ability to interfere with multiple host defense mechanisms through one of two ways: manipulation of the ubiquitin proteasome system (UPS) or altered intracellular protein trafficking.

In particular, Vpu is a small integral membrane protein that is expressed late in the viral life cycle and found only within HIV-1 and some related simian immunodeficiency virus (SIV) isolates. Vpu antagonizes multiple cellular targets that are involved in innate and adaptive immunity, including the restriction factor BST-2, a family member of cellular "intrinsic" proteins that serve to restrict viral replication immediately following viral infection. In this work, we show that Vpu downregulates the chemokine receptor CCR7 on the surface of HIV-1-infected primary $CD4^+$ T cells by sequestering the protein in a perinuclear compartment (the *trans*-Golgi Network: TGN). This compromises the migratory potential of T cells in a CCR7-dependent manner and may have major implications in HIV-1 pathogenesis.

Additionally, we clarify the mechanisms by which Vpu relies on cullin-RING Ligases (CRLs), one of the largest classes of E3 Ubiquitin Ligases within the UPS, to downregulate host proteins from the cell surface. Through the use of a neddylation

inhibitor, a post-translational modification necessary for CRL activity, we establish that Vpu-mediated BST-2, CCR7 and Natural T and B cell antigen (NTB-A) downregulation is CRL-independent. This provides further support that Vpu is a multifunctional accessory protein that has evolved several ways to interfere with its cellular targets.

To all those that will come after me, believe in yourself and anything becomes possible.

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CHAPTER 1

MODULATION OF INTRINSIC, INNATE, AND ADAPTIVE IMMUNE FACTORS BY HIV-1 VPU

1.1 Introduction

After viral entry into cells, viruses must subvert the cellular landscape in order to provide a favorable environment conducive for producing new progeny. As intracellular obligate parasites, viruses utilize host cellular machinery to effectively transcribe their genome, translate the RNA into proteins, and assemble the components into virions for the infection of new target cells. In most cases, viral replication and dissemination can be controlled and eventually cleared through the effects of the host immune system. Pathogens such as Human Immunodeficiency Virus (HIV), however, have evolved specific countermeasures to evade host immunity, leading to chronic life-long infections that are difficult if not impossible to eradicate.

Genomically, all retroviruses encode common structural and enzymatic genes required for cell entry, reverse transcription, integration, proteolytic processing and viral packaging (Gag, Pol, and Env) (Kirchhoff, 2010). Members of the more complex Lentivirus family, which includes HIV, encode additional genes. Some of these are regulatory, functioning as transcriptional regulators or assisting with the nuclear export of viral mRNAs (Tat and Rev). The other remaining genes (*nef*, *vif*, *vpu*, *vpr,* and / or *vpx*) encode for "accessory" proteins that, in some cases, are dispensable for viral infection *in vitro*. Instead, accessory proteins are essential in evading the host immune response *in vivo*, leading to efficient viral persistence and pathogenesis (Malim and Emerman, 2008). The acquisition of certain accessory genes within HIV and other primate lentiviruses most likely reflects an evolutionary tactic to enhance species-specific viral fitness against a number of host-selective pressures, including proteins that immediately act on inhibiting viral replication and dissemination ("restriction factors"). As such, all

accessory proteins possess an inherent ability to serve as viral adaptors, whereby they can recruit specific cellular complexes to assist in antagonizing host immune proteins and/or modify the host environment to maintain effective immune evasion (Collins and Collins, 2014).

Among one of the most common viral counteraction mechanisms is subversion of the ubiquitin proteasome system (UPS). Within eukaryotic cells, the UPS is essential in regulating protein degradation to maintain cellular homeostasis. Typically, proteins are "marked" for degradation through the post-translational addition of ubiquitin onto lysine, serine, or threonine residues, resulting in polyubiquitin chains that provide a signal for the protein to be degraded by the 26S proteasome (Soucy et al., 2009). The process of ubiquitination involves three main steps and is carried out in an enzymatic fashion. First, ubiquitin is activated by ubiquitin-activating enzyme (UAE/E1) in an ATP-dependent manner (Haas and Rose, 1982). Second, the activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2) via a transthiolation reaction. Finally, an E2 can associate with an ubiquitin ligase (E3) to mediate the polyubiquitination of a target substrate recruited by the E3.

One of the largest and best-characterized E3 ubiquitin ligase complexes are Cullin RING Ligases (CRLs). CRLs play a role in regulating many diverse cellular processes, some of which include transcription, signal transduction, development, and multiple aspects of the cell cycle (Bosu and Kipreos, 2008). CRL complexes consist of a cullin scaffold (humans possess seven cullins) onto which a substrate receptor, substrate adaptor, and RING-box protein 1 (Rbx1) can load (Petroski and Deshaies, 2005). An E2 can then interact with Rbx1 to facilitate the transfer of ubiquitin onto the substrate

(Figure 1.1).

Activation of CRLs is accomplished through the addition of the ubiquitin-homologous NEDD8 (neural precursor cell expressed developmentally down-regulated protein 8) onto cullins, a post-translational process termed neddylation (Rabut and Peter, 2008). This induces a conformational change within cullins whereby they can assemble into a full CRL complex through dissociation from CAND1 or GLMN1, which holds them in an inactive-bound form (Pierce et al., 2013; Tron et al., 2012; Zheng et al., 2002). Conversely, CRLs can be inactivated though deneddylation, a function of the eight-subunit COP9 signalosome (CSN) complex (Schmaler and Dubiel, 2010).

With regards to HIV, accessory proteins can act as viral adaptors to recruit CRLs for the targeted degradation of specific proteins. Usually, these include restriction factors that serve as an "intrinsic" first line of defense against viral infection. For instance, HIV-1 Vif recruits a cullin5-containing (CRL5) complex to induce the proteasomal degradation of APOBEC3G (apolipoprotein G mRNA-editing enzyme, catalytic polypeptide-like 3G) (Yu, 2003). In the absence of Vif, APOBEC3G induces lethal G to A hypermutations within the retroviral genome due to its cytidine deaminase activity, severely impairing viral replication (Harris et al., 2003; Mangeat et al., 2003; Sheehy et al., 2002; Zhang et al., 2003). Similarly, the HIV-2 Vpx protein recruits a cullin4-containing (CRL4) complex to induce SAMHD1 (SAM domain and HD domain-containing protein 1) proteasomal degradation (Hrecka et al., 2011; Laguette et al., 2011), restoring the intracellular pool of deoxynucleoside triphosphates for efficient cDNA synthesis (Lahouassa et al., 2012). Furthermore, HIV-1 Vpu degrades CD4 by linking the protein to a cullin1-containing (CRL1) complex to increase viral fitness (Figure 1.1)

(Margottin et al., 1998).

This chapter will provide an overview of the recent progress within the Vpu field and our current understanding of the strategies employed by this multifunctional viral factor to utilize both CRL-dependent and -independent mechanisms to promote immune evasion and pathogenesis.

1.2 Acquisition of Vpu and HIV phylogeny

Lentiviruses chronically infect a wide variety of mammalian species, including primates, felids, and wild and domesticated ungulates. In particular, primate lentiviruses are found in over 40 different African nonhuman primates (NHP) (Hahn et al., 2000; Pandrea et al., 2008), and current knowledge proposes that *vpu* was obtained from an SIV (Simian Immunodeficiency Virus) presently found within some members of the *Cercopithecus* family (Bailes et al., 2003). This *vpu*-containing SIV was then transmitted to chimpanzees and formed a hybrid with an SIV ancestor found within red-capped mangabeys (Bailes et al., 2003). Due to overlapping territorial ranges (Central and West Africa) and the predatory relationship chimpanzees have with red-capped mangabeys, it is likely that a chimpanzee was co-infected with both simian viruses. Consequently, viral recombination may have occurred, resulting in the emergence of SIV_{chz} (*Pan troglodytes troglodytes: Ptt*). This subsequently led to viral spread among chimpanzees, transmission into humans and gorillas, and the eventual appearance of HIV-1 and SIV_{gor} (*Gorilla gorilla gorilla*) (Gao et al., 1999; Sharp and Hahn, 2010).

There are two types of HIV that cause AIDS within humans: HIV-1 and HIV-2. HIV-1 consists of four lineages: Group M ("Main" – the major cause of the worldwide AIDS pandemic), Group O ("Outlier"), Group N ("Non M, Non O"), and Group P. Each

lineage arose through independent zoonotic (cross-species) transmission events from either SIV_{chz} or SIV_{gor} (Group P) (Plantier et al., 2009). HIV-2 emerged through cross-species transmission from a retrovirus within sooty mangabeys (SIV_{sm}) and is mainly restricted to West and Central Africa (along with HIV-1 Groups N, O and P) (Hahn et al., 2000; Sharp and Hahn, 2010).

1.3 Vpu expression and structure

Vpu and envelope (*Env)* are expressed from a single bicistronic mRNA, whereas all other HIV-1 products are produced through a complex pattern of alternative splicing or proteolyic processing by the Gag and Pol polyproteins (Schwartz et al., 1990). The Vpu gene product encodes for an 81-amino-acid type 1 integral membrane phospho-protein that contains two major structural domains: an N-terminal hydrophobic membrane anchor (residues 1-27) and a large 54-residue C-terminal amphipathic region that extends into the cytoplasm (Figure 1.2) (Maldarelli et al., 1993; Strebel et al., 1988). Vpu has the ability to homo-oligomerize and is mainly localized within intracellular membrane regions corresponding to the endoplasmic reticulum (ER), the *trans*-golgi network (TGN), and endosomal compartments (Dube et al., 2009; Klimkait et al., 1990; Maldarelli et al., 1993). Synthetic peptides encompassing the Vpu cytoplasmic domain identified two discrete α-helical structures separated by a flexible linker containing a pair of serine residues (Federau et al., 1996; Henklein et al., 1993; Kochendoerfer et al., 2004; Wittlich et al., 2009; Wray et al., 1995; Zheng et al., 2003). These two phosphoacceptor sites, serine 52 and 56, lie within a highly conserved C-terminal stretch of residues (47-58) and are constitutively phosphorylated by the ubiquitous casein kinase II (CKII) (Schubert et al., 1994; Tiganos et al., 1998).

1.4 Vpu-induced degradation of CD4

Primate lentiviruses induce the rapid and sustained cell surface downregulation of cluster of differentiation 4 (CD4) to prevent lethal superinfection and premature apoptosis of virally infected cells (Wildum et al., 2006). Interestingly, HIV-1 devotes Nef, Env and Vpu to target CD4 via three distinct mechanisms. Nef, an early gene product, targets pre-existing cell-surface CD4 molecules for lysosomal degradation via a clathrin-dependent process (Aiken et al., 1994; Chaudhuri et al., 2007; Rhee and Marsh, 1994). The Env precursor protein, gp160, retains CD4 within the ER and inhibits its transport to the plasma membrane (PM) (Bour et al., 1991; Crise et al., 1990; Jabbar and Nayak, 1990). Vpu induces the proteasomal degradation of *de novo* synthesized CD4 from the ER, reducing its half-life from 6 hours to approximately 15 minutes to enable Env maturation and full viral assembly (Bour et al., 1991; Buonocore and Rose, 1990; Willey et al., 1992a, b).

Vpu binds CD4 at a short cytoplasmic membrane proximal motif, LSEKKT (residues 414-419) (Bour et al., 1995; Lenburg and Landau, 1993; Vincent et al., 1993; Yao et al., 1995). Although the exact residues are unknown, NMR studies have suggested Vpu's cytoplasmic α-helices (C-terminal domain) seem to play an important role in CD4 interaction (Margottin et al., 1996; Singh et al., 2012). In support of this, a Vpu mutant harboring a scrambled primary amino acid sequence within the transmembrane (TM) domain still bound CD4 and induced its degradation (Schubert et al., 1996). Interestingly, a recent study identified a conserved residue within the Vpu TM domain, tryptophan 22 (W22), that when mutated to leucine inhibited CD4 degradation but not interaction (Magadan and Bonifacino, 2012). Magadan and colleagues showed that W22, which is

highly conserved among HIV-1 and some SIV_{chz} strains, instead prevents Vpu oligomerization (Magadan and Bonifacino, 2012; Maldarelli et al., 1993). This keeps Vpu in its monomeric, active form to presumably retain a favorable interaction with CD4, spatially orienting the immune receptor for eventual polyubiquitination of its cytosolic tail at lysine and serine/threonine residues (Binette et al., 2007; Magadán et al., 2010).

Proteasomal degradation of CD4 relies on the recruitment of Skp1-Cullin1-F-box (SCF) E3 Ubiquitin Ligase complex. This was elucidated when a yeast two-hybrid approach identified an interaction between Vpu and the human beta transducin-repeat containing protein (β-TrCP), where CD4, Vpu and β-TrCP existed in a ternary complex (Margottin et al., 1998). β-TrCP is a member of the F-box protein (FBP) family, currently composed of 68 additional proteins (Skaar et al., 2013). FBPs serve as substrate adaptors to mediate the formation of an SCF E3 Ubiquitin Ligase Complex that results in the degradation of a wide variety of cellular proteins (Mahon et al., 2014). β-TrCP contains an F-box motif necessary for interaction with Skp1 and WD40 repeats that recognize canonical phosphodegron motifs present within substrates (Skaar et al., 2013). Accordingly, mutation of Vpu's phosphoserine residues inhibits interaction with β-TrCP, preventing CD4 degradation (Margottin et al., 1998). Silencing of β-TrCP1 or the paralogous β-TrCP2 (HOS) also prevents the ability of Vpu-mediated CD4 degradation (Butticaz et al., 2007). Furthermore, dominant-negative mutants or depletion of cellular pools of the ER-associated degradation (ERAD) components VCP (p97), UFD1L, or NLP4 inhibit Vpu from degrading CD4 (Binette et al., 2007; Magadán et al., 2010). Taken together, these latter findings support how Vpu extracts CD4 from the ER for eventual degradation by a cytosolic ubiquitin ligase complex (Figure 1.3).

1.5 Vpu-mediated counteraction of BST-2

1.5.1 BST-2: structure and function

Bone marrow stromal antigen 2 (BST-2; tetherin) inhibits viral egress of nearly all enveloped viruses, including retroviruses, filoviruses, rhapdoviruses, paramyxoviruses, arenaviruses, and herpes viruses (Neil, 2013). Accordingly, Vpu counteracts BST-2 in HIV-1 infected cells to promote efficient viral release (Neil et al., 2008; Van Damme et al., 2008). Constitutive BST-2 expression is cell-type specific and Type 1 IFN inducible (Geraghty et al., 1994; Neil et al., 2007; Sakai et al., 1995).

BST-2 was initially discovered within terminally differentiated B cells obtained from multiple myeloma patients (Goto et al., 1994; Ohtomo et al., 1999). The protein is a 30-36 kDa type II single pass TM protein that forms stable cysteine-linked dimers (Andrew et al., 2009; Goto et al., 1994; Ohtomo et al., 1999; Perez-Caballero et al., 2009). At the PM, BST-2 resides within cholesterol-rich microdomains (lipid rafts) and also localizes within internal membrane compartments: the TGN and early and recycling endosomes (Kupzig et al., 2003; Masuyama et al., 2009). Structurally, BST-2 has an unusual topology, consisting of a short N-terminal cytoplasmic tail, a TM domain, and an ectodomain (extracellular core) that is anchored by a C-terminal glycosyl-phosphatidylinositol (GPI) moiety (Kupzig et al., 2003). X-ray crystallography of the recombinant BST-2 ectodomain identified a 90 Å parallel homodimer constituting a disulfide-linked coiled-coil domain structure (Hinz et al., 2010; Schubert et al., 2010). Upon viral budding BST-2 dimers adopt an “axial” configuration wherein pairs of TM domain or GPI anchors are inserted into assembling virions, while the remaining pairs of membrane anchors remain in the infected cell’s plasma membrane (Perez-Caballero et

al., 2009; Venkatesh and Bieniasz, 2013). Interestingly, an "artificial" BST-2 composed of heterologous protein domains inhibited HIV-1 and Ebola viral release, identifying that the configuration of BST-2, rather than the primary sequence, is important for antiviral activity (Perez-Caballero et al., 2009). Moreover, species-specific adaptation to counteract BST-2 occurs among all primate lentiviruses through the actions of either Vpu (HIV-1) (Neil et al., 2008; Van Damme et al., 2008), Env (HIV-2; SIV_{agm} Tan; SIV_{mac} ΔNef isolates) (Gupta et al., 2009; Hauser et al., 2010; Le Tortorec and Neil, 2009; Serra-Moreno et al., 2011) or Nef (most SIV isolates) (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009).

1.5.2 Vpu-mediated BST-2 counteraction: traffic jam in the TGN

It is now widely accepted that Vpu-mediated surface downregulation of BST-2 is a key mechanism in viral antagonism. Rather than altering the distribution of BST-2 on the PM (i.e., within lipid rafts), Vpu interferes with the protein's normal trafficking pattern (Dube et al., 2011; Lopez et al., 2012). BST-2 normally cycles between the PM, TGN, and endosomes, with a fraction sorted for lysosomal degradation through the coordinated action of the endosomal sorting complex required for transport (ESCRT) machinery (Habermann et al., 2010; Janvier et al., 2011; Masuyama et al., 2009; Rollason et al., 2007). Internalization of BST-2 from the PM relies on clathrin-mediated endocytosis, particularly Adaptor-Protein Complex 2 (AP-2) (Masuyama et al., 2009; Rollason et al., 2007). Adaptor-Protein Complex 1 (AP-1) mediates retrieval of BST-2 from late endosomes back to the TGN (Masuyama et al., 2009; Rollason et al., 2007). In both cases, a dual tyrosine motif (Y_6xY_8; where x is any amino acid) within the cytoplasmic domain of BST-2 mediates binding with AP-1/AP-2 (Rollason et al., 2007).

On the other hand, Vpu and BST-2 interact through their transmembrane domains (I_{34}, L_{37} and L_{41} (BST-2), and A_{14}, W_{22} and A_{18} (Vpu)), leading to the formation of an anti-parallel helix-helix interface (Figure 1.2) (Kobayashi et al., 2011; Skasko et al., 2011; Vigan and Neil, 2010). Consequently, scrambling of the Vpu TM domain inhibits Vpu from antagonizing BST-2 (Schubert et al., 1996; Van Damme et al., 2008). Interestingly, Vpu does not increase the constitutive endocytosis rate of BST-2 but rather inhibits *de novo* and recycled BST-2 from trafficking to the PM, sequestering the protein in a perinuclear compartment (Dubé et al., 2010; Dube et al., 2011; Dube et al., 2009; Lau et al., 2011; Mitchell et al., 2009; Schmidt et al., 2011). This is attributed to Vpu isolates possessing a highly conserved region (E_{59} VSAL_{63}V) within the membrane-distal half of their cytoplasmic domain (Kueck and Neil, 2012; McNatt et al., 2013). Specifically, this motif mimics a canonical acidic dileucine-based sorting signal ((D/E)xxxL(L/I/M)) that mediates interaction with AP complexes (Canagarajah et al., 2013), which Vpu utilizes to simultaneously bind BST-2 and AP-1 to prevent *de novo* and recycled BST-2 from reaching the cell surface (Figure 1.4) (Jia et al., 2014).

Apart from BST-2 downmodulation, Vpu also accelerates the lysosomal, degradation of BST-2 in an ECSRT and $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex-dependent manner (Caillet et al., 2011; Douglas et al., 2009; Iwabu et al., 2009; Janvier et al., 2011; Mitchell et al., 2009). However, we and others have shown that Vpu-induced surface downregulation of BST-2 is independent of β-TrCP and cullin activity (Goffinet et al., 2010; Ramirez et al., 2015; Tervo et al., 2011). This clarifies that Vpu-induced BST-2 degradation is a consequence, rather than a cause, of BST-2 surface downregulation, and that Vpu utilizes both CRL-dependent and -independent mechanisms to counteract the

antiviral function and overall cellular levels of BST-2.

1.5.3 BST-2 and Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

Despite a well-documented role in inhibiting cell-free virus, the immunological implications of BST-2's ability to tether nascent virions at the cell surface had not been well understood. In particular, tethered particles leave HIV-1 vulnerable to recognition and opsonization by circulating antibodies. One mechanism of clearing antibody-opsonized cells is ADCC. Classical ADCC involves the binding of FcγRIIIa expressed on Natural Killer (NK) cells with the F_c regions on antibodies, leading to the release of NK cytotoxic granules to lyse target cells. Two studies concurrently identified that Vpu modulates BST-2 surface expression to evade ADCC (Alvarez et al., 2014; Arias et al., 2014). Alvarez et. al. showed that HIV-1ΔVpu-infected cells displayed enhanced IgG opsonization and greater NK cell FcγRIIIa signaling and degranulation, correlating these functions with enhanced BST-2 expression (Alvarez et al., 2014). Meanwhile, the Evans group co-cultured an NK cell line constitutively expressing FcγRIIIa with CEM target cells containing a tat-inducible luciferase reporter gene. In the presence of antibodies derived from HIV-1-viremic patients, HIV-1ΔVpu cells were 60-fold more susceptible to ADCC than HIV-1-infected cells. Moreover, this effect was enhanced through Type 1 IFN treatment but inhibited upon knockdown of BST-2 (Arias et al., 2014). Therefore, these results suggest that BST-2 serves an important (and until recently underappreciated) role in bridging both innate and adaptive antiviral immunity.

1.6 Other recently discovered targets of Vpu

1.6.1 NTB-A and PVR

NK cells play a vital role in combating the early phases of viral infection through multiple mechanisms. These include the production of cytokines to inhibit viral replication and dissemination, the participation in ADCC to induce target cell apoptosis (as discussed earlier), or the release of cytolytic granules (degranulation) onto infected cells (Sowrirajan and Barker, 2011). As such, many viruses (including HIV-1) have evolved ways to subvert and incapacitate NK cells from exerting these antiviral functions. In particular, Vpu was recently shown to downregulate NK T and B cell antigen (NTB-A) (Shah et al., 2010). NTB-A is a Type I transmembrane protein and a member of the signaling lymphocytic activation molecule (SLAM) receptor family. It is expressed on all NK and $CD4^+$ T cells and acts in a homotypic manner as a co-activating receptor on NK cells (Cannons et al., 2011). Successful NK degranulation requires the coordinated action of three major classes of receptors: i.) adhesion receptors ii.) activation receptors and iii.) co-activation receptors (Bryceson et al., 2006). Thus, by decreasing cell surface NTB-A (Fogli et al., 2008; Ward et al., 2007), Vpu impairs the ability of NK cells to properly release their cytolytic granules and induce the lysis of HIV-1-infected cells (Shah et al., 2010). This finding also supports why NK cells display inefficient degranulation despite Vpr upregulating ligands (ULBP1/2) for the NK activation receptor NKG2D (Ward et al., 2009). Interestingly, although Vpu binds NTB-A via transmembrane domain interactions, Vpu does not require CRL activity to downmodulate NTB-A (Ramirez et al., 2015; Shah et al., 2010). Rather, Vpu affects the glycosylation pattern of NTB-A, thereby affecting its transport to the plasma membrane

and retaining NTB-A in the TGN (Bolduan et al., 2013). This novel mechanism further alludes to the versatility of Vpu and its interference with cellular targets.

Recently, it was shown that PVR (poliovirus receptor, CD155) is downregulated by both Nef and Vpu (Matusali et al., 2012). PVR is a ligand for the DNAM-1 adhesion molecule and is expressed on NK cells, $CD8^+$ T cells, as well as other cell types. While Nef seems to decrease total levels of PVR, Vpu sequesters the ligand in a perinuclear compartment (Bolduan et al., 2014; Matusali et al., 2012). Matusali and colleagues showed that HIV-1ΔNef-infected Jurkat cells were more susceptible to NK cell-mediated lysis and that this effect was partly reduced by blocking with an α-DNAM-1 antibody, suggesting a role for PVR in these functions (Matusali et al., 2012). The contribution Vpu alone plays in modulating PVR and NK cell-mediated lysis was not, however, shown. Therefore, as DNAM-1 is also involved in cell adhesion and migration, the exact role that PVR downregulation plays in HIV-1 pathogenesis will need to be further elucidated, particularly in primary cells.

Finally, in addition to activating receptors, NK cells also possess inhibitory receptors (iNKRs) that prevent the inadvertent killing of healthy cells. Among the major classes of iNKRs are the Killer Immunoglobulin (Ig) Like Receptors (KIRs), whose ligands include the major histocompatibility complex (MHC) class I molecules (Sowrirajan and Barker, 2011). Cell surface expression of MHC-I molecules is present on all nucleated cells, but is usually downregulated by viruses such as HIV-1 as a means to evade cytotoxic T lymphocyte (CTL) responses (Collins et al., 1998; Schwartz et al., 1996). Indeed, one of the major roles of HIV-1 Nef is to downregulate the MHC-I alleles HLA-A and B but not HLA-C or E (Cohen et al., 1999). Since HLA-C and E

preferentially protect humans from NK cytotoxicity, this selective MHC downregulation provides HIV-1 with the means to effectively avoid recognition by cytotoxic $CD8^+$ T cells and NK cells (Cohen et al., 1999). Given that NTB-A and PVR are also downregulated by Vpu and Nef, Vpu therefore assists Nef in the coordinated disarming of NK cells against HIV-1 infection.

1.6.2 CD1d

Invariant Natural Killer T cells (iNKT) are a unique subset of lymphocytes that play key roles within the innate immune system through the secretion of antiviral and immunoregulatory cytokines. They possess markers typically associated with NK cells as well as a semi-invariant T cell receptor (TCR) repertoire: Vα24 and Jα18 (α chain) paired with Vβ11 (β chain) (Bendelac et al., 2007). iNKT cells bind nonclassical antigen-presenting molecules of the CD1 family that present glycolipid antigens rather than peptides (Godfrey and Kronenberg, 2004). This leads to iNKT cell activation and subsequent effector function. Humans possess four different CD1 molecules that present endogenous or exogenous lipid-antigens to iNKT cells: CD1a, CD1b, CD1c (Group I), and CD1d (Group II) (Brigl and Brenner, 2004; Moody, 2006). Expression of Group I CD1 molecules is mostly limited to Antigen Presenting Cells (APCs), whereas CD1d is more widely expressed on APCs, B cells, monocytes, macrophages, and in some circumstances, activated T cells (Dougan et al., 2007).

Both Nef and Vpu contribute to impairing iNKT cell activation through surface downregulation of CD1d (Sandberg et al., 2012). Nef accelerates the internalization rate of CD1d molecules from the PM in an AP-2-dependent manner, leading to CD1d retention in the TGN (Chen et al., 2006; Cho et al., 2005). Vpu, however, inhibits the

recycling of CD1d back to the PM, leading to CD1d accumulation within early endosomes (Moll et al., 2010). Mutation of Vpu's serine residues did not abrogate Vpu-mediated CD1d downregulation, suggesting cullin activity and β-TrCP are dispensable for this function (Sandberg et al., 2012). Interestingly, a cytoplasmic distal C-terminal motif (APW) that is conserved between Group M Vpu proteins was recently identified as a critical determinant in Vpu-mediated CD1d downregulation (Bächle et al., 2015). Further structural and functional studies will be needed to determine whether this motif mediates binding between Vpu and CD1d or to possibly another cellular co-factor.

1.6.3 CCR7 and CD62L

Coordinated lymphocyte trafficking between the blood, lymphatics, and secondary lymphoid organs (SLO) is essential for the proper development of an immune response against pathogens and is primarily controlled through expression of the homing molecules CCR7 and CD62L (L-selectin). CCR7 is a chemokine receptor that possesses a seven-transmembrane domain-spanning region and heterotrimeric G-protein signaling activity. Various subpopulations of T cells constitutively express CCR7, whereas the protein is upregulated upon maturation by antigen-presenting dendritic cells (DCs) (Forster et al., 2008). Continual lymphocyte recirculation between the bloodstream and lymphatic tissues is regulated by CCR7's two chemokine ligands (CCL19 and CCL21), which are expressed in a homeostatic manner within primary and secondary lymphoid organs (Legler et al., 2014). The lectin-like receptor CD62L serves as an adhesion molecule, binding glycoproteins (i.e., CD34, GlyCAM-1) present on high endothelial venules (HEVs). This helps in the capture of leukocytes from the bloodstream and facilitates their subsequent "rolling" along HEVs, initiating a cascade of events that

culminates in the transmigration of immune cells into SLO (Khan and Kubes, 2003).

Recently, we and others have shown that HIV-1 modulates surface expression of both CCR7 and CD62L in $CD4^+$ T cells (Ramirez et al., 2014; Trinite et al., 2014; Vassena et al., 2015). Vpu physically interacts with CCR7 and is both necessary and sufficient to reduce surface levels of the chemokine receptor (Ramirez et al., 2014). Rather than inducing degradation, Vpu relocalizes CCR7 within the TGN (Ramirez et al., 2015; Ramirez et al., 2014). As a consequence, HIV-1-infected primary $CD4^+$ cells expressing Vpu are impaired in their ability to migrate *in vitro* in a CCL19-dependent manner (Ramirez et al., 2014).

Interestingly, a coordinated effort between Nef and Vpu is necessary to downregulate CD62L (Vassena et al., 2015). Vassena et al. showed that both Nef and Vpu inhibit the transport of *de novo* CD62L towards the PM, resulting in the sequestration of CD62L within a perinuclear compartment (Vassena et al., 2015). Importantly, the adherent and signaling properties attributed to CD62L expression were hindered in primary $CD4^+$ T cells infected with HIV-1 (Vassena et al., 2015). Future studies within humanized mouse models will likely be necessary to determine the full biological implications of HIV-1-mediated CCR7 and CD62L downregulation and how these functions may influence viral propagation, dissemination, and / or persistence.

1.6.4 Tetraspanins

Humans encode 33 tetraspanins, integral membrane proteins that possess four transmembrane-spanning domains. Tetraspanins have a propensity to form homo- and hetero-dimers, -trimers or -tetramers with themselves or other proteins (integrins and other tetraspanins), resulting in the organization of specialized membrane domains

known as tetraspanin-enriched microdomains (TEMs) (Hemler, 2005). TEMs regulate many diverse cellular processes, including cell adhesion, motility, fusion, activation, and proliferation (Yanez-Mo et al., 2009). In addition to their multifaceted roles within cell biology, tetraspanins have also been associated with infectious diseases. Indeed, reports have shown that some tetraspanins (CD9, CD53, CD63, CD81, CD82, tetraspanin 14) are enriched within HIV-1 budding sites and can be incorporated into viral particles (Thali, 2011). HIV-1 Gag has been implicated in specifically recruiting tetraspanins to areas of HIV-1 assembly, perhaps to provide a favorable environment for viral egress (Hogue et al., 2011; Krementsov et al., 2010). Paradoxically, other reports have shown the incorporation of tetraspanins into HIV-1 particles renders the virus less infectious through inhibiting fusion and entry into target cells (Krementsov et al., 2009; Sato et al., 2008; Symeonides et al., 2014; Weng et al., 2009). Moreover, although tetraspanins accumulate at viral budding sites, a reduction in the overall levels of tetraspanins has been observed (Krementsov et al., 2009).

Two studies reported that Nef and Vpu alter the cell surface expression of members of the tetraspanin family, altering their trafficking through sequestration in a perinuclear compartment (Haller et al., 2014; Lambele et al., 2015). Lambele and colleagues focused on CD81, a ubiquitous tetraspanin, and showed that Vpu degraded the protein in multiple cell types in a proteasomal- and lysosomal-dependent manner (Lambele et al., 2015). Moreover, HIV-1ΔVpu or ΔNef virions produced in CD81-expressing CEMss cells were less infectious than HIV-1wt virions (Lambele et al., 2015). In a similar manner, Haller et al. showed that HIV-1ΔVpu or ΔNef viruses displayed reduced viral spread, cell migration, and actin rearrangement (Haller et al., 2014). Thus,

it seems that both Nef and Vpu regulate overall tetraspanin levels to provide an environment conducive to efficient viral dissemination.

1.7 Conclusions

Despite its small size, Vpu is a multifunctional viral protein that interferes with numerous host immune factors through discrete mechanisms. First, Vpu acts as a viral adaptor by "mimicking " a canonical phosphodegron motif via its phosphoserine residues, recruiting β-TrCP and the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex to induce CD4 proteasomal or BST-2 lysosomal degradation (Roy et al., 2014). Second, Vpu alters intracellular protein trafficking, resulting in the sequestration of its cellular targets within a perinuclear compartment and/or their inability to recycle towards the PM (Figure 1.5). The exact residues, domains and/or cellular co-factors necessary for this latter mechanism of Vpu are still being defined, although some critical motifs have emerged. In particular, residues within the distal cytoplasmic domain of Vpu seem to mediate binding to AP-1 (ELV), which helps sequester and inhibit the recycling of BST-2 towards the PM, resulting in its accelerated degradation (Jia et al., 2014; Kueck and Neil, 2012). A conserved APW motif within the C-terminal domain of Vpu regulates CD1d cell surface downregulation (Bächle et al., 2015). Whether these motifs have any effects on other recently discovered targets of Vpu awaits further investigation. Moreover, the exact biological and functional roles that downregulation of recently described Vpu targets (in particular, tetraspanins, CCR7, and CD62L) has in promoting viral pathogenesis remains to be determined. Furthermore, another interesting point to consider is the relative redundancy of both Vpu and Nef to modulate a number of host cell receptors, albeit via different mechanisms. Given their different expression profiles (Nef as an early gene,

Vpu as a late gene) and their subcellular locations (Nef on the PM, Vpu within the ER, Golgi and endosomes), some studies have suggested HIV-1 evolved to utilize these accessory factors to modulate host vesicular trafficking at all stages of viral replication, providing the virus with the genetic and functional tools to adapt to an ever-changing microenvironment (Haller et al., 2014).

Finally, among the four lineages of HIV-1 (M, N, O and P), only HIV-1 Group M has reached pandemic levels. Using mathematical modeling, Iwami and colleagues deduced HIV-1 M Vpu conferred a 2.38-fold increase in the prevalence of HIV-1 human-to-human transmission (Iwami et al., 2015). This activity was lost in the absence of Vpu, with individuals developing intrinsic herd immunity through the antiviral effects of BST-2, supporting the notion that BST-2 acts as a restriction factor (Iwami et al., 2015). Further indirect functional studies in support of this include Vpu alleles from HIV-1-infected patients maintaining CD4 and BST-2 antagonistic potential (Pickering et al., 2014). Moreover, Vpus from Groups N, O, and P do not possess the combined abilities to strongly downregulate CD4, NTB-A, or CD1d as well as efficiently counteract BST-2 (Sauter et al., 2011; Sauter et al., 2009; Sauter et al., 2012; Yang et al., 2011). With the recent discovery of other cellular targets downmodulated by Vpu, it will be very interesting to see whether other primate lentiviral Vpus display an inefficient ability to perform these functions. In any case, a continual understanding of immune evasion mechanisms mediated by Vpu will undoubtedly help in our understanding of HIV-1 pathogenesis, but perhaps also in the rational design of novel therapeutics.

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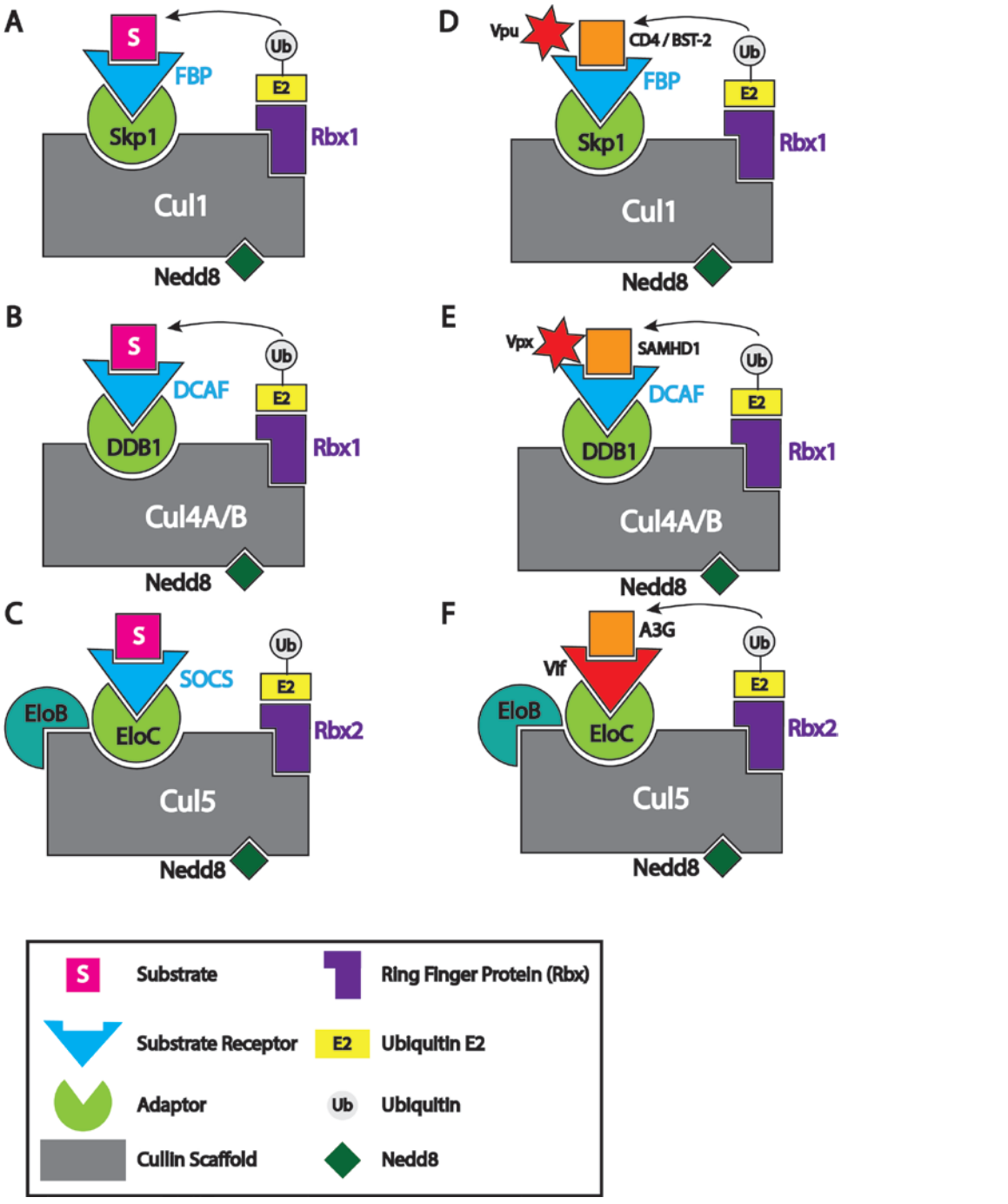

Figure 1.1 Manipulation of Cullin-RING Ligases (CRLs) by HIV accessory proteins. A-C.) Cullins are modular scaffold proteins. They are comprised of an Rbx protein at their C-terminus and different adaptor molecules and substrate receptors at their N-terminus. Binding of Nedd8 (neddylation) induces a conformational change within CRL-complexes, activating them to properly facilitate the transfer of ubiquitin onto substrate molecules. **D-F.)** The HIV accessory proteins Vpu, Vpx, and Vif act as viral adaptors to utilize CRL-machinery for the degradation of host immune factors. Figure adapted from Mahon et al., 2014. A3G: APOBEC3G.

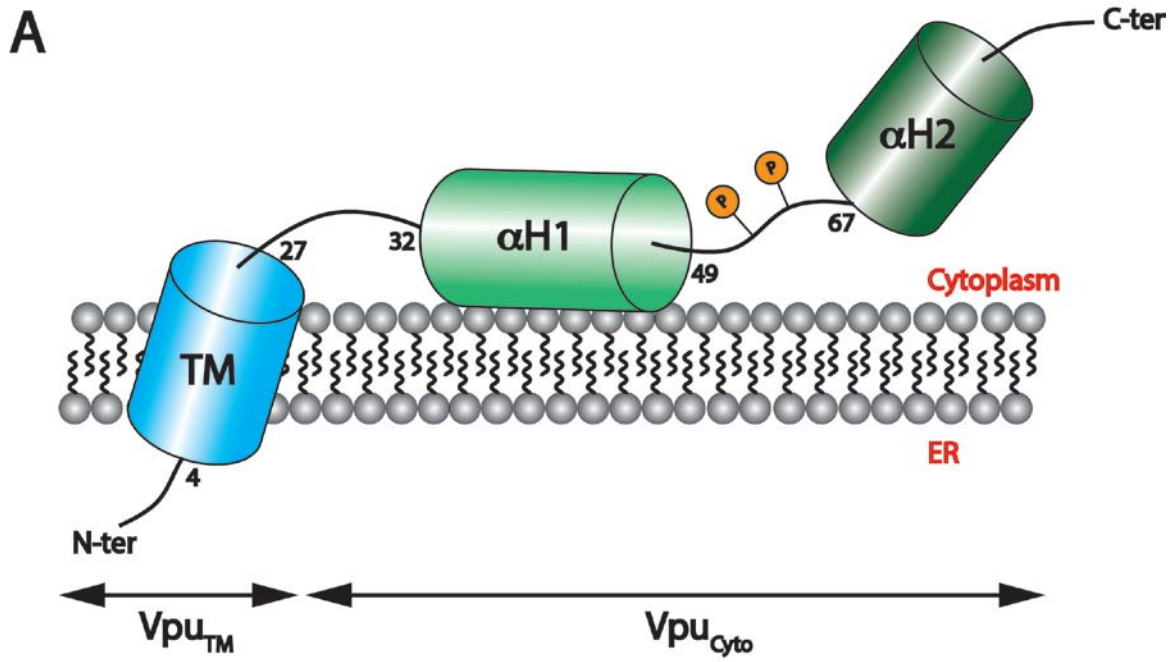

Figure 1.2: Schematic representation of HIV-1 Vpu
Vpu is an 81-amino-acid Type 1 integral membrane protein. Residues 4-27 encompass a membrane anchor region (transmembrane domain). The cytoplasmic portion includes two α-helices, which are separated by a highly conserved region that includes two constitutively phosphorylated serine residues. Vpu localizes to the ER, TGN and endosomal compartments. Modified from Dube et al., 2010.

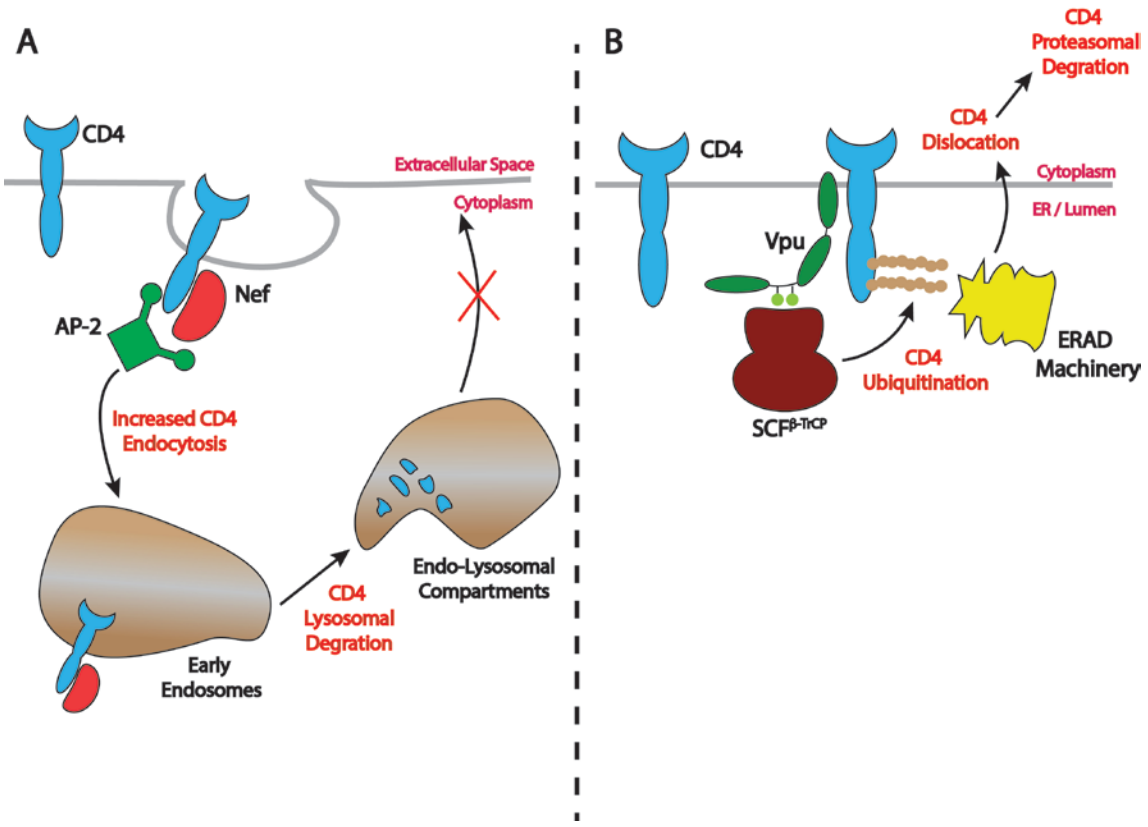

Figure 1.3: Antagonism of CD4 by HIV-1 Nef and Vpu.
A.) Nef increases the internalization rate of CD4 molecules on the PM in an AP-2-dependent manner. This results in CD4 / Nef complexes within early endosomes and eventual CD4 lysosomal degradation. **B.)** Vpu interacts with CD4 within the ER and induces CD4 ubiquitination through recruitment of an $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex. The ERAD machinery subsequently dissociates CD4 from the ER (and Vpu), leading to its degradation via the 26S proteasome. PM: plasma membrane; AP-2: adaptor-protein complex 2; ER: endoplasmic reticulum; ERAD: ER-associated degradation.

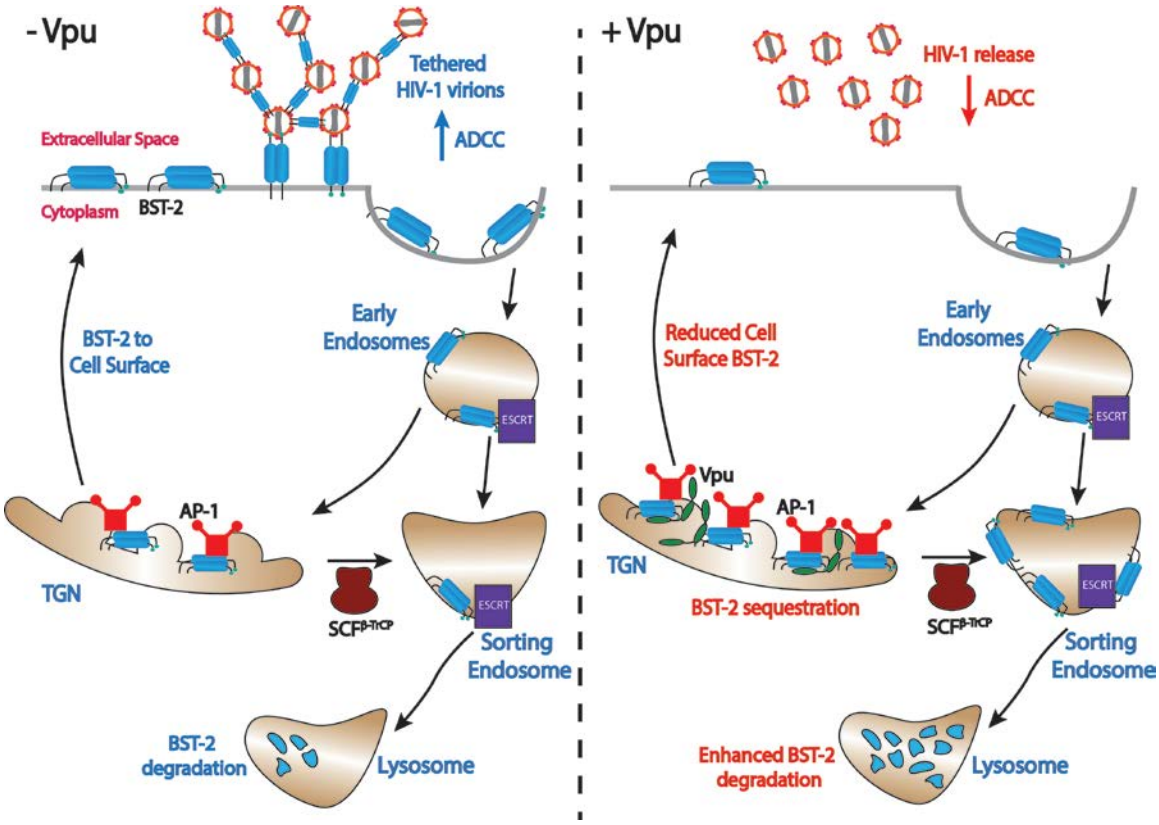

Figure 1.4: Vpu-mediated counteraction of BST-2.
BST-2 is a cell-type-specific, Type I IFN-inducible protein that tethers all enveloped viruses (including HIV-1) to the cell surface to inhibit viral egress and promote antibody-dependent cell-mediated cytotoxicity (ADCC). BST-2 normally recycles between early endosomes, the TGN and the PM. A fraction of BST-2 is ubiquitinated by the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex and degraded in the lysosome in an endosomal sorting complexes required for transport (ESCRT)-dependent manner. In the presence of Vpu, *de novo* and recycled BST-2 are retained within the TGN. Vpu interacts with AP-1 to mistraffick BST-2 to sorting endosomes, resulting in enhanced BST-2 degradation and reduction of the protein on the cell surface. As a result, this promotes HIV-1 release and decreases ADCC. TGN: *trans*-golgi network; PM: plasma membrane

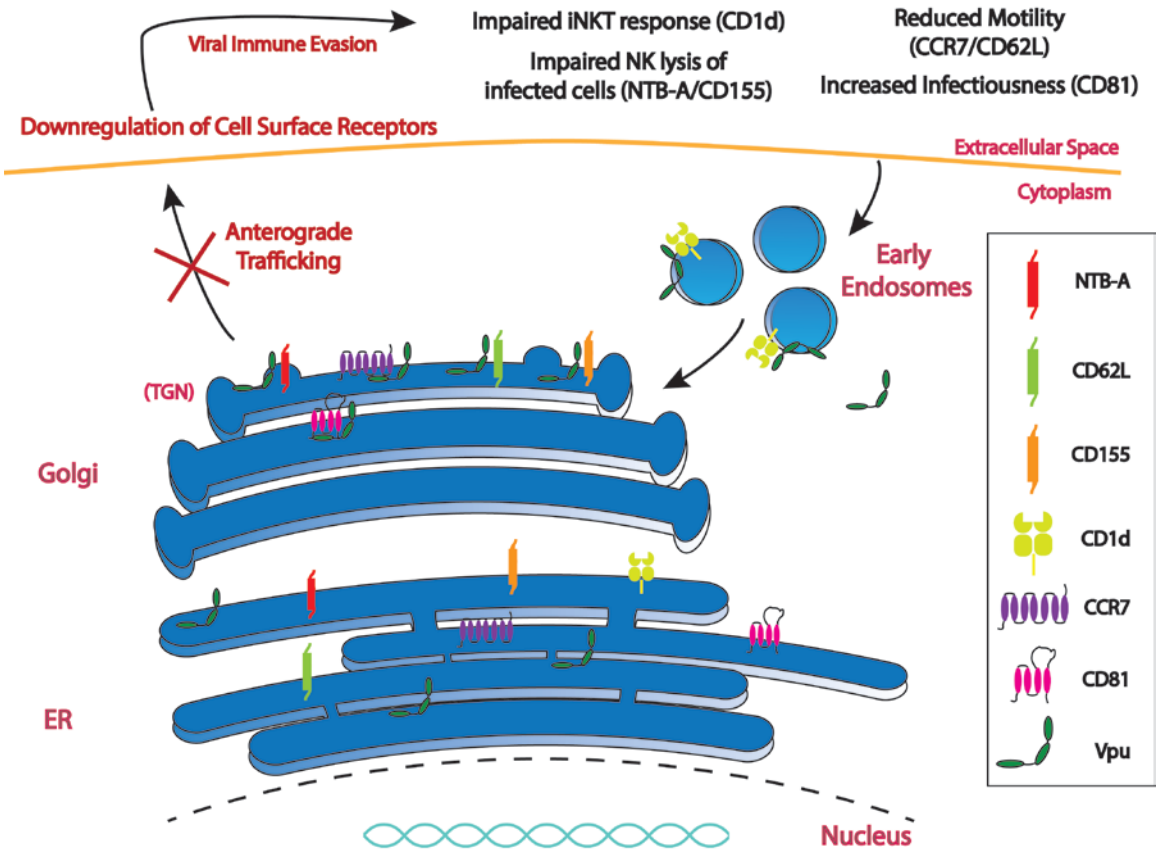

Figure 1.5: Sequestration of Vpu cellular targets.
Vpu interferes with host protein vesicular trafficking, thereby reducing expression of immune factors at the cell surface. In general, Vpu retains NTB-A, CCR7, CD62L, CD155, and CD81 within a perinuclear compartment (most often, the TGN). Vpu also hinders the recycling of CD1d towards the PM in early endosomes. As a result, multiple levels of innate and adaptive immunity are compromised, leading to viral persistence and pathogenesis.

CHAPTER 2

DOWNMODULATION OF CCR7 BY HIV-1 VPU RESULTS IN IMPAIRED MIGRATION AND CHEMOTACTIC SIGNALING WITHIN $CD4^+$ T CELLS

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Downmodulation of CCR7 by HIV-1 Vpu Results in Impaired Migration and Chemotactic Signaling within CD4$^+$ T Cells

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SUMMARY

The chemokine receptor CCR7 plays a crucial role in the homing of central memory and naive T cells to peripheral lymphoid organs. Here, we show that the HIV-1 accessory protein Vpu downregulates CCR7 on the surface of CD4$^+$ T cells. Vpu and CCR7 were found to specifically interact and colocalize within the *trans*-Golgi network, where CCR7 is retained. Downmodulation of CCR7 did not involve degradation or endocytosis and was strictly dependent on Vpu expression. Stimulation of HIV-1-infected primary CD4$^+$ T cells with the CCR7 ligand CCL19 resulted in reduced mobilization of Ca^{2+}, reduced phosphorylation of Erk1/2, and impaired migration toward CCL19. Specific amino acid residues within the transmembrane domain of Vpu that were previously shown to be critical for BST-2 downmodulation (A14, A18, and W22) were also necessary for CCR7 downregulation. These results suggest that BST-2 and CCR7 may be downregulated via similar mechanisms.

INTRODUCTION

HIV-1 encodes four accessory genes, *vpu*, *nef*, *vif*, and *vpr*, that have numerous effects on the host cell. These effects include downregulation of cell-surface molecules and evasion of restriction factors and innate immune responses (reviewed in Kirchhoff, 2010; Malim and Emerman, 2008). The HIV-1 Vpu protein has a predicted length that ranges from 77 to 86 amino acid residues. Vpu is translated from a *vpu-env* bicistronic mRNA (Schwartz et al., 1990; Strebel et al., 1988) during a late phase of the viral life cycle and is not thought to be incorporated into budding virions (Nomaguchi et al., 2008). Structurally, Vpu consists of three major domains: a short N-terminal luminal tail (3–12 amino acids), a single hydrophobic transmembrane domain (TMD; 27 amino acids), and a C-terminal amphipathic portion (54 residues) that extends into the cytoplasm (Maldarelli et al., 1993; Wray et al., 1995). The C-terminal region consists of two α-helices connected by a short motif in which two conserved serine residues (serine 52 and serine 56) are phosphorylation sites for casein kinase II and are responsible for the recruitment of β-TrCP-1 and β-TrCP-2 (Strebel, 2007).

Vpu sequesters de novo synthesized CD4 in the endoplasmic reticulum (ER), targeting it for proteasomal degradation (Willey et al., 1992). This function is dependent on the binding of β-TrCP to Vpu's cytoplasmic phosphoserine residues (Butticaz et al., 2007; Margottin et al., 1998). Vpu-mediated downmodulation of BST-2/Tetherin has been shown to be partly dependent on the interaction of Vpu with β-TrCP (Iwabu et al., 2009), although whether this interaction leads to degradation of BST-2 is still debated (Dubé et al., 2010; Mangeat et al., 2009). Vpu interacts with BST-2 within the *trans*-Golgi network (TGN) and in recycling endosomes (Douglas et al., 2009; Dubé et al., 2010; Mitchell et al., 2009) rather than within the ER, as is the case with CD4 (Willey et al., 1992). Vpu has been shown to cooperate with Nef in the downregulation of CD1d from the surface of HIV-1-infected dendritic cells (DCs), thereby limiting the ability of CD1d to patrol the endocytic system in search of lipid antigens to present to invariant natural killer T (iNKT) cells (Moll et al., 2010).

Shah et al. (2010) recently found that Vpu also downmodulates the surface expression of the NK cell coactivating receptor NK-T and B cell antigen (NTB-A) on infected CD4$^+$ T cells. As a consequence, degranulation by NK cells, which requires signaling through NTB-A, is impaired. Downregulation of NTB-A by Vpu protects the infected cells from lysis by NK cells (Shah et al., 2010). Interestingly, it appears that recruitment of β-TrCP is not required for either Vpu-mediated CD1d or NTB-A surface downmodulation, suggesting that Vpu acts as a multifunctional viral protein that is able to interfere in different ways with different host factors (Sandberg et al., 2012).

In this work, we describe the ability of HIV-1 to downregulate the C-C chemokine receptor-7 (CCR7) from the surface of primary CD4$^+$ T cells in a Vpu-dependent manner. CCR7 belongs to a family of seven-transmembrane-spanning chemokine receptors that mediate their signals through the activation of heterotrimeric Gα_i proteins. CCR7 is mainly expressed by mature dendritic cells (Ohl et al., 2004), naive B cells (Reif et al., 2002), and naive and central memory CD4$^+$ T cells (Sallusto et al., 1999). Studies in CCR7-deficient mice underscored the central role of CCR7 as a major homing receptor that directs the migration of cells into the lymph nodes, where priming and assembly of immune responses take place (Förster et al., 1999). Additionally, recent studies have revealed previously unrecognized functions of CCR7 in promoting T cell recirculation in peripheral tissues (Debes et al., 2005; Höpken et al., 2010). CCR7 signaling is triggered by the chemokines CCL19 and CCL21 (Rot and von Andrian, 2004), which are constitutively expressed by reticular stromal cells in lymphoid organs (Luther et al., 2000). Binding of either ligand to the receptor culminates in G protein activation, calcium flux, and chemotactic responses (Willimann et al., 1998; Yoshida et al., 1998). We show that HIV-1 infected cells, through the action of Vpu, display reduced expression of CCR7 and a reduced ability to signal and migrate in response to CCL19.

RESULTS

HIV-1 Downregulates the Chemokine Receptor CCR7 on the Surface of Primary CD4$^+$ T-Lymphocytes

We previously reported that HIV-1 infection of in vitro cultured central memory T cells (T_{CM}) generates a population of productively infected cells (Bosque and Planelles, 2009). We wished to examine whether any phenotypic differences induced by HIV-1 infection occurred in these cells. To that end, we infected primary CD4$^+$ lymphocytes (generated as described in Experimental Procedures) with a replication-deficient HIV-1 molecular clone (termed DHIV) carrying GFP in place of Nef (DHIV-GFPΔNef; Figure S1) and analyzed the expression of GFP versus different surface markers at 2 days postinfection. As shown in Figure 1A, both uninfected and infected cells expressed similar levels of the activation marker CD45RO, the chemokine receptor CXCR4, and the costimulatory molecule CD27, all of which are highly expressed on cultured T_{CM}. As expected, infected cells downregulated CD4 as a consequence of Vpu expression (Willey et al., 1992). Unexpectedly, we found that the levels of the chemokine receptor CCR7 were 49% lower (based on mean fluorescence intensity [MFI] values) in infected cells relative to uninfected cells (Figure 1A).

We then investigated whether this was a general effect of HIV-1 on chemokine receptors. We infected T_{CM} cells with a molecular clone of HIV-1 that encodes all of the accessory genes. In this case, cells were stained for surface expression of the chemokine receptors CCR7, CXCR4, CXCR3, CCR4, CCR6, and CCR5, followed by intracellular staining of p24Gag viral antigen. As shown in Figure 1B, among the tested receptors, HIV-1 was only able to downregulate CCR7. Contrary to previous findings showing that Nef downmodulates the chemokine receptor CXCR4 (Hrecka et al., 2005; Venzke et al., 2006), we did not observe CXCR4 downregulation.

Vpu Mediates Cell-Surface CCR7 Downregulation in CD4$^+$ T Cells

Next, we tested whether any accessory protein had a potential role in manipulating CCR7 expression. To that end, we infected cells with HIV-1 viruses lacking each accessory gene and analyzed CCR7 expression 2 days postinfection. As shown in Figure 2A, CCR7 was downmodulated from the cell surface by HIV-1ΔVpr, HIV-1ΔVif, and HIV-1ΔNef to the same extent as it was by wild-type (WT) HIV-1 (panels i–v). However, HIV-1ΔVpu failed to downregulate CCR7, indicating that Vpu was necessary for this function (panel vi).

We then examined whether Vpu was sufficient for CCR7 surface downregulation. CCRF-CEM T cells, which constitutively express CCR7 and CD4, were nucleofected with expression vectors encoding either Vpu-GFP or GFP alone (Shah et al., 2010). CCR7 surface expression was reduced in Vpu-GFP, but not GFP-transfected, cells (Figure 2B, compare panels i and ii), indicating that Vpu is sufficient to downmodulate CCR7. As expected, CD4 surface levels were also lower in Vpu-GFP-expressing, but not GFP-expressing, cells (Figure 2B, panels iii and iv; Willey et al., 1992).

To address whether HIV-1 infection reduced the total levels of CCR7 (as opposed to only surface levels), cells were fixed, permeabilized, and costained with CCR7 and p24Gag antibodies. As a control, we stained for CD4, whose degradation is triggered by Vpu via the ER-associated degradation (ERAD) pathway (Binette et al., 2007; Magadán et al., 2010; Schubert et al., 1998; Willey et al., 1992). As shown in Figures 2C (panels i and iii) and 2D, the total levels of CCR7 were not significantly different between infected and uninfected cells (see "Total" in Figures 2C and 2D), suggesting that Vpu did not induce CCR7 degradation but, more likely, promoted its redistribution within the cell. In contrast, HIV-1 infection drastically reduced the surface and total levels of CD4 (Figure 2B, panels ii and iv) due to the combined effect of both Vpu and Nef degrading the protein (Kirchhoff, 2010).

To directly assess whether Vpu induces CCR7 degradation, we conducted both a cycloheximide (CHX) study and a pulse-chase analysis. We used CHX, a blocker of protein synthesis, so that we could evaluate the fate of total levels of protein in the absence of de novo synthesis. As shown in Figure S2A, when HIV-1-infected primary cells were incubated in the presence of CHX for 24 hr, the total levels of CCR7 remained constant between infected and uninfected cells. Therefore, the decrease in surface CCR7 induced by Vpu cannot be explained by protein degradation.

As an independent method to examine the possible degradation of CCR7, we performed a pulse-chase analysis in 293T cells by cotransfecting CCR7-Flag with an expression vector encoding GFP or a Vpu-GFP fusion protein (Shah et al., 2010). At 24 hr posttransfection, cells were pulse labeled with [^{35}S] for 30 min and chased for up to 24 hr. CCR7 was then immunoprecipitated using an anti-Flag antibody and the lysates were separated by SDS-PAGE, followed by autoradiography. We observed a band at 43 kDa corresponding to CCR7. We detected only a minor difference in CCR7 protein levels in the absence or presence of Vpu (100% versus 86%, respectively) after 24 hr (Figures S2B and S2C). Therefore, although these results do not exclude

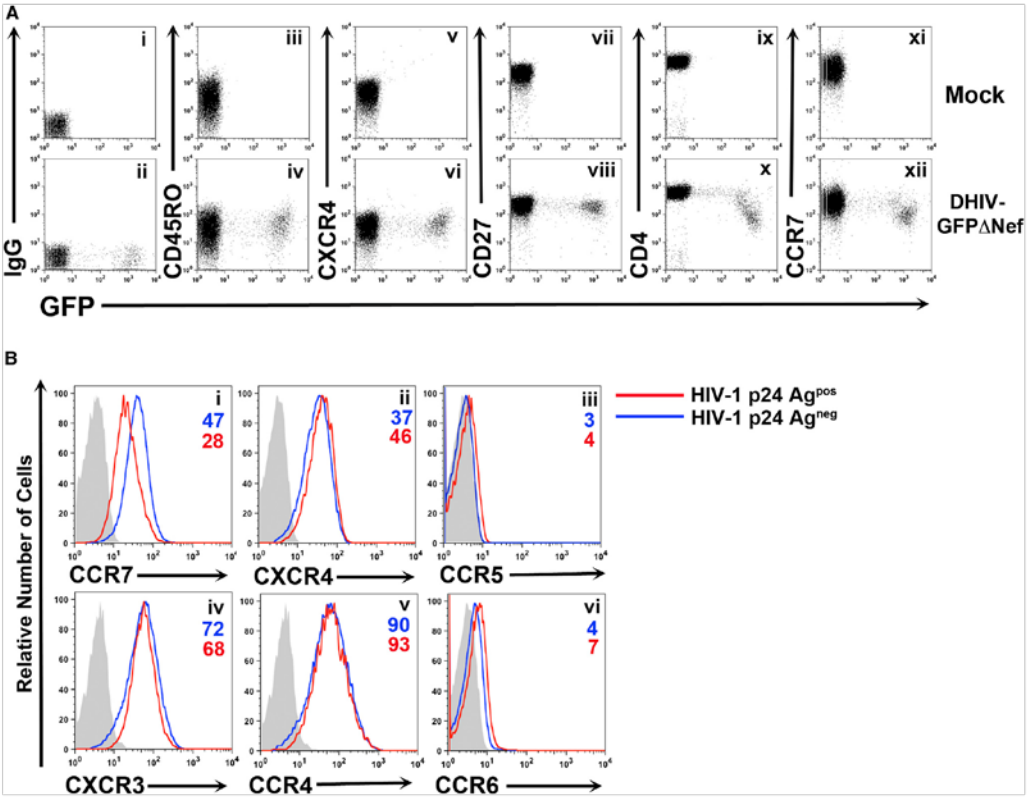

Figure 1. HIV-1 Downregulates the Chemokine Receptor CCR7 from the Surface of Infected Primary CD4⁺ T Cells

(A) Surface levels of CD45RO (iii and iv), CXCR4 (v and vi), CD27 (vii and viii), CD4 (ix and x), and CCR7 (xi and xii) versus GFP expression were analyzed 2 days postinfection in uninfected (Mock) and infected (DHIV-GFPΔNef) cultured CD4⁺ T_{CM} cells. An immunoglobulin G (IgG) matched control was used to establish positive surface marker expression (i and ii). Unless otherwise noted, all figures involving primary CD4⁺ T cells are representative of three separate experiments performed in three different donors.

(B) Primary CD4⁺ T cells were either mock infected or infected with DHIV. At 2 days postinfection, cells were surface stained for the chemokine receptor CCR7 (i), CXCR4 (ii), CCR5 (iii), CXCR3 (iv), CCR4 (v), or CCR6 (vi), followed by intracellular staining for HIV-1 p24Gag. A comparison between $p24Gag^{neg}$ cells (blue line) and $p24Gag^{pos}$ cells (red line) is depicted in each histogram along with an IgG matched isotype control (gray shaded histogram).

See also Figure S1.

a minor contribution of degradation of CCR7, we conclude that degradation is not the major mechanism by which Vpu induces downregulation of CCR7 from the cell surface.

Downmodulation of CCR7 by Vpu Occurs with Replication-Competent HIV-1

To directly examine under more physiological conditions (i.e., in a spreading infection) whether Vpu could downmodulate CCR7, we infected primary CD4⁺ T cells with either HIV-1_{NL4-3} or the mutant HIV-1_{NL4-3}ΔVpu, in which the start codon of Vpu was mutated to a stop codon. At 2, 3, 5, and 7 days postinfection, the cells were surface stained for CCR7 or BST-2, followed by intracellular staining for p24 Gag. Replication-competent HIV-1 efficiently downregulated CCR7 from the cell surface, and this effect became more significant as the time of infection increased (Figures 3A, panels i–iv, and 3B). By comparison, infection of cells with HIV-1_{NL4-3}ΔVpu was unable to induce CCR7 surface downmodulation (Figures 3A, panels v–viii, and 3B). As a control, Vpu also efficiently downregulated BST-2 from the cell surface (Figure S3).

CCR7 and Vpu Colocalize within the TGN

Vpu has previously been shown to colocalize with BST-2 (Van Damme et al., 2008) and preferentially sequester the host protein within a perinuclear compartment, specifically the TGN (Dubé et al., 2009, 2010; Hauser et al., 2010; Vigan and Neil, 2010).

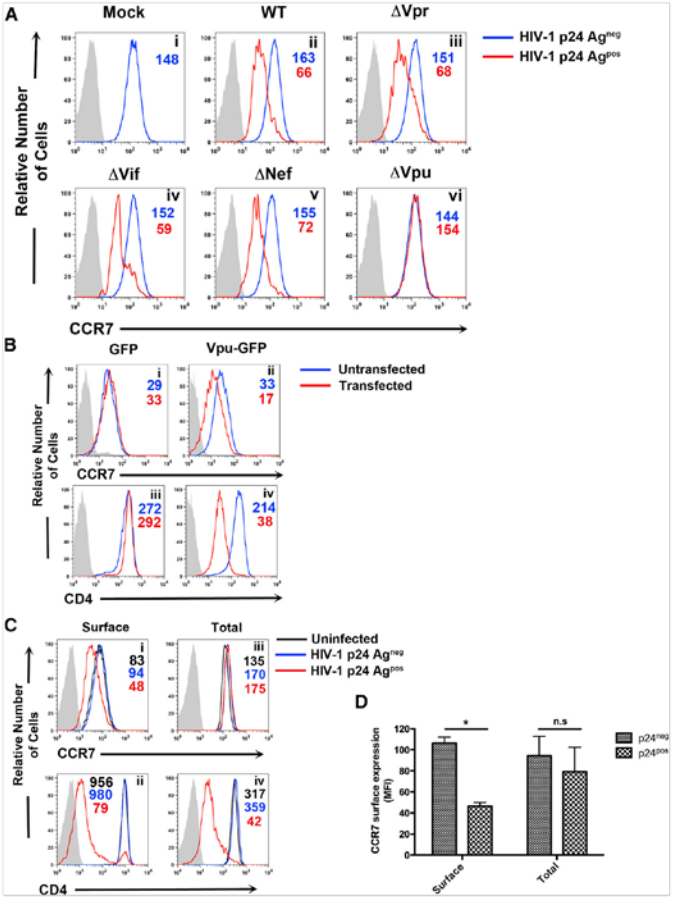

Figure 2. HIV-1 Vpu Is Necessary and Sufficient for Surface Downmodulation, but Not Degradation, of CCR7

(A) Primary CD4$^+$ T cells were either mock infected (i) or infected with DHIV (ii), DHIVΔVpr (iii), DHIVΔVif (iv), DHIVΔNef (v), or DHIVΔVpu (vi). Two days later, cells were assessed for surface levels of CCR7 in p24Gag^{neg} (blue line) and p24Gag^{pos} cells (red line). Gray-shaded histograms represent IgG matched isotype controls.

(B) CCRF-CEM cells were nucleofected with 2 μg of either a GFP or Vpu-GFP expression vector. At 24 hr posttransfection, the relative surface levels of CCR7 (i and iii) and CD4 (ii and iv) were measured. The histograms depict a comparison between untransfected (blue line) and transfected (red line) cells relative to the IgG matched control (gray-shaded histogram). The figure is representative of three independent experiments.

(C) The relative surface levels of CCR7 (i) and CD4 (ii) were assessed 2 days after infection with DHIV, as in (A). In addition, cells were permeabilized and costained with antibodies for either CCR7 (iii) or CD4 (iv) along with antibody for p24Gag. HIV-1 p24Gag^{pos} cells and p24Gag^{neg} cells are represented by red and blue lines, respectively. Uninfected cells (black line) were used as a control along with an IgG matched isotype (gray-shaded histogram).

(D) MFI values of the surface and total levels of CCR7 from three independent experiments. The data were normalized by setting MFI values from uninfected (mock) cells to 100% and are depicted graphically as mean ± SEM (*$p < 0.05$).

See also Figure S2.

Therefore, to determine whether Vpu colocalizes and/or sequesters CCR7, we transfected HeLa cells with a CCR7 fusion construct bearing a C-terminal mCherry tag (CCR7-mCherry) along with Vpu-GFP. In the absence of Vpu-GFP, CCR7-mCherry localized both at the cell surface and intracellularly (Figure 4A, top row). The amount of colocalization between CCR7-mCherry and TGN46, a TGN marker, in the absence of Vpu, was minimal (Figures 4A, upper panels, and 4B). However, in cells cotransfected with CCR7-mCherry and Vpu-GFP, both proteins were highly colocalized together, as quantified by Pearson's correlation coefficient (PCC; Figure 4B), and specifically within the TGN cellular compartment (Figures 4A, lower panels, and 4B; Barlow et al., 2010). Moreover, the degree of colocalization between CCR7-mCherry and TGN46 (PCC = 0.38) increased when Vpu-GFP was present (PCC = 0.58), suggesting that Vpu sequesters CCR7 within the TGN. These findings are highly reminiscent of how Vpu induces BST-2 surface downregulation (Dubé et al., 2010; Vigan and Neil, 2010).

Vpu Does Not Increase the Endocytosis Rate of CCR7 in the Presence of CCL19

Since we observed that the steady-state levels of CCR7 remained constant in HIV-1-infected cells, we tested whether Vpu may be increasing the internalization rate of the chemokine receptor. Primary CD4$^+$ T cells infected with HIV-1_{NL4-3} were stained with an antibody against CCR7 at 4°C and then placed back at 37°C for various time points to allow for internalization. The cells were then stained with an allophycocyanin (APC)-conjugated secondary antibody followed by p24Gag antigen, and analyzed by flow cytometry. As shown in Figure S4, we did not observe endocytosis of CCR7 in uninfected cells (mock, solid black line), p24Gag^{neg} cells (solid blue line), or p24Gag^{pos} cells (solid red line). This indicates that Vpu does not increase the constitutive endocytosis rate of CCR7. Moreover, the stability of the chemokine receptor on the surface is consistent with previous reports showing that CCR7 is highly stable on the cell membrane unless it is provided with one of its chemokine ligands, such as CCL19 (Otero et al., 2006). As a positive control for endocytosis of

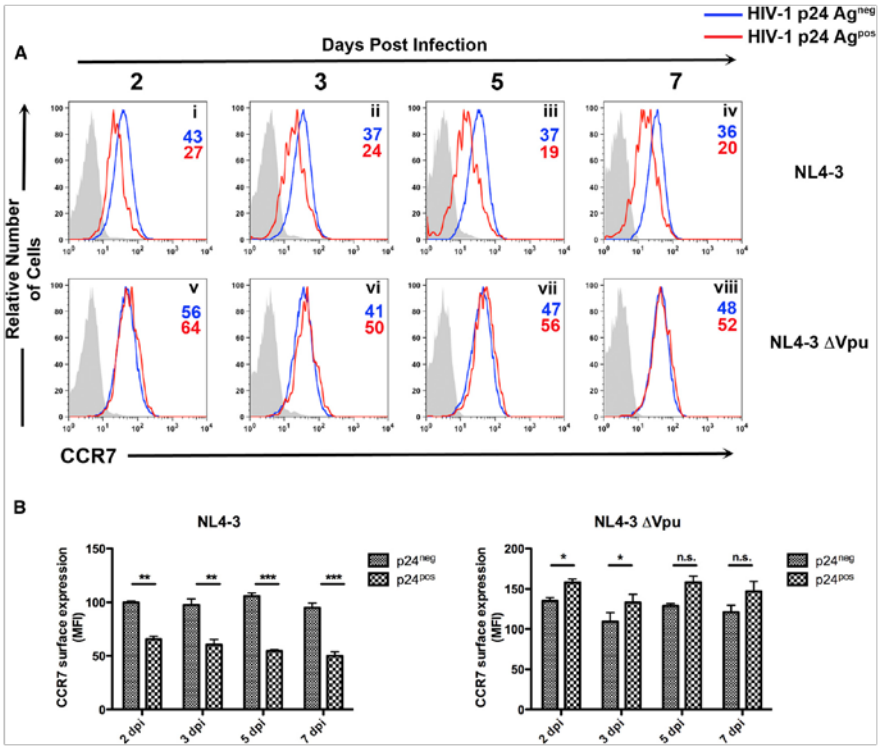

Figure 3. Vpu Downregulates CCR7 in the Context of a Spreading Infection

(A) Primary CD4+ T cells were infected with either HIV-1_{NL4-3} or HIV-1_{NL4-3}ΔVpu at a multiplicity of infection (moi) of 0.1. At 2, 3, 5, and 7 days postinfection, cells were surface stained for CCR7 and permeabilized for detection of p24Gag. Histograms represent $p24Gag^{neg}$ cells (blue line), $p24Gag^{pos}$ cells (red line), or an IgG matched isotype (gray-shaded histogram).

(B) MFI values of the surface levels of CCR7 in HIV-1_{NL4-3} (left) or HIV-1_{NL4-3}ΔVpu (right) infected cells. The data were normalized by setting the MFI values from uninfected (mock) cells to 100% and are depicted graphically as mean ± SEM. The data are representative of three independent experiments in three separate donors (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S3.

CCR7, we stimulated cells with CCL19 (Figure S4, dashed black, blue, and red lines). It is noteworthy that CCL19-induced endocytosis of CCR7 was also unaffected by the presence of Vpu.

The TMD of Vpu Is Required for Downregulation of CCR7

Vpu triggers CD4 proteasomal degradation by linking this protein to the Skp1-Cullin-F-box (SCF)/β-TrCP E3 ubiquitin ligase complex (Kerkau et al., 1997; Margottin et al., 1998). Vpu-mediated BST-2 removal from the surface of HIV-1-infected cells also requires the interaction of Vpu with β-TrCP (Mitchell et al., 2009). Two phosphorylated serines (serines 52 and 56) in the DpSGXXpS motif (where "p" denotes phosphorylation of the following amino acid residue and "X" denotes any amino acid residue) present at the C terminus of Vpu recruit β-TrCP (Everard-Todeschi et al., 2006; Wu et al., 2003). To address whether Vpu interaction with β-TrCP is required for CCR7 downregulation, we used an HIV-1 mutant in which both Vpu serine residues were mutated to asparagine (VpuS52,56N). We anticipated that if an interaction with the SCF/β-TrCP complex were required for CCR7 downregulation, mutation of the serine residues would completely abolish this phenotype, as is the case for downregulation of CD4 (Willey et al., 1992). As shown in Figure 5A (panels ii and iv), VpuS52,56N was still able to downregulate CCR7 (MFI = 71 and 42 for p24− and p24+ cells, respectively; 41% downregulation), although somewhat less efficiently than WT Vpu (MFI = 76 and 29 for p24− and p24+ cells, respectively; 62% downregulation). Therefore, VpuS52,56N retained most of its ability to downregulate surface CCR7. We interpret these results to mean that interaction with the β-TrCP-containing E3 ubiquitin ligase complex is not required for CCR7 surface downmodulation by Vpu.

The TMD of Vpu (residues 4–27) is highly conserved among strains of the pandemic HIV-1 group M (Vigan and Neil, 2010,

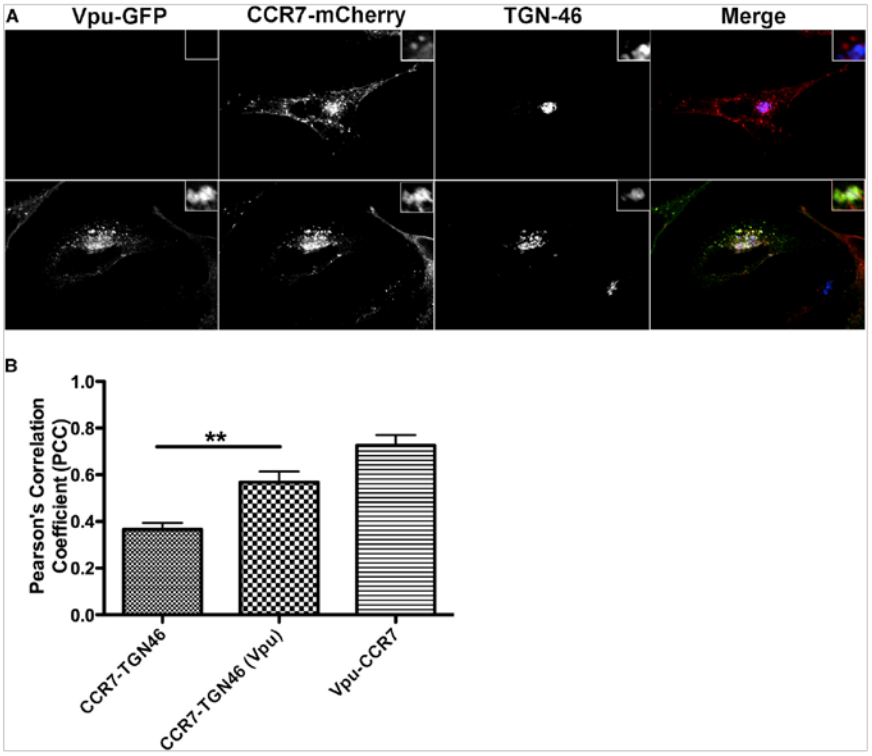

Figure 4. Vpu Colocalizes with CCR7 within the TGN

(A) HeLa cells were transiently transfected with either CCR7-mCherry alone (top row) or in combination with Vpu-GFP (bottom row). At 24 hr posttransfection, cells were fixed, permeabilized, and stained with a TGN-specific antibody (TGN46). Images were acquired using a spinning-disc confocal microscope. Red, CCR7-mCherry; green, Vpu-GFP; blue, TGN46.

(B) Relative colocalization levels between CCR7-TGN46, Vpu-TGN46, or Vpu and CCR7 were quantified using Pearson's correlation coefficient (PCC). Data are graphically depicted as mean ± SEM. The PCC values are representative of ten individual cells (**$p < 0.01$).

See also Figure S4.

2011). Moreover, this region is required for downregulation of NTB-A (Shah et al., 2010), BST-2 (McNatt et al., 2013; Van Damme et al., 2008), and CD4 (Magadán and Bonifacino, 2012; Magadán et al., 2010; Tiganos et al., 1998). To determine whether the TMD of Vpu played a role in CCR7 downmodulation, we infected $CD4^+$ T cells with an HIV-1 mutant encoding Vpu with a scrambled TMD (VpuRD) (Schubert et al., 1996). Infection of cells with HIV-1VpuRD virus failed to induce CCR7 downregulation (Figure 5B, panels ii and iii). We then investigated the specific residues within the TM region that may be critical for Vpu to downmodulate CCR7. Previous studies have shown that the A14, W22, and to a lesser extent A18 residues within Vpu's TMD are important for the downmodulation and interaction of Vpu with BST-2 (Skasko et al., 2012; Vigan and Neil, 2010). Mutation of alanine 14 and tryptophan 22 to phenylalanine and alanine, respectively, completely abolished Vpu-dependent CCR7 downregulation (Figure 5B, panels iv and vi). As previously shown for Vpu-dependent downregulation of BST-2 (Vigan and Neil, 2010), the change of residue 18 of Vpu also had an intermediate effect on CCR7 downregulation (panel v). The A14,18F and A14,18F/W22A mutations also abolished CCR7 downregulation (panels vii and viii). Mutation of serine 23 to alanine (panel ix) or isoleucine 17 to alanine (panel x), however, did not affect CCR7 downmodulation. The above data indicated that CCR7 downregulation requires specific residues in the TMD of Vpu, and these residues are the same as those previously shown to be important for BST-2 downregulation (Vigan and Neil, 2010).

CCR7 Coimmunoprecipitates with Vpu

To address whether Vpu-mediated downregulation of CCR7 required a physical interaction between the viral protein and the chemokine receptor, we transfected 293T cells with a

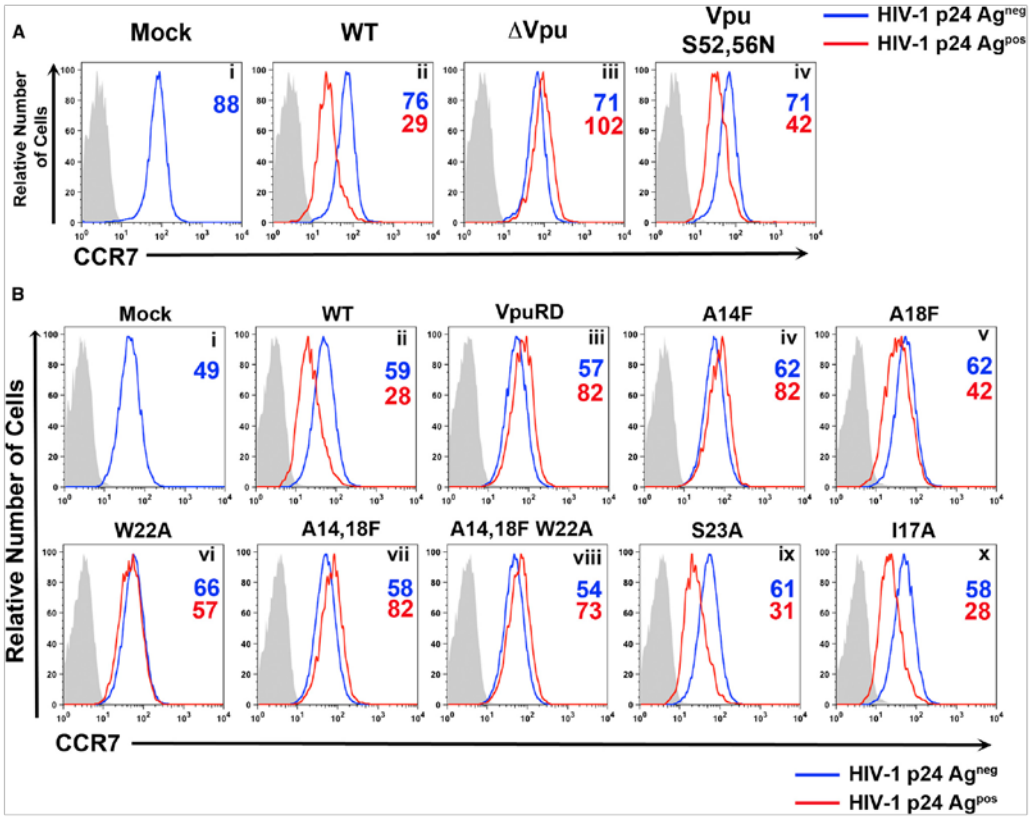

Figure 5. CCR7 Surface Downregulation Requires Vpu's TMD, but Not its Conserved Serines

(A) Primary CD4$^+$ T cells were either mock infected (i) or infected with DHIV (ii), DHIVΔVpu (iii), or DHIV-VpuS52,56N (iv), in which Vpu's conserved serines were mutated to asparagines. All cells were surface stained for CCR7 expression followed by intracellular p24Gag staining, as in Figure 1B.

(B) Primary CD4$^+$ T cells were either mock infected (i) or infected with DHIV (ii) or DHIV-VpuRD (iii), in which the TMD of Vpu was scrambled. Additionally, cells were infected with the indicated Vpu TM mutants (iv–x). Cells were stained and analyzed as described in (A).

plasmid expressing either GFP or Vpu-GFP, including mutants (VpuA14F-GFP, VpuRD-GFP, and VpuS52,56N-GFP) alone or in combination with CCR7-Flag. At 24 hr posttransfection, CCR7-Flag was immunoprecipitated from whole-cell lysates, followed by immunoblotting with anti-GFP. Figure 6 (lane 6) shows that Vpu coimmunoprecipitated with CCR7. Surprisingly, both VpuRD and VpuA14F, mutants that failed to downregulate CCR7, also coimmunoprecipitated with CCR7 (lanes 8 and 10). Interestingly, VpuS52,56N, which also coimmunoprecipitated with CCR7 (lane 12), did not show the upper two bands, which we interpret to be the phosphorylated forms of Vpu at serine 52 and/or serine 56.

The above results indicate that the interaction between CCR7 and Vpu, while necessary, is not sufficient for downmodulation of CCR7 surface levels. Further studies are needed in order to identify other potential requirements, beyond CCR7-Vpu binding, that may exist. One possibility is that interaction(s) of Vpu with additional cellular proteins may be required for the downmodulation of CCR7.

CCL19-Mediated Mobilization of Intracellular Calcium and Erk1/2 Phosphorylation Are Impaired in HIV-Infected CD4$^+$ T cells

Binding of either CCL19 or CCL21 to CCR7 initiates a signaling cascade that leads to the release of calcium from intracellular stores (Wu et al., 2000) and activates extracellular-signal-regulated kinase 1/2 (Erk1/2) (Tilton et al., 2000). In our preliminary tests, primary CD4$^+$ T cells migrated in response to CCL19 more efficiently than they did to CCL21 in vitro (Figure S5). Therefore, to further examine the potential effects of Vpu on CCR7

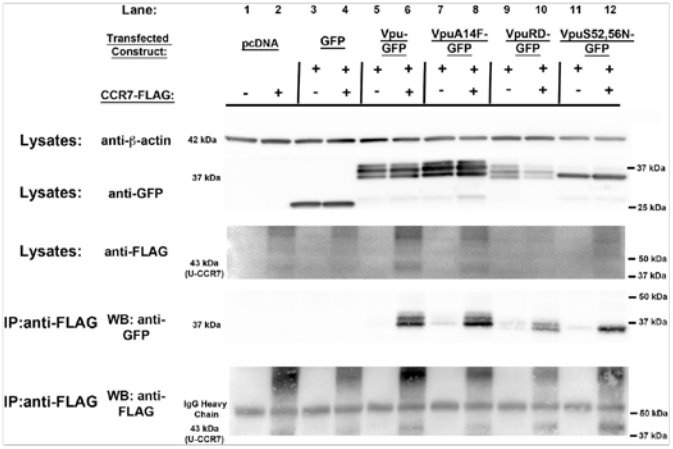

Figure 6. CCR7 Interacts with Vpu

HEK293T cells were transfected with GFP, Vpu-GFP, VpuA14F-GFP, VpuRD-GFP, and VpuS52,56N-GFP either with an empty vector or in combination with CCR7-Flag. At 24 hr post-transfection, cells were lysed and immunoprecipitated using anti-Flag antibody. Lysates were analyzed by western blot and probed for β-actin (42 kDa), GFP (37 kDa), and Flag (43 kDa; unglycosylated form of CCR7 [U-CCR7]) by loading 10 μg of lysate per sample. Antibodies probed against GFP and Flag were used to analyze immunoprecipitates by western blot. IgG heavy chain: 53 kDa. Results are representative of two different experiments.

function, we decided to use CCL19 for the next set of experiments. We first asked whether decreased surface expression of CCR7 in HIV-infected cells impaired signaling by CCL19. To that end, we compared the efficiency of calcium mobilization in infected and uninfected cells after stimulation with CCL19. Cultured T_{CM} cells were infected with a recombinant HIV-1_{NL4-3} construct encoding the murine heat-stable antigen (HSA/CD24) in place of Vpr (Jamieson and Zack, 1998). As shown in Figure 7A, mock-infected and HIV-HSA^{neg} (uninfected) cells responded in a similar fashion to CCL19, with 44.3% and 49.4% of the cells, respectively, increasing the $[Ca^{2+}]_i$ at the lower dose utilized. In contrast, only 28.8% of HIV-HSA^{pos} (infected) cells upregulated $[Ca^{2+}]_i$ in response to the same stimulation.

It is noteworthy that the basal levels (Figure 7A, "No treatment") of $[Ca^{2+}]_i$ were higher in infected cells (2.06%) than in uninfected ones (0.42%). The reason for this difference is unknown. It is possible that the binding of the staining antibody against murine HSA could trigger a modest increase in the $[Ca^{2+}]_i$. The response to ionomycin was comparable in each sample analyzed, indicating that cells similarly incorporated the fluorescent dye and that calcium mobilization in response to other stimuli was preserved and not affected by infection.

Phosphorylation of Erk1/2 is another event triggered by CCL19 binding to CCR7. We predicted that levels of phosphorylated ERK1/2 would be compromised in HIV-infected cells after stimulation with CCL19. To address this, we infected activated $CD4^+$ T cells with viruses that expressed Vpu (DHIV-GFPΔNef) or did not express Vpu (DHIV-GFPΔNefΔVpu). As expected, unstimulated cells infected with either DHIV-GFPΔNef or DHIV-GFPΔNefΔVpu showed no induction of p-ERK1/2 (Figure 7B, gray and black lines). Phorbol myristate acetate (PMA) treatment (positive control) led to p-ERK1/2 levels that were comparable between GFP^{pos} and GFP^{neg} cells infected with either virus. When cells were stimulated with CCL19, the GFP^{neg} population had fairly similar levels of p-ERK1/2 regardless of whether the cultures were infected with DHIV-GFPΔNef (red line; MFI = 46) or DHIV-GFPΔNefΔVpu (blue line; MFI = 60). In contrast, GFP^{pos} cells infected with DHIV-GFPΔNef had reduced levels of p-ERK1/2 (red line; MFI = 113) compared with those infected with DHIV-GFPΔNefΔVpu (blue line; MFI = 217).

Interestingly, the levels of both PMA and p-ERK1/2 were generally higher (regardless of whether the virus was DHIV-GFPΔNef or DHIV-GFPΔNefΔVpu) in GFP^{pos} cells than in GFP^{neg} cells. This may reflect the fact that HIV infection (in particular, the viral protein Tat) may induce cellular stress, leading to the phosphorylation of Erk1/2 (Herbein and Khan, 2008). Therefore, it is likely that phosphorylation of ERK1/2 during HIV-1 infection occurs in response to multiple signals. Taken together, the above results suggest that Vpu-mediated downregulation of CCR7 upon viral infection results in an impaired ability of infected primary $CD4^+$ T cells to respond to CCL19.

Vpu Decreases the Capacity of $CD4^+$ T Cells to Migrate toward CCL19

Based on the above results, we predicted that Vpu downregulation of CCR7 would result in decreased cellular migration in a CCL19 chemokine gradient. To test our hypothesis, we infected primary $CD4^+$ T cells with either HIV-1_{NL4-3} or HIV-1_{NL4-3}ΔVpu. At 5 days postinfection, cells were placed in the upper chamber of a transwell plate and allowed to migrate toward either medium alone or chemokine ligands specific for CCR7 (CCL19) or CXCR4 (SDF1α). The cells in the lower chambers of the transwell plates were then fixed and permeabilized, stained for p24Gag, and enumerated. As shown in Figure 7C, HIV-1_{NL4-3}-infected cells showed a decreased ability to migrate toward CCL19 relative to noninfected (NI) cells as well as cells infected with HIV-1_{NL4-3}ΔVpu. Migration defects were not observed in response to an SDF1α gradient. Interestingly, cells infected with HIV-1_{NL4-3}ΔVpu showed a slightly enhanced ability to migrate toward CCL19, but not toward SDF1α, relative to NI cells. Taken together, these results indicate that Vpu negatively modulates the chemotactic potential of primary $CD4^+$ T cells to migrate specifically toward CCL19.

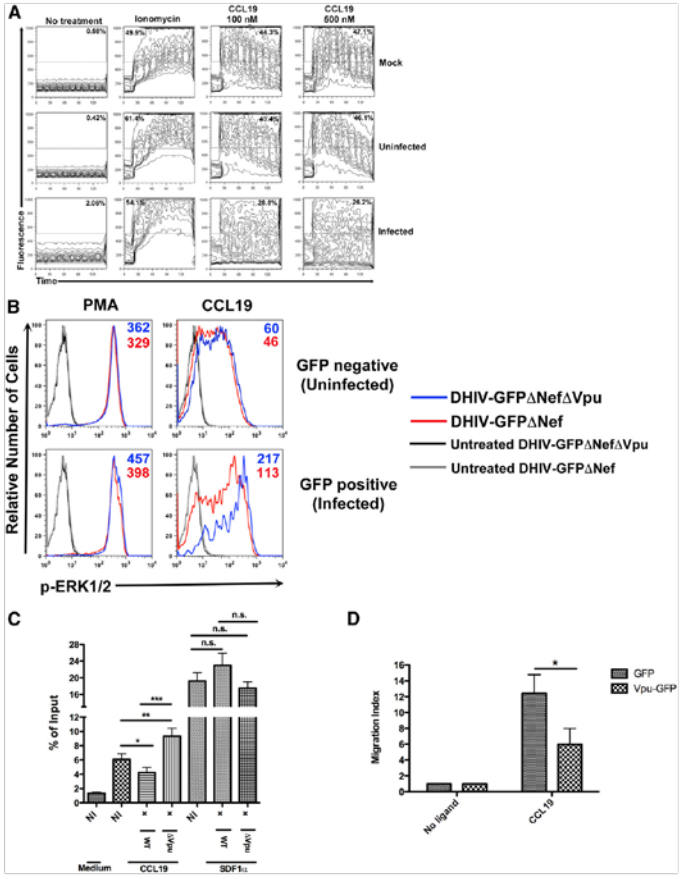

Figure 7. CCL19-Mediated Chemotaxis and Chemotactic Signaling Responses Are Impaired in HIV-1-Infected Primary CD4$^+$ T Cells

(A) Primary CD4$^+$ T cells were either mock infected or infected with DHIV-HSA. At 2 days postinfection, the cells were loaded with Fluo3-AM followed by surface staining for HSA. Cells were left untreated ("No treatment") or stimulated with ionomycin (20 ng/ml) or with either 100 nM or 500 nM CCL19. Changes in fluorescence were recorded over time by flow cytometry. The figure is representative of two independent experiments in two different donors.

(B) Primary CD4$^+$ T cells were either mock infected or infected with the virus DHIV-GFPΔNef or DHIV-GFPΔNefΔVpu. Forty-eight hours later, the cells were stimulated with either 5 ng/ml PMA or 50 ng/ml CCL19 for 5 min and immediately stained to detect ERK1/2 phosphorylation. The histograms depicted are split into GFP− (uninfected) and GFP+ (infected) rows. The blue line depicts cells infected with Vpu− virus, and the red line depicts cells infected with Vpu+ virus. At least 5 × 10^4 viable GFP+ and GFP− cells were collected via flow cytometry. The figure is representative of two independent experiments performed in two different donors.

(C) HIV-1_{NL4-3} or HIV-1_{NL4-3}ΔVpu was used to infect primary CD4$^+$ T cells at an moi of 0.1. At 5 days postinfection, cells were placed in the upper chamber of a transwell, and either medium alone or medium containing CCL19 or SDF1α was put into the lower chamber. After 1 hr, the percentages of T cells that migrated towards the lower chamber in response to medium or ligand relative to total cells stained (input) were calculated. Data are depicted as the mean ± SEM and are representative of four independent experiments performed in duplicate and in different donors (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(D) CCRF-CEM cells transfected with GFP or Vpu-GFP plasmids were placed in the upper chamber of a transwell, and either medium or CCL19 was put into the lower chamber. After 3 hr, the number of GFP-expressing T cells that were attracted toward the medium was calculated and is depicted as a migration index (MI). Data are represented as mean ± SEM of three independent experiments performed in duplicate (*$p < 0.05$).

See also Figure S5.

Finally, to also determine whether Vpu was sufficient to induce an impaired chemotactic response, CCRF-CEM cells were nucleofected with expression plasmids encoding GFP or Vpu-GFP. Twenty-four hours later, the cells were subjected to in vitro transmigration assays. As shown in Figure 7D, Vpu-GFP-expressing cells showed a reduced capacity to migrate toward CCL19 relative to GFP-expressing cells (migration index [MI] of 6 versus 12), indicating that Vpu alone was still able to cause a CCR7-specific defect in cellular migration.

DISCUSSION

Naive and central memory CD4$^+$ T cells are characterized by the ability to continuously transition through secondary lymphoid organs, where cognate antigen is expressed by professional antigen-presenting cells. In order for T cells to correctly home to peripheral lymphoid sites, cellular migration is orchestrated by chemokines and chemokine receptors. The chemokine receptor CCR7 and its two known ligands, CCL19 and CCL21, are crucial factors in this process. Previous reports showed that HIV-1 infection interferes with T cell recirculation, mostly by accelerating T cell differentiation and promoting a CCR7^{low} phenotype (Pantaleo and Harari, 2006; Younes et al., 2003). Perez-Patrigeon et al. (2009) found that chemotaxis triggered by CCL19 was impaired in naive, central memory, and effector memory T cells from HIV-infected patients, although they did not find differences in CCR7 surface expression levels between T cells of HIV-1-infected patients and those of healthy subjects.

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Thus, the findings by Perez-Patrigeon et al. may be in contradiction to our observations in this study. However, one notable difference between the two studies is that Perez-Patrigeon et al. did not analyze CCR7 surface levels in a manner that discriminated between infected and uninfected cells. It is also worth noting that a recent report disputed the involvement of CCR7 in the trafficking of memory $CD4^+$ T cells from blood into the lymph nodes (Vander Lugt et al., 2013).

Surprisingly, immunoprecipitation of CCR7 from cells expressing WT Vpu, VpuRD, or VpuA14F (with the latter two being unable to downregulate CCR7) showed a physical interaction in all cases. We surmised that the interaction between the two proteins, although necessary, was not sufficient toward Vpu-mediated modulation of CCR7. According to the solid-state nuclear magnetic resonance structure of the HIV-1_{NL4-3} Vpu TMD in lipid membranes (Marassi et al., 1999; Park et al., 2003; Skasko et al., 2012), residues A14, A18, and W22 form a diagonal line on the TM α-helix. Although previous studies implicated these residues as potential points of contact between Vpu and BST-2 (Kobayashi et al., 2011; Rong et al., 2009; Skasko et al., 2012; Vigan and Neil, 2010), McNatt et al. (2013) recently showed that such residues are responsible for maintaining the overall structure of Vpu TMD, rather than constituting points of interaction. Our experiments confirm that residues A14, W22, and to a lesser extent A18 in Vpu are important for CCR7 downmodulation.

Alteration of immune cell functionality is a hallmark of many viral infections. Specifically, CCR7 expression levels on DCs have been found to be downregulated by cytomegalovirus infection (Moutaftsi et al., 2004) and by HHV-8 (Cirone et al., 2012), with a consequent decrease in the ability of cells to migrate to peripheral lymphoid organs and coordinate the immune response. The data presented here suggest that in a similar fashion, HIV-1 may inhibit migration of $CD4^+$-infected T cells to peripheral lymphoid tissues, possibly hindering the initiation of effective immune responses.

EXPERIMENTAL PROCEDURES

Cells and Plasmids

For details on in vitro cultured T_{CM} cells, see Supplemental Experimental Procedures. The T-lymphoblastoid CCRF-CEM cell line was maintained in RPMI complete (supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-L glutamine). Human embryonic kidney 293T (HEK293T) and HeLa cells were cultured in Dulbecco's modified Eagle's medium complete. For a detailed description of the plasmids used in this work, see Supplemental Experimental Procedures. Studies involving primary $CD4^+$ T cells were covered under protocol #IRB_00067637 approved by the University of Utah Institutional Review Board.

Transfections, Coimmunoprecipitation, and Immunoblots

For overexpression of Vpu, CCRF-CEM cells were nucleofected with pAcGFP or pAcGFP-Vpu using the Amaxa Nucleofector Kit C (Lonza). For coimmunoprecipitation, HEK293T cells were transfected via calcium phosphate with the indicated plasmids and processed as explained in Supplemental Experimental Procedures.

Flow Cytometry

Detection of both surface antigens and intracellular p24Gag was previously described (Ward et al., 2009). Total levels of CCR7 or CD4 and p24Gag within cells were detected by simultaneous staining with anti-APC-CCR7 or anti-APC-CD4 antibodies along with mouse-FITC-anti-p24. Surface levels of BST-2 were analyzed by staining cells with anti-BST2 (NIH AIDS Reagent Program; Dr. Klaus Strebel) and then staining cells with a goat anti-rabbit secondary antibody coupled to Alexa 647 (Molecular Probes, Invitrogen).

To measure relative levels of p-ERK1/2, cells were stimulated with CCL19 for 5 min at 37°C. Cells were immediately fixed in 2% formaldehyde (Polysciences) and permeabilized in 90% ice-cold methanol. They were then labeled with anti-p-ERK1/2 (Thr202/Tyr204), followed by staining with a goat anti-rabbit secondary antibody coupled to Alexa 647.

Calcium Mobilization Assay

Intracellular calcium mobilization was measured in primary $CD4^+$ T cells infected with DHIV-HSA virus, encoding HSA/CD24 in place of Vpr, according to the procedure described in Supplemental Experimental Procedures.

Immunofluorescence Microscopy

HeLa cells were transfected and stained as described in Supplemental Experimental Procedures. Images were acquired on an Olympus FV-1000 using a 60× oil lens, and quantification was performed using the Velocity 3D image analysis software (Perkin-Elmer).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.05.015.

AUTHOR CONTRIBUTIONS

P.W.R. and M.F. designed and performed the experiments, analyzed data, and wrote the manuscript. B.S. and E.B. provided materials. A.B.D.-S. designed and performed experiments. C.R. analyzed data. A.B. and V.P designed experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTAL INFORMATION

Downmodulation of CCR7 by HIV-1 Vpu Results in Impaired Migration and Chemotactic Signaling Within $CD4^+$ T Cells

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Figure S1

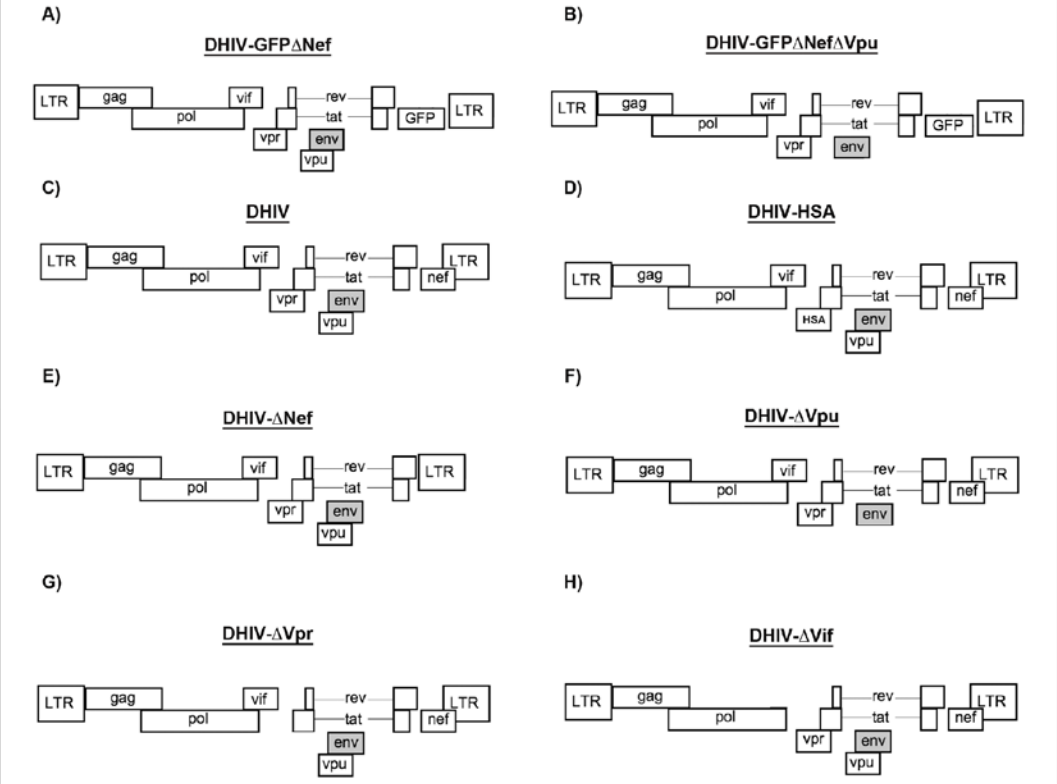

Figure S1: DHIV Constructs. (Related to Figure 1) The HIV-1_{NL4-3} sequence was cut between two BglII sites to efficiently delete envelope/gp120 (gray box) but maintain in-frame Tat, Rev and RRE ORFs. This mutant lentiviral vector was thus termed "defective" HIV, or DHIV. All constructs, with the exception of HIV-1_{NL4-3} and HIV-1_{NL4-3}ΔVpu, used in this study were derived from the DHIV backbone through either: i.) Introduction of a frameshift mutation within each of the accessory genes ; ii.) The replacement of Vpr with the mouse heat stable antigen (HSA) gene or iii.) The GFP gene in place of Nef. A.) DHIVGFPΔNef, B.) DHIVGFPΔNefΔVpu, C.) DHIV D.) DHIV-HSA E.) DHIV-ΔNef F.) DHIV-ΔVpu G.) DHIV-ΔVpr H.) DHIV-ΔVif.

Figure S2

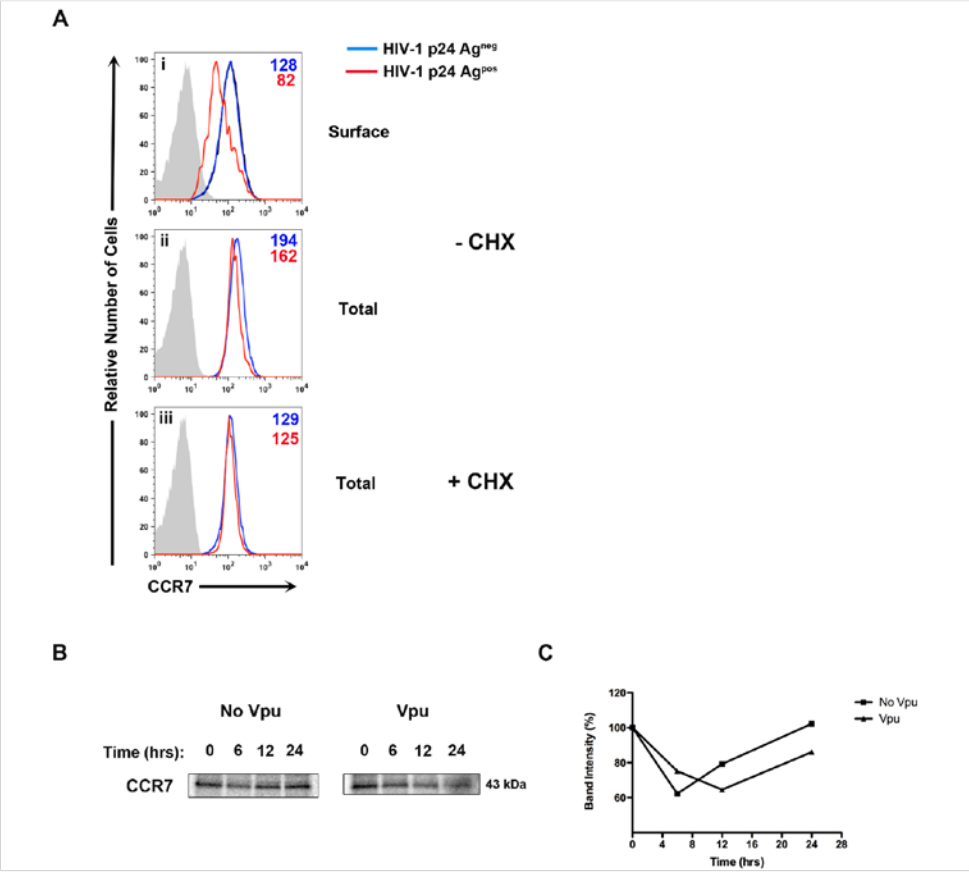

Figure S2: Vpu does not degrade CCR7. (Related to Figure 2) A.) Activated $CD4^+$ T cells were infected with DHIV. Two days later, cells were incubated in either the absence or presence of cyclohexamide (10 μg/ml) to block new protein synthesis. Twenty-four hours later, cells were either surface stained for CCR7 or fixed and permeabilized to measure total levels of CCR7. HIV infection was assessed through intracellular staining and detection of p24Gag. A comparison between uninfected $p24Gag^{neg}$ cells (blue line) and infected $p24Gag^{pos}$ cells (red line) are depicted in each histogram along with an IgG matched isotype control (gray shaded histogram). Figure is representative of two independent experiments performed in two different donors. B.) 293T cells were co-transfected with CCR7-Flag and expression vectors encoding either GFP or a Vpu-GFP fusion protein. 24 hours later, cells were pulse labeled with [^{35}S]-methionine for 30 minutes. Cells were then chased for up to 24 hours and lysates collected at each time point indicated. CCR7 was then immunoprecipitated using an anti-Flag antibody, lysates separated by SDS-PAGE and analyzed by autoradiography. C.) Quantification of the kinetics of CCR7 protein turnover derived from B.

Figure S3

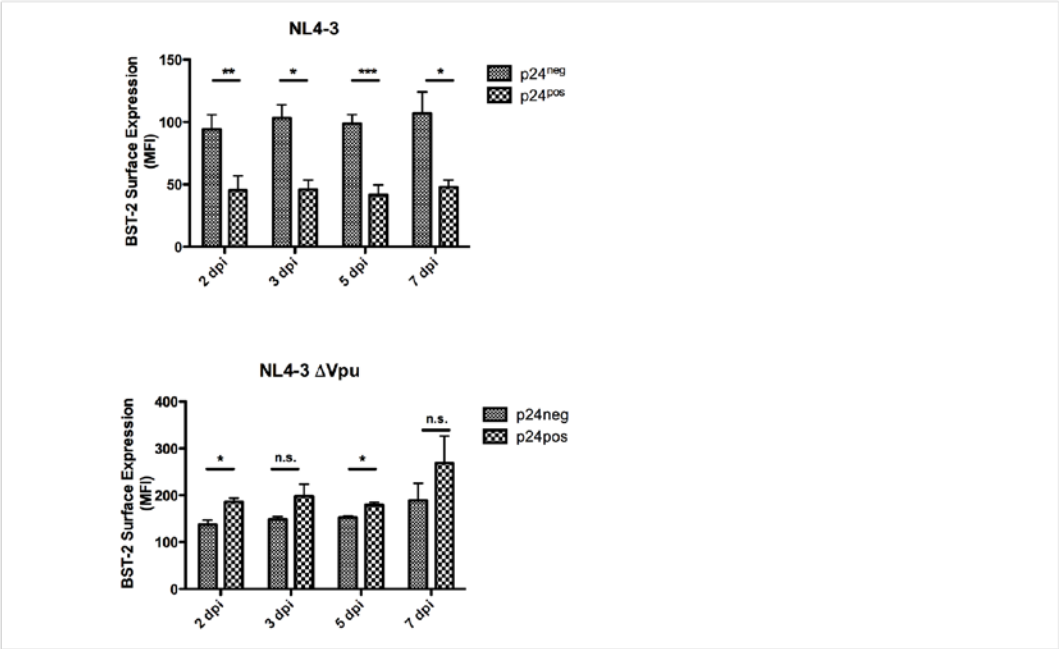

Figure S3: BST-2 is downregulated from the surface of HIV-1_{NL4-3} infected cells. (Related to Figure 3) Primary $CD4^+$ T cells were infected with either HIV-1_{NL4-3} or HIV-$1_{NL4-3}\Delta$Vpu at an MOI=.1. At two, three, five and seven days post infection, cells were surface stained for BST-2, followed by intracellular staining for HIV-1 p24Gag. Data was normalized by setting MFI values from uninfected (mock) cells to 100% and is depicted graphically as +/- mean SEM and is representative of three independent experiments performed in three separate donors.
* = $p < 0.05$ ** = $p < 0.01$ *** = p,0.001

Figure S4

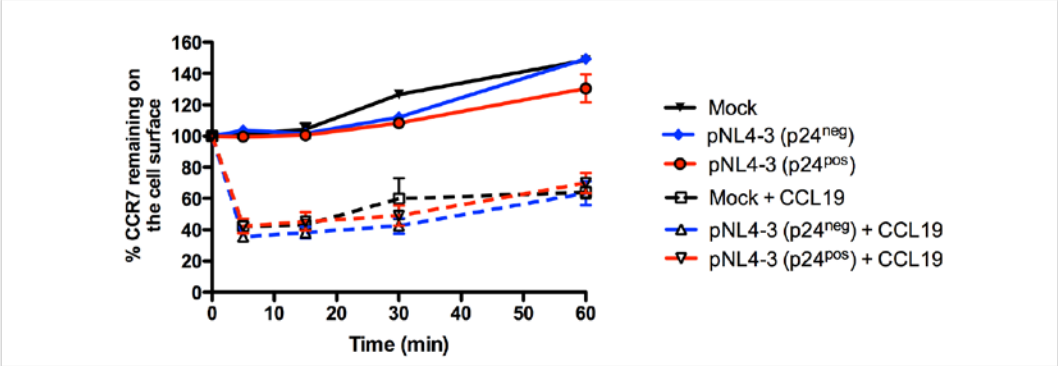

Figure S4: Vpu does not increase the endocytosis rate of CCR7. (Related to Figure 4) Primary $CD4^+$ T cells were infected with HIV-1_{NL4-3} at an MOI=.1. Seven days post infection, cells were or were not treated with CCL19 (1µg/ml) for 10 minutes at 37°C. Next, cells were stained with an antibody against CCR7 to label all surface bound receptor for 30 minutes at 4°C. Cells were then washed with cold PBS, split into different tubes and either placed back at 4°C (Time 0) or at 37°C for 5,15,30 or 60 minutes to induce receptor endocytosis. At each time point, cells were immediately placed on ice, fixed and placed at 4°C. When all time points were collected, cells were stained with an Alxa-Fluor647 (AF647) conjugated secondary antibody followed by permeabilization for detection of $p24Gag^{Ag}$. Data was graphed as: (the MFI value at each time point (unstimulated or CCL19 stimulated cells) / MFI value of unstimulated cells at Time 0) x 100. Solid color lines = Unstimulated cells ; Dashed color lines = CCL19 stimulated cells. Mock = solid / dashed black line ; $p24Gag^{neg}$ = solid / dashed blue line ; $p24Gag^{pos}$ = solid / dashed red line. Figure is representative of two independent experiments performed in two different donors.

Figure S5

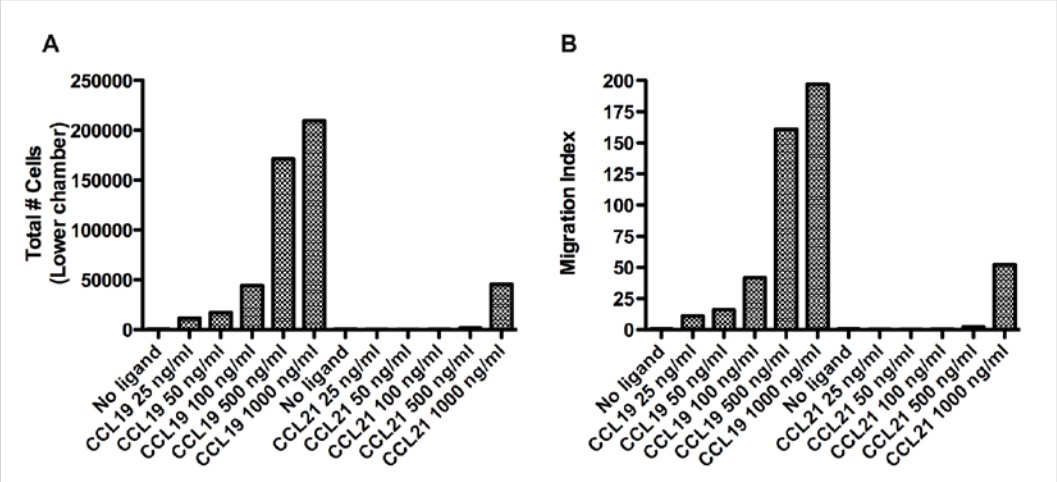

Figure S5: *In vitro* cultured T_{CM} do not respond to CCL21 as efficiently as they do to CCL19. (Related to Figure 7) 4 x 10^5 activated primary $CD4^+$ T cells were incubated for 1 hour at 37°C in transwell chambers in chemotaxis medium (RPMI 1640 + .5% BSA). Either the number of total cells present within the lower chambers (A) or a migration index ((B) ; number of total cells present in lower chambers in response to ligand / number of cells present in lower chamber with medium alone) is depicted.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Plasmids: The plasmid pcDNA3.1 (Invitrogen) was used as an empty vector control. pAcGFP-N1 (Clontech, Mountain View, CA) and pAcGFP-Vpu were previously described (Shah et al., 2010). The vectors expressing VpuA14F-GFP, VpuRD-GFP and VpuS52,56N-GFP were generated within the pAcGFP-Vpu by site-direct mutagenesis PCR (Stratagene) and confirmed by sequencing. All DHIV viruses mutated in Vpu were generated within the DHIV backbone by QuikChange XL site-directed mutagenesis PCR (Stratagene) and confirmed by sequencing. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 (Cat. # 114) from Dr. Malcolm Martin. pNL4-3ΔVpu was constructed by placing a stop codon at the start of the Vpu sequence. CCR7 cDNA (Sino Biological, Inc.) was PCR amplified and tagged at the carboxy terminus by subcloning into pGEM-mCherry (Addgene, Cambridge, MA). To construct pBSXC-CCR7-mCherry, pGEM-CCR7-mCherry was digested with XhoI and EcoRI and the 1.8kb fragment was ligated into the BSXC vector. To construct pCMVG-CCR7-Flag, CCR7 was isolated from pBSXC-CCR7-mCherry by digestion with SpeI and XhoI and then subcloned into pCMVG-GAS2L1 (previously digested with NotI and SalI).

Generation of *in vitro* cultured T_{CM}: Naïve $CD4^+$ T cells were isolated from peripheral blood mononuclear cells of healthy, anonymous donors, using the appropriate isolation kit (Miltenyi Biotec). Cells were cultured for three days in complete medium (RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-L glutamine) in the presence of αCD3/αCD28 immuno-beads (Invitrogen), αIL-4 (Peprotech) and αIL-12 (Peprotech) as previously described (Bosque and Planelles,

2009). At day 3, activating and polarizing stimuli were removed and cells were cultured in presence of IL-2 (30 UI/ml) for 2 additional days. Cells were then infected with the specific viruses used in each experiment and then cultured with IL-2 until the time of analysis.

Viruses and infections: The production of pseudotyped viruses was accomplished by co-transfection of 20 μg of DHIV and 5 μg of CXCR4-tropic envelope plasmid (named pLET-LAI) by calcium phosphate mediated transfection of HEK293T cells. Eighteen hours later, transfection medium was removed and replaced by fresh complete medium. Cellular supernatants containing the viral particles were recollected 48 hours post transfection, aliquotted and immediately stored at -80°C. Viral titer was assessed using the RETROtek p24 ELISA kit (ZeptoMatrix, Buffalo, NY). For the production of replication competent HIV-1_{NL4-3} and HIV-$1_{NL4-3}\Delta$Vpu viruses, 25μg of plasmid were transfected into 293T cells as described above. SupT1 cells were then infected to determine an optimal infectious dose to use in primary cells. At day 5 post activation, primary $CD4^+$ T cells generated as explained above were infected by spinoculation: 10^6 cells were infected with 500 ng/mL p24 for 2 hours at 2900 rpm and 37°C in 1 mL. Alternatively, cells were infected with HIV-1_{NL4-3} and HIV-$1_{NL4-3}\Delta$Vpu at an MOI=.1 via spinoculation. Virus was then removed and cells were resuspended in complete medium supplemented with IL-2 at a concentration of 10^6 cells/ml. Medium was replaced every 2-3 days.

Co-immunoprecipitation and immunoblots: Twenty-four hours post transfection, cells were lysed for two hours on ice in NETN buffer (100 mM NaCl, .5 mM EDTA , 20mM

Tris-Hcl , .0.5% NP-40) containing protease and phophatase inhibitors (Roche). Protein concentration was measured by Bradford Assay (Pierce). Anti-Flag was then conjugated to Dynabeads Protein G (Invitrogen) for 30 min at RT with rotation. Next, cell lysates (100 μg) were incubated with the anti-flag/dynabead mixture for 1 hr at 4°C with rotation to allow for precipitation. Immunoprecipitates and 10 μg total-cell lysates were then run on a 4-12% denaturing polyacrylamide precast gel (BioRad, Hercules, CA). Proteins were transferred to a methanol-soaked polyvinylidene difluoride (PVDF) membrane, followed by blocking in 5% skim milk solution in Tris-buffered saline with .1% Tween 20 (Calbiochem). Membranes were probed overnight with primary antibodies diluted in 2% skim milk solution at 4°C with rotation. The next day, membranes were probed with secondary antibodies for 2 hrs in 2% skim milk solution. Specific antibody reactions were detected using Immobilon Western reagents (Millipore) and membranes developed in a GeneGnome bioimaging processor (Syngene, Frederick, MD).

Metabolic labeling and immunoprecipitation: 293T cells were cotransfected with 1μg pCMVG-CCR7-Flag and either 1μg pAcGFP-N1 or pAcGFP-Vpu. Twenty-four hours post-transfection, cells were starved for 30 minutes in methionine- and cysteine-free DMEM medium (Life Technologies). Cells were then pulse labeled with 100μCi/ml [^{35}S] for 30 minutes at 37°C and then immediately harvested (Time 0) or complete DMEM medium (Life Technologies) added to cells for the start of the chase period, which lasted 24 hours. At the time points indicated, cells were washed in PBS and pelleted by centrifugation, followed by lysis in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8], 159 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid) containing protease and phophatase inhibitors (Roche) on ice for 5 minutes. Soluble

protein was then collected following centrifugation and lysates stored at -80°C. To immunoprecipitate CCR7, protein concentration was first measured by Bradford Assay (Pierce). Anti-Flag antibody (Sigma) was then conjugated to Dynabeads Protein G (Invitrogen) for 30 min at RT with rotation. Next, cell lysates (150 μg) were incubated with the anti-flag/dynabead mixture for 1.5 hrs at 4°C with rotation to allow for precipitation. Immunoprecipitates were then run on a 4-12% denaturing polyacrylamide precast gel (BioRad, Hercules, CA). Gels were fixed (20% MeOH ; 7.5 % Glacial Acetic Acid) for 30 minutes and dried for two hours on a Gel Dryer (BioRad). Finally, gels were developed in a storage phosphor screen (Molecular Dynamics) and scanned using a Typhoon Phosphorimager (GE Healthcare), followed by densitometric quantification performed with the ImageQuant software (Molecular Dynamics).

Immunofluorescence Microscopy: HeLa cells were grown on glass coverslips and transfected with CCR7-mcherry and Vpu-GFP plasmids using the Lipofectamine 2000 reagent (Life Technologies). 24 hrs later, cells were washed, fixed with 4% paraformaldehyde and permeabilized/blocked (10% rabbit serum and 0.2% Triton-X, in PBS), each for 30 minutes at room temperature (RT). Cells were then immunostained for 1 hour at RT using sheep anti-human TGN46 (Antibody Serotec) followed by a rabbit secondary antibody coupled to Alexa 647 (Jackson Immunoresearch) for 30 minutes at RT. Cells were counterstained with Hoescht and mounted on slides using FluorSave Reagent (Calbiochem).

Calcium mobilization assay: Primary $CD4^+$ T cells were infected with a DHIV-HSA virus encoding the heat-stable antigen (HSA/CD24) in place of Vpr, Two days post

infection, 3 million cells were loaded with 4 μg/ml Fluo3-AM (Life Technologies) in 1 ml loading buffer (Hank's Balanced Salt Solution + 1% FBS) for 30 minutes at 37°C in the dark. Cells were washed and stained with APC-Rat Anti-Mouse CD24 (BD-Pharmingen) for 15 minutes at room temperature in the dark, followed by two washing steps. Cells were split at 500,000 cells/tube and resuspended in 500 μl of loading buffer.

Migration assays: Chemotaxis of activated primary $CD4^+$ T cells was measured by migration of cells through a polycarbonate filter of 5 μm pore size in transwell chambers (Corning Costar, Lowell MA). Cells (4×10^5; 100 μl) were added to the upper chamber and either medium alone (RPMI supplemented with 0.5% BSA) or medium plus ligand (50 ng/ml CCL19 or 25 ng/ml SDF1α ; 600 μl total) were added to the lower chambers. After a 1 hour incubation at 37°C, cells from the lower chamber were fixed, permeabilized and stained for p24Gag. For cell enumeration, 10^5 AccuCount Fluorescent Particles (Spherotech) were collected via flow cytometry to determine the total number of cells that migrated relative to the input (direct staining of 4×10^5 cells). CEM-CCRF cells were nucleofected with 2μg of either GFP or Vpu-GFP. Twenty-four later, chemotaxis was measured as described above except 3×10^5/100 μl cells were added to the upper chamber, a concentration of 1000 ng/ml CCL19 was used and cells were incubated for 3 hours at 37°C. Data is depicted as a Migration Index (MI) score, which was calculated using the following formula: # of cells in sample / # cells in control (absence of ligand).

Antibodies used for flow cytometry: The following human mAb were used: phycoerythrin-conjugated (PE)-anti-CD45RO, PE-anti-CXCR4, PE-anti-CD27, allophycocyanin-conjugated (APC)-anti-CCR7 (Caltag, Burlingame, CA), APC-anti-CD4

(Life Technologies), fluoresceinisothiocyanate-conjugated (FITC)-anti-CCR5 (BD Biosciences) mouse-(FITC)-anti-p24 antibody (clone KC57, Beckman Coulter), anti-p-ERK1/2 (Thr202/Tyr204) rabbit monoclonal antibody (Cell Signaling), goat anti-rabbit secondary antibody coupled to Alexa 647 (Molecular Probes, Invitrogen), goat anti-mouse secondary antibody coupled to Alexa 647 (Molecular Probes, Invitrogen). The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-Bst-2 (Cat. # 11722) from Drs. Klaus Strebel and Amy Andrew.

Statistics: A paired Student's t test was used to perform statistical analysis, with P values of <0.05 considered statistically significant.

SUPPLEMENTAL REFERENCES

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Shah, A.H., Sowrirajan, B., Davis, Z.B., Ward, J.P., Campbell, E.M., Planelles, V., and Barker, E. (2010). Degranulation of natural killer cells following interaction with HIV-1-infected cells is hindered by downmodulation of NTB-A by Vpu. Cell Host Microbe *8*, 397-409.

CHAPTER 3

HIV-1 VPU UTILIZES BOTH CULLIN-RING LIGASE (CRL) DEPENDENT AND INDEPENDENT MECHANISMS TO DOWNMODULATE HOST PROTEINS

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HIV-1 Vpu utilizes both cullin-RING ligase (CRL) dependent and independent mechanisms to downmodulate host proteins

Peter W Ramirez[1†], Ana Beatriz DePaula-Silva[1†], Matt Szaniawski[1], Edward Barker[2], Alberto Bosque[1] and Vicente Planelles[1*]

Abstract

Background: Hijacking of the cullin-RING E3 ubiquitin ligase (CRL) machinery is a common mechanism employed by diverse groups of viruses for the efficient counteraction and degradation of host proteins. In particular, HIV-1 Vpu usurps the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex to mark CD4 for degradation by the 26S proteasome. Vpu also interacts with and downmodulates a number of other host proteins, including the restriction factor BST-2. However, whether Vpu primarily relies on a cullin-dependent or -independent mechanism to antagonize its cellular targets has not been fully elucidated.

Results: We utilized a sulphamate AMP analog, MLN4924, to effectively block the activation of CRLs within infected primary $CD4^+$ T cells. MLN4924 treatment, in a dose dependent manner, efficiently relieved surface downmodulation and degradation of CD4 by NL4-3 Vpu. MLN4924 inhibition was highly specific, as this inhibitor had no effect on Nef's ability to downregulate CD4, which is accomplished by a CRL-independent mechanism. In contrast, NL4-3 Vpu's capacity to downregulate BST-2, NTB-A and CCR7 was not inhibited by the drug. Vpu's from both a transmitted founder (T/F) and chronic carrier (CC) virus preserved the ability to downregulate BST-2 in the presence of MLN4924. Finally, depletion of cellular pools of cullin 1 attenuated Vpu's ability to decrease CD4 but not BST-2 surface levels.

Conclusions: We conclude that Vpu employs both CRL-dependent and CRL-independent modes of action against host proteins. Notably, we also establish that Vpu-mediated reduction of BST-2 from the cell surface is independent of β-TrCP and the CRL- machinery and this function is conserved by Vpu's from primary isolates. Therefore, potential therapies aimed at antagonizing the activities of Vpu may need to address these distinct mechanisms of action in order to achieve a maximal effect.

Keywords: CRL, Vpu, MLN4924, Nef, CD4, BST-2, NTB-A, CCR7

Background

Cullin-RING ligases (CRLs) constitute an important group of ubiquitin ligases and play a prominent role in the efficient regulation of protein turnover and homeostasis [1]. In particular, a recurring theme among viruses from distant families is their common ability to usurp CRL complexes with the aim of evading host control mechanisms. Notably, the HIV-1 accessory protein Vif hijacks a cullin-5 containing ubiquitin ligase complex (CRL5) to target cytidine deaminases of the APOBEC3 family for proteasomal degradation [2–5]. Similarly, the HIV-2 accessory protein Vpx relies on a CRL4 complex to degrade the restriction factor SAMHD1 [6, 7].

Activation of CRLs is dependent on a process known as neddylation. This post-translational modification involves the covalent addition of the NEDD8 protein, a relative of ubiquitin, onto a lysine residue on the cullin backbone. Neddylation induces a conformational change

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in the CRL complex that turns the enzyme catalytically active, allowing the transfer of ubiquitin to a substrate [8]. MLN4924, a potent inhibitor of the E1 neddylation enzyme NAE (Nedd8-activating enzyme), blocks the activity of all CRLs but does not affect non-cullin ubiquitin ligases [9]. Previous studies have shown that MLN4924 can potently block Vif-mediated proteasomal degradation of APOBEC3G [10]. Furthermore, in the context of HIV-2, MLN4924 inhibited the degradation of SAMHDI induced by Vpx, phenocopying the absence of Vpx in HIV-2 and restoring efficient restriction of the virus in myeloid cells [11–13].

The HIV-1 accessory protein Vpu, in addition to counteracting the restriction factor BST-2/tetherin [14, 15] and downregulating CD4, antagonizes multiple immune system molecules. Binding of Vpu's phospho-serine residues to the F-box protein β-TrCP forms an $SCF^{\beta\text{-TrCP}}$ (CRL1) complex that targets CD4 for proteasomal degradation [16, 17]. With regards to BST-2, counteraction is thought to be triggered by Vpu's acidic di-leucine motif manipulating Adaptor-Protein 1 (AP-1) to mislocalize BST-2 towards a perinuclear compartment (*trans*-Golgi network-TGN) [18, 19]. However, the requirement for β-TrCP in BST-2 antagonism by Vpu has remained controversial. For other Vpu targets, specifically NTB-A and CCR7, a cullin-independent mechanism of downregulation has been proposed [20, 21].

In this study, we asked whether pharmacological inhibition of the $SCF^{\beta\text{-TrCP}}$ complex by MLN4924 would reveal whether cullin activity is important for Vpu to downmodulate its cellular targets. We hypothesized that downregulation of BST-2, CCR7 and NTB-A by Vpu would not be impacted by MLN4924 treatment. Moreover, we predicted that Vpu downregulation (and degradation) of CD4 would be relieved by MLN4924. Finally, we sought to determine whether Vpu-mediated cell surface downregulation of BST-2 is a function that can be dissociated from BST-2 degradation and that is cullin-independent.

Results

Pharmacological inhibition of CRL-activity disables NL4-3 Vpu's ability to downregulate CD4, but not BST-2, CCR7 or NTB-A

To determine whether Vpu can act as multifunctional protein capable of downregulating host proteins in the absence of neddylation and a functional $SCF^{\beta\text{-TrCP}}$ complex, primary $CD4^+$ T cells were infected with either an HIV-$1_{NL4\text{-}3}$-derived, replication-defective virus carrying GFP in place of Nef (DHIVGFP(Vpu+/Nef−); Fig. 1a), or with an isogenic virus lacking both Nef and Vpu (DHIVGFP(Vpu−/Nef−); Fig. 1b) [21]. All viruses were pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G). We utilized the above *nef*- and *env*-deficient viruses such that the known activities of Nef and Env on CD4 would not interfere with that of Vpu (reviewed in [22]). Two days post infection, cells were incubated in either DMSO (solvent) or MLN4924 and protein surface expression analyzed by flow cytometry 24 h later. As expected, the virus devoid of Nef and Vpu (DHIVGFP(Vpu−/Nef−)) showed similar surface levels of CD4, BST-2, CCR7 and NTB-A when comparing GFP-negative (uninfected) and -positive (infected) cells (Fig. 2a, panels ii, viii, xiv, xx). Downregulation of CD4, BST-2, CCR7 and NTB-A was apparent in cells that were infected with DHIVGFP(Vpu+/Nef−) and treated with DMSO (Fig. 2a, panels iii, vix, xv, xxi). However, MLN4924 relieved downmodulation of CD4 in a dose-dependent manner (Fig. 2a, panels iii–vi, b). In contrast, downregulation of BST-2, CCR7 and NTB-A was unaffected by MLN4924 treatment (Fig. 2a, panels vii–xxiv, b). These results indicate that Vpu utilizes both cullin-dependent and -independent mechanisms for down modulating host proteins.

MLN4924 relieves NL4-3 Vpu, but not Nef mediated, degradation of CD4

To determine whether MLN4924 also prevented the degradation of CD4, primary $CD4^+$ T cells were infected as described in Fig. 2 but were instead permeabilized, fixed and stained for total levels of CD4. Figure 3 shows that inhibition of neddylation rescued CD4 from Vpu-induced degradation (Fig. 3a, panels ix–xii, b). As a further control to show specificity of cullin inactivation by MLN4924, primary $CD4^+$ T cells were infected with either env-defective HIV-1 (DHIV; Fig. 1e), lacking Vpu but expressing Nef (DHIV Vpu−/Nef+; Fig. 1f) or an isogenic virus lacking Nef and expressing Vpu (DHIV Vpu+/Nef−; Fig. 1g). Nef accelerates the endocytosis of target CD4 molecules present on the plasma membrane via clathrin and Adaptor-Protein 2 (AP-2), ultimately shuttling CD4 for lysosomal degradation in a multivesicular body (MVB) dependent manner [23, 24]. We therefore hypothesized that a virus encoding only Nef (DHIV Vpu−/Nef+) would be able to downmodulate CD4 in a manner that would be insensitive to MLN4924 treatment. This expectation was corroborated as shown in Figs. 3c (panels vii and viii), d.

Primary Vpu isolates maintain the ability to downregulate BST-2 in the absence of CRL-activity

A recent study showed that Vpu alleles from field strains of HIV-1 have the capacity to modulate host proteins, in particular CD4 and BST-2, to a greater extent than the prototypical HIV-$1_{NL4\text{-}3}$ Vpu [25]. We therefore wished to determine whether the CRL-dependent and independent mechanisms observed with HIV-$1_{NL4\text{-}3}$ Vpu

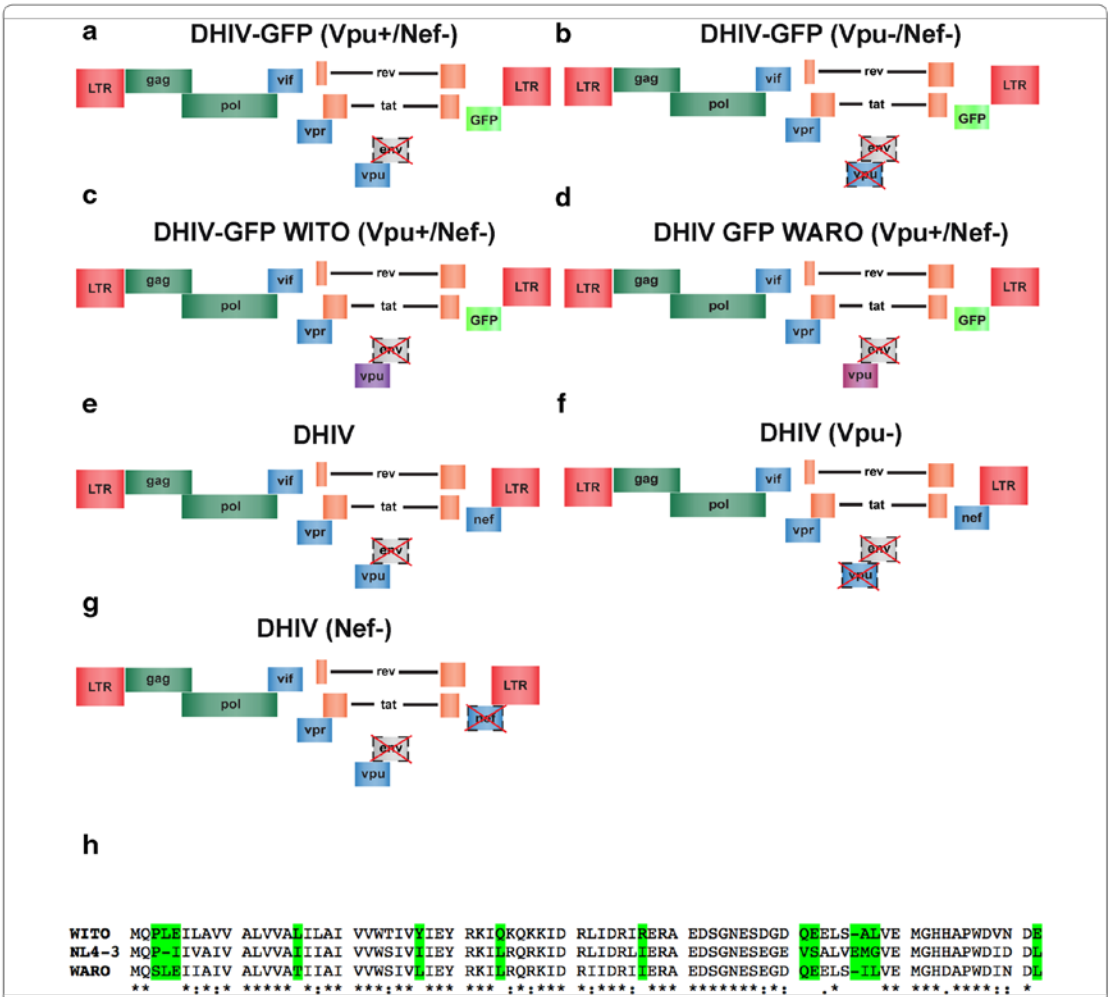
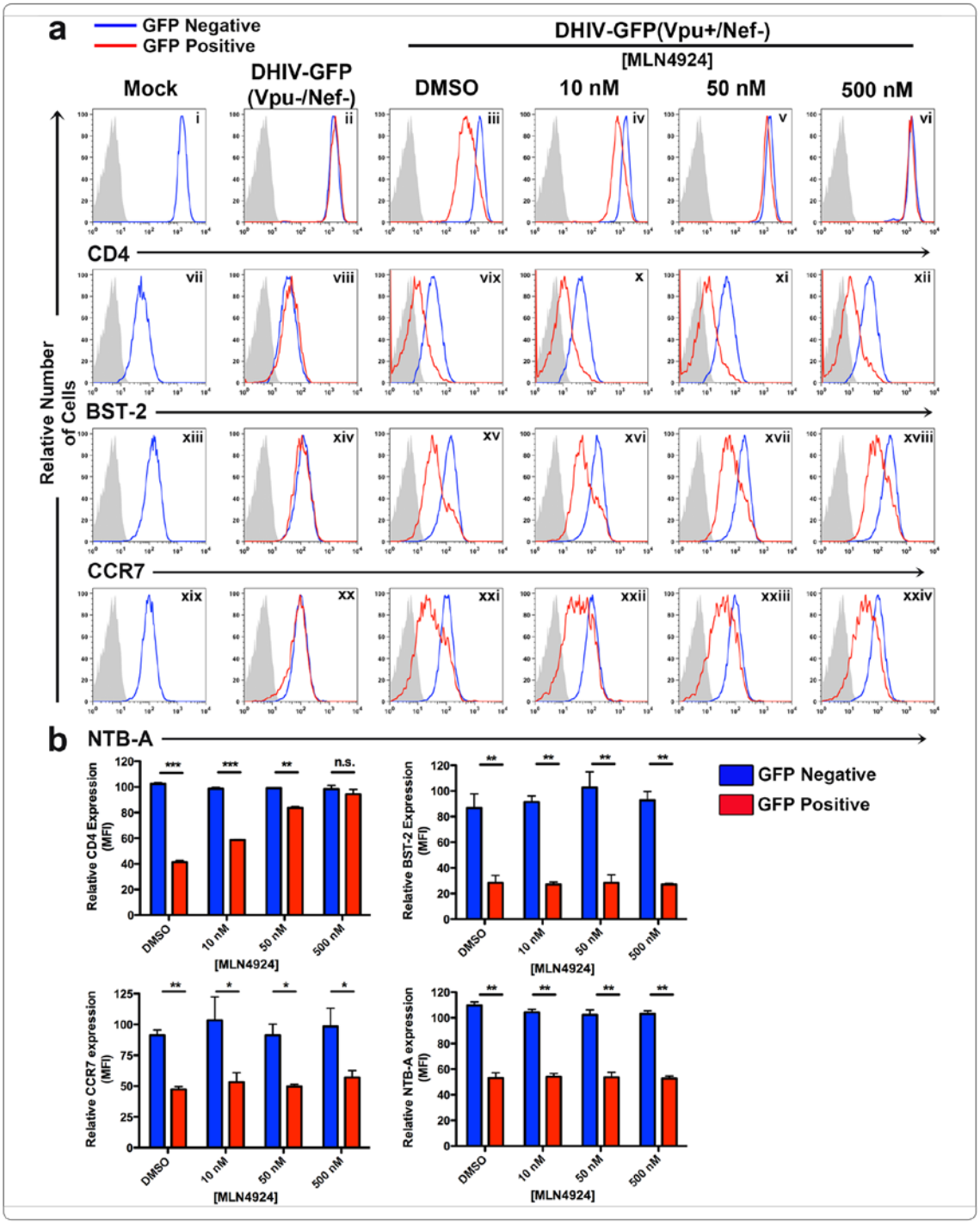


Fig. 1 Lentiviral constructs and Vpu amino acid alignments. **a** The lentiviral vector DHIV, or "defective" HIV, was derived from the HIV-1_{NL4-3} sequence and cut between two BglII sites to efficiently delete envelope/gp120 (*gray box with dashed lines, red* X marks) but maintain in-frame Tat, Rev and RRE ORFs. The constructs used in this study were derived from the DHIV backbone and are as follows: *i* the GFP gene in place of Nef. *ii* Replacement of NL4-3 Vpu with a primary Vpu isolate or *iii* introduction of a frame shift mutation within Vpu and Nef. **a** DHIV-GFP (Vpu+/Nef−). **b** DHIV-GFP (Vpu−/Nef−). **c** DHIV-GFP WITO (Vpu+/Nef−). **d** DHIV-GFP WARO (Vpu+/Nef−). **e** DHIV. **f** DHIV (Vpu−). **g** DHIV (Nef−). **h** Amino acid sequence alignment of Vpu proteins from a transmitted founder (T/F;WITO) and chronic carrier (CC; WARO) compared to NL4-3 Vpu. An *asterisk* indicates fully conserved residues; *colon* represents amino acid conservation with strongly similar properties; *period* designates amino acid conservation with weakly similar properties. *Highlighted* residues mark amino acid differences between the three strains.

(Fig. 2a) would be maintained with Vpu alleles from primary isolates. To address this, we replaced Vpu in DHIV-GFP(Vpu+/Nef−) with Vpu's from either a transmitted founder (T/F; WITO) [26–30] or from a chronic carrier (CC; WARO) [31] virus. These new viruses were termed DHIV-GFP WITO (Vpu+/Nef−; Fig. 1c) and DHIV-GFP WARO (Vpu+/Nef−; Fig. 1d). We observed that Vpu's from both a T/F (DHIV-GFP WITO) and CC (DHIV-GFP WARO) exhibited an enhanced ability to downregulate CD4 when compared to HIV-1_{NL4-3} Vpu (Fig. 4a, panels iii–v, b upper left). This effect was previously reported by Jafari et al., who proposed that primary Vpu isolates may adopt an optimized tertiary structure better suited to counteract CD4 as a result of differences between their


a
GFP Negative
GFP Positive
DHIV-GFP(Vpu+/Nef-)
[MLN4924]
Mock
DHIV-GFP (Vpu-/Nef-)
DMSO
10 nM
50 nM
500 nM
CD4
BST-2
CCR7
Relative Number of Cells
b
NTB-A
Relative CD4 Expression (MFI)
Relative BST-2 Expression (MFI)
Relative CCR7 expression (MFI)
Relative NTB-A expression (MFI)
[MLN4924]
GFP Negative
GFP Positive

(see figure on previous page.)
Fig. 2 HIV-1 Vpu utilizes both cullin dependent and independent mechanisms to downregulate host proteins. **a** Primary $CD4^+$ T cells were either mock infected or infected at an MOI of 1 with DHIVGFP(Vpu+Nef−) or DHIVGFP(Vpu−Nef−). 2 days post infection, either DMSO or increasing concentrations of MLN4924 were added to cell cultures. 24 h later, surface expression of CD4, BST-2, CCR7 or NTB-A was analyzed by flow cytometry. Histograms depict a comparison of GFP negative (*blue line*) and GFP positive (*red line*) cells along with an IgG isotype control (*gray* shaded histogram). Unless otherwise noted, all experiments involving primary $CD4^+$ T cells are representative of three separate experiments performed in three different healthy donors. **b** Relative mean fluorescence intensity (MFI) values of surface expression of CD4, BST-2, CCR7 or NTB-A from DHIVGFP(Vpu+Nef−) infected cells (**a**). Data was normalized by setting the MFI values from uninfected (mock) cells to 100% and is depicted graphically as ±SEM. Unless otherwise noted, all experiments including statistics were performed through a pairwise Student's t test comparing GFP positive and GFP negative cells to assess significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

amino acid sequence and that of HIV-1_{NL4-3} Vpu [16, 32, 33] (Fig. 1h). MLN4924 treatment had a dramatic effect on the ability of both a T/F and CC Vpu to downregulate CD4 (Fig. 4a, panels viii–x, b lower left). Both a T/F and CC Vpu were able to decrease the cell surface density of BST-2 to about the same extent as HIV-1_{NL4-3} Vpu (Fig. 4a, panels xiii–xv, b upper right). However, the addition of MLN4924 in cells expressing Vpu did not restore surface levels of BST-2 to any significant degree (Fig. 4a, panels xviii–xx, b lower right). These results reinforce the fact that primary Vpu isolates also possess the ability to counteract host proteins by CRL-independent mechanisms.

siRNA mediated knockdown of cullin 1 reduces CD4, but not BST-2, surface levels in the presence of Vpu

Although MLN4924 is primarily used and known as an NAE (and thus pan-CRL) inhibitor, at IC_{50} values of 1.5 and 8.2 μM the drug can also block the functions of the NAE-related enzymes, ubiquitin-activating enzyme (UAE) and SUMO-activating enzyme (SAE) [9]. Therefore, as an alternative approach to chemical inhibition, we assessed Vpu function in cells depleted of cullin 1. HeLa-CD4 cells were transfected twice (24 h apart) with either a non-targeting siRNA or siRNA targeting cullin 1, followed by infection with either DHIV-GFP WARO (Vpu+/Nef−) or DHIV-GFP (Vpu−/Nef−). As shown in Fig. 5, knockdown of cullin 1 attenuated Vpu's capacity to downregulate CD4 from the cell surface (Fig. 5a, compare panels ii and v), but had no effect on downregulation of BST-2 (Fig. 5a, panels viii and xi). We conclude that Vpu's ability to decrease surface levels of BST-2 is independent of CRL-activity.

Discussion

Vpu acts as a functional protein that interferes with cellular targets through multiple mechanisms. The di-serine motif of Vpu renders Vpu capable of recruiting the E3 ubiquitin ligase complex substrate adaptor β-TrCP for target ubiquitination and eventual proteasomal (for CD4) [17, 34, 35] or endosomal sorting complexes required for transport (ESCRT) mediated endo-lysosomal degradation (for BST-2) [36–40]. Previous studies have also shown, however, that Vpu-mediated surface downregulation of BST-2 can be uncoupled from BST-2 degradation [41–43]. The explanation lies in the fact that Vpu induces the mislocalization of BST-2 within a perinuclear compartment (i.e. *trans*-golgi network (TGN)) [36, 41, 44–46]. As a consequence, both recycled and newly synthesized BST-2 are retained within the TGN, thereby decreasing total levels of BST-2 at the cell surface [45–47]. A recent report by Jia et al. denoted interaction of Vpu with the clathrin Adaptor-Protein complex 1 (AP-1) [19]. Binding of AP-1 and Vpu occurs through a conserved motif, $E_{59}xxxL_{63}V_{64}$ (ELV), within Vpu's C-terminal domain, previously reported to be important in BST-2 surface downmodulation and viral release [18]. In our present study, we found that pharmacological inhibition of CRL- activity or knock down of cullin 1 hindered Vpu's capacity to downregulate CD4, but not BST-2. Therefore, our findings support a model whereby cullin activity (and β-TrCP) are dispensable for Vpu to downregulate BST-2.

Numerous studies have shown that the di-serine motif of Vpu, which mediates interaction with β-TrCP when phosphorylated, is required for downmodulation and/or degradation of both CD4 and BST-2 [17, 48–52]. Thus, mutation of the di-serine motif abrogates degradation of CD4 [16, 53], confirming a role for β-TrCP. On the other hand, Tervo et al. found that depletion of β-TrCP2 or the simultaneous depletion of β-TrCP1 and 2 did not block the ability of Vpu to promote HIV-1 release and failed to restore surface levels of BST-2 [43]. These results, taken together, suggest that the di-serine motif of Vpu is directly involved in interaction with β-TrCP, but that mutations in this motif affect the downregulation of BST-2 through possibly a more general effect on Vpu protein conformation or perhaps the binding of another cellular factor implicated in the mislocalization of host proteins [43]. Therefore, our observation that downregulation of BST-2, CCR7 and NTB-A is CRL-independent does not contradict the notion that the di-serine motif of Vpu is required for this function [15, 21, 54].

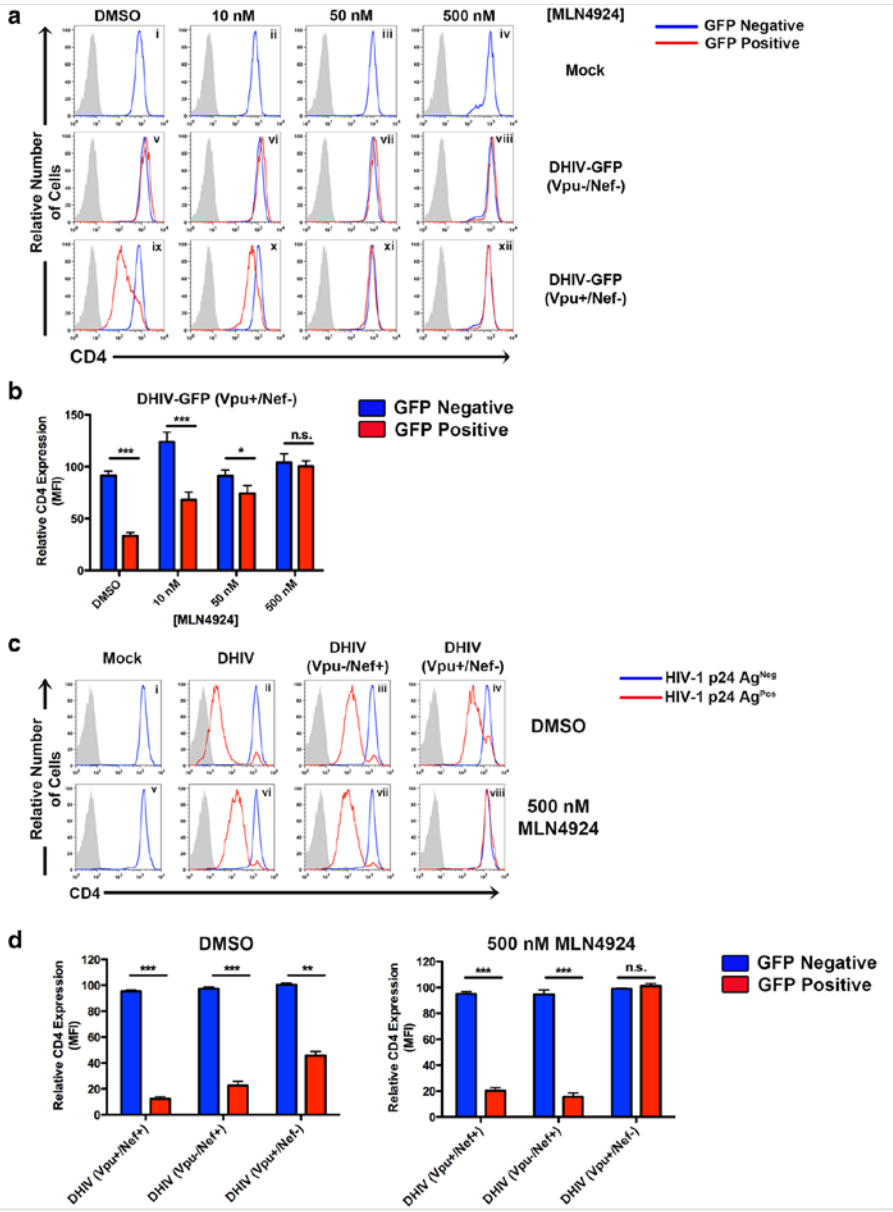


Fig. 3 MLN4924 alleviates Vpu- but not Nef-mediated degradation of CD4. **a** Cultured T_{CM} were infected as described in Fig. 2a. To assess total levels of CD4, cells were permeabilized and stained 24 h after addition of MLN4924 and analyzed by flow cytometry. Histograms are color-coded as described in Fig. 2a. **b** MFI values of total (intracellular) CD4 expression levels from DHIVGFP(Vpu+Nef−). Data was normalized and statistical significance obtained as described in Fig. 2b. *n.s.* not significant. **c** Primary $CD4^+$ T cells were either mock infected or infected at an MOI of 1 with DHIV WT, DHIV Vpu−/Nef+ or DHIV Vpu+/Nef−. 2 days post infection, cell cultures were treated with either DMSO or 500 nM MLN4924. 24 h post MLN4924 treatment, cells were assessed for surface levels of CD4 between $p24Gag^{neg}$ (*blue line*) and $p24Gag^{pos}$ (*red line*). *Gray* shaded histograms represent an IgG matched isotype control. Shown is one representative experiment out of three. **d** Relative CD4 surface levels were quantified from data obtained in Fig. 3c and are depicted graphically as ±SEM of either cells treated with DMSO (*left*) or 500 nM MLN4924 (*right*). Statistical significance between $p24Gag^{neg}$ and $p24Gag^{pos}$ populations was determined as in Fig. 2b.

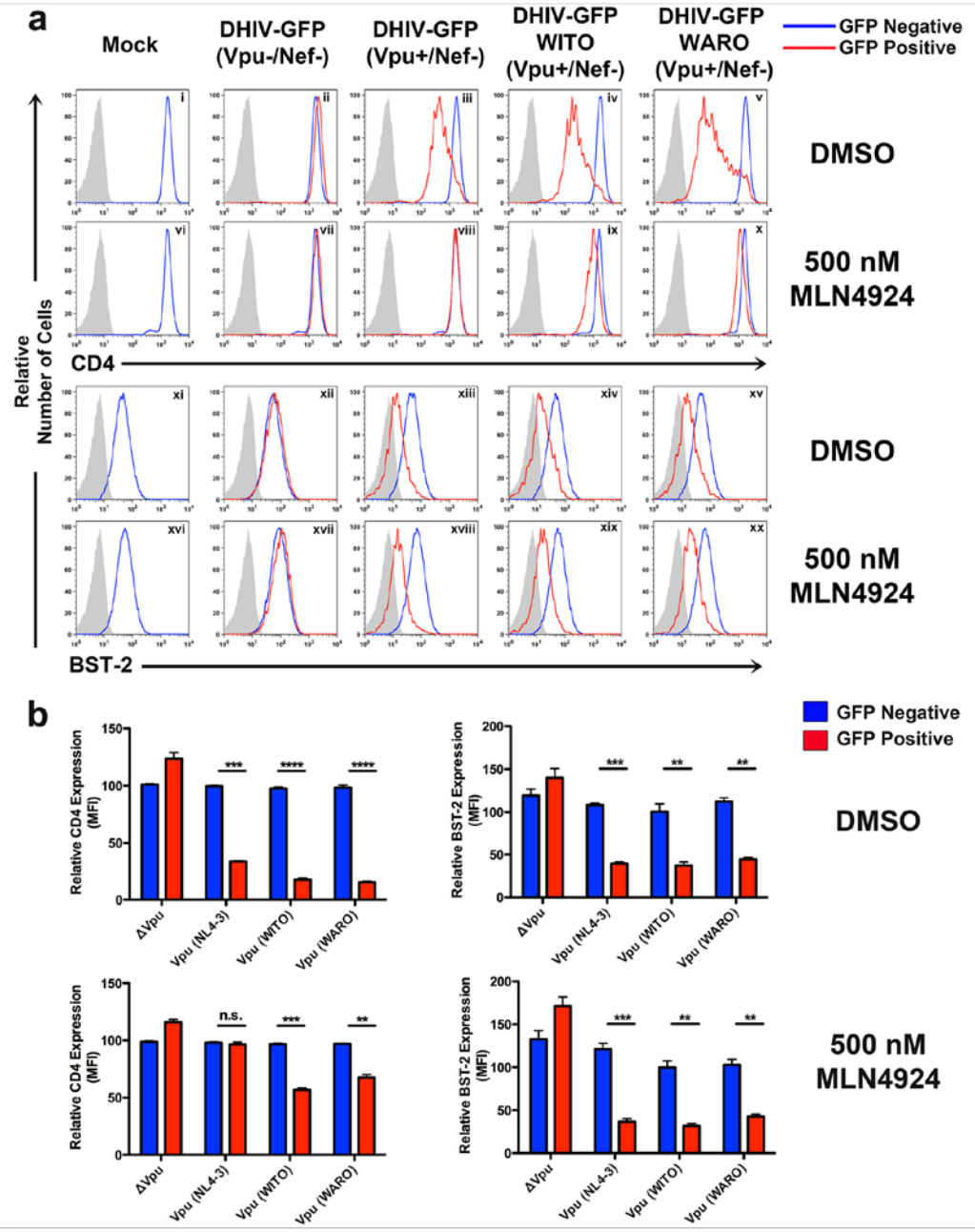


Fig. 4 Primary Vpu isolates maintain the ability to decrease surface expression of CD4 and BST-2 in a CRL-dependent and independent manner. **a** Primary CD4$^+$ T cells were either mock infected or infected at an MOI = 1 with DHIV-GFP (Nef−/Vpu−), DHIV-GFP (Nef−/Vpu+) or viruses encoding Vpu taken from either a transmitted/founder (T/F) (DHIV-GFP WITO (Nef−/Vpu+)) or a chronic carrier (CC) primary isolate (DHIV-GFP WARO (Nef−/Vpu+)). The cells were treated with either DMSO or 500 nM MLN4924 48 h post infection. 24 h post MLN treatment, the cellular GFP negative and positive populations (*red* and *blue* histograms) were assessed for surface levels of CD4 and BST-2 via flow cytometry. An IgG control was included to set the baseline for positive staining (*gray* shaded histogram). **b** Relative CD4 and BST-2 surface levels were quantified, normalized, and scored statistically as described in Fig. 2b.

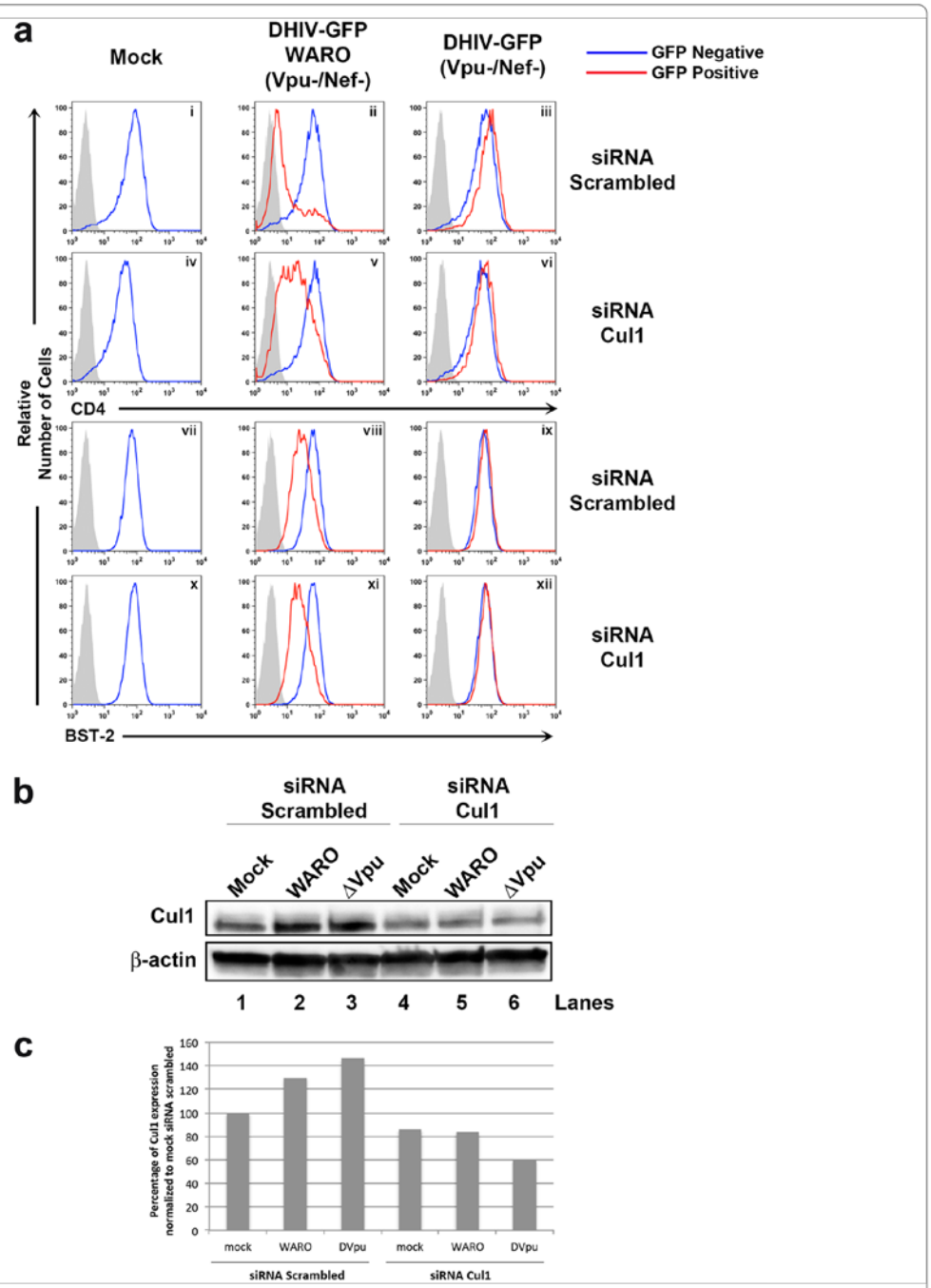


Fig. 5 siRNA knockdown of cullin 1 hinders surface downmodulation of CD4, but not BST-2, by Vpu. **a** HeLa-CD4 cells were transfected twice with pooled control or cullin 1 siRNAs. 4 h post the second transfection, the cells were infected with VSV-G pseudotyped DHIV-GFP (Nef−/Vpu−) or DHIV-GFP WARO (Nef−/Vpu+). Cells were subsequently stained to detect surface levels of CD4 and BST-2 between GFP negative (*blue line*) and GFP positive (*red line*) populations 48 h post infection. *Gray* shaded histogram represents an IgG matched isotype control. **b** A portion of cells from **a** were lysed and subjected to Western blot to determine the knockdown efficiency of cullin 1. **c** Quantification of cullin 1 normalized to β-actin from **b**.

Structurally, phosphorylation of Vpu at serine 52 and 56 induces a conformational change in Vpu's C-terminus: the formation of a β-strand within residues 50–59 and displacement of the third alpha helix (h3) away from the phosphorylation site [55]. The structural rearrangements induced by phosphorylation result in the emergence of acidic side chains surrounding serine 52 and 56, creating an electronegative binding region upon which a protein exhibiting electropositive potential can bind [55]. In particular, phosphorylated serines 52 and 56, glycine 53, asparagine 54 and the hydrophobic residues isoleucine 46 and leucine 45 were shown to be involved in the binding to the F-box protein β-TrCP [56]. Our observations with MLN4924 and cullin knockdown argue against a role for cullin mediated ubiquitination in the downregulation of BST-2 from an independent line of experimentation other than mutagenesis.

Given that Vpu does not induce the degradation of CCR7 or NTB-A but rather retention within the TGN, our data suggest that these molecules are also downregulated in a CRL-independent manner (Fig. 2a) [20, 21, 54]. A recent report by Bachle et al. identified a conserved C-terminal AWF motif present within HIV-1 subtype B Vpu isolates that influences the ability of Vpu to downregulate the lipid antigen receptor CD1d [57]. Therefore, whether Vpu's AWF or ELV motifs, which are CRL-independent, are required for downmodulation of CCR7 and NTB-A remains to be determined. It will also be compelling to investigate the requirement for β-TrCP and the $SCF^{\beta\text{-}TrCP}$ complex in downmodulation of other reported targets of Vpu, such as CD1d, CD155, CD62L and members of the tetraspanin family [58–61].

Conclusions

This work shows that cullin inactivation, through either pharmacological inhibition or depletion of cullin 1, does not render Vpu unable to downregulate BST-2, CCR7 or NTB-A. This highlights that facts that Vpu is multifunctional and that therapeutic targeting of neddylation, while potent and specific against CD4 downmodulation by Vpu, would still allow other targets to be downregulated.

Methods

Antibodies and reagents

Antibodies used in this study were as follows: APC-labeled mouse anti-human CCR7 (clone 150303; R & D Systems Inc.), mouse anti-human NTB-A-APC (clone 292811; R & D Systems Inc.), APC-labeled mouse anti-human CD4 (clone S3.5; Life Technologies), rabbit anti-human BST-2 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; Drs. Klaus Strebel and Amy Andrew (Cat. # 11721)), goat-anti-rabbit Alexa Fluor (AF) 647 (Molecular Probes, Invitrogen), mouse APC-conjugated isotype control (clone 20102; R & D Systems Inc.), rabbit AF-647-conjugated isotype control (Cell Signaling Technology), rabbit anti-human cullin 1 (abcam) and mouse anti-human β-actin (Sigma Aldrich). MLN4924 was purchased from Cayman Biologicals. The dry solvent was then resuspended in DMSO at a stock concentration of 20 mM, further aliquotted and diluted at 200 μM and used as indicated. ON-TARGETplus SMARTpool siRNAs targeting human cullin 1 or control non-targeting siRNAs were purchased from Dharmacon.

Cells and plasmids

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Atlas Biologicals) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Life Technologies). HeLa-$CD4^+$ clone 1022 (obtained through the NIH AIDS Reagent Division of AIDS, NIAID, NIH (Cat. #1109; Dr. Bruce Chesebro) were cultured in RPMI complete media in the presence of 1 μg/ml G418 (Life Technologies) while CCRF-CEM and primary $CD4^+$ T cells were cultured in RPMI complete media only. All cells were maintained at 5% CO_2 at 37°C. For all experiments involving primary $CD4^+$ T cells, coverage was maintained under protocol #IRB_00067637 approved by the University of Utah Institutional Review Board. The generation of cultured T_{CM} has been described previously [21].

The DHIV plasmids used in this manuscript have been described previously (Ramirez et al.; Cell Reports 2014). To construct DHIV-GFP WITO (Vpu+/Nef−) and DHIV-GFP WARO (Vpu+/Nef−), we first re-ligated DHIV-GFP (Vpu+/Nef−) after Xho1 and Sma1 digestion to create a unique EcoR1 site. A novel Mlu1 site after Vpu (ACC TGT to ACGCGT) was then introduced using site-directed mutagenesis (Stratagene) according to the manufacturers' instructions with the following primers: FWD 5′-CAG TCTATTATGGGGTACGCG TGTGGAAGGAAGCAACC and REV 5′-GGTTGCT TC CTTCCACACGCGTACCCCATAATAGACTG. To replace HIV-$1_{NL4\text{-}3}$ Vpu with Vpu's from primary isolates, we obtained full length transmitted founder (T/F; pWITO.c/2474; Cat# 11739, Dr. John Kappes and Dr. Christina Ochsenbauer) or chronic carrier (CC; pWARO; Cat # 12419, Dr. Beatrice Hahn) HIV-1 infectious molecular clones (IMC) from the NIH AIDS Reagent Program. HIV-1_{WITO} Vpu was PCR amplified using the following primers: FWD 5′-GCAGGAGTGGAAGCCAT AATAA*GAATTC* and REV 5′-ACA*ACGCGT*CTACTC ATCATTAACATCCCAAGGAGC (EcoR1 and Mlu1 sites italicized, respectively) and subcloned into DHIV-GFP(Vpu+/Nef−) to create DHIV-GFP WITO(Vpu+/

Nef−). DHIV-GFP WARO(Vpu+/Nef−) was constructed in a similar fashion with the following HIV-1_{WARO} Vpu primers: FWD 5′-GGAGTGGAAGCCATAATAA*GAATTC*TGC and REV 5′-ACG*ACGCGT*CTACAGATCA TTAATATCCCAAGGAGCATC. All constructs were confirmed via sequencing.

Flow cytometry

Surface levels of CD4, BST-2, CCR7 and NTB-A were assessed by staining cells with their appropriate antibodies at 4°C for 30 min in buffer (1 × PBS + 3% FBS). An additional step including a secondary antibody was necessary to detect BST-2 surface levels. A viability dye, eFlour 450 (eBioscience) was then used to distinguish live from dead cells. Fixation was achieved using 0.5% Paraformaldehyde (PFA).

In experiments involving surface analysis of CD4 and detection of intracellular p24, cells were first probed with anti-APC-CD4, stained with eFluor 450, permeabilized (Cytofix/Cytoperm: BD Biosciences) and then stained with mouse-anti-FITC-p24. Total levels of CD4 in primary $CD4^+$ T cells were measured by staining cells with eFlour 450, permeabilization and then probing with anti-APC-CD4. All data was collected on a BD FACS CantoII and analyzed with FlowJo software.

Viruses and infections

Viral stocks were generated by co-transfection of 20 μg DHIV along with 5 μg of a construct expressing vesicular stomatitis virus G-protein (VSV-G) by calcium phosphate mediated transfection into HEK293T cells. Media (DMEM) was replaced after 16 h and the cellular supernatant collected, aliquotted and stored at −80°C 48 h post-transfection. Viral titers and MOI were determined via infection of CCRF-CEM cells. Primary $CD4^+$ T cells generated as described above were infected 5 days post-activation at an MOI of 1 via spinoculation: 10^6 cells (1 ml final volume) for 2 h at 37°C in the presence of 8 μg/ml Polybrene (Sigma). After infection, cells were then resuspended in RPMI complete medium supplemented with IL-2 (30 U/ml).

siRNA mediated cullin 1 depletion

HeLa cells (5×10^5) were transfected twice (24 h apart) with either control siRNA or siRNAs against cullin 1 at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. The medium was changed 4 h after each transfection. 4 h post the second transfection, the cells were either mock infected or infected with DHIV-GFP (Vpu−/Nef−) or DHIV-GFP WARO (Vpu+/Nef−) for 4 h at 37°C at an MOI = 0.8. After 48 h, the cells were either processed for flow cytometry or lysed and subjected to Western blot to detect cellular levels of cullin 1 between samples.

Abbreviations

CRL: cullin-RING ligase; RING: really interesting new gene; AP-1: Adaptor-Protein 1; Vpu: viral protein u.

Authors' contributions

PWR contributed in the design of the study, conceived and performed experiments, analyzed data and wrote the manuscript. ABDS designed and performed experiments and analyzed data. MAS performed experiments. EB and AB participated in study design. VP participated in study design, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 4

DISCUSSION

4.1 Strategies mediated by HIV-1 for viral persistence

HIV-1 has emerged as one of the most successful infectious disease agents to afflict humanity in the modern world, with a global death toll of over 39 million (World Health Organization (WHO), as of 2013). For a virus that causes chronic life-long infections (35 million individuals are currently living with the disease), many factors contribute to why HIV-1 causes a general lack of host immunological control. Some of these include: **(1) Latency**. As a retrovirus, HIV-1 integrates into the host genome of target cells. The absence of viral gene expression during this relatively "dormant" state and the cellular reservoirs harboring HIV-1 (long lived resting memory $CD4^+$ T cells) contribute to the difficulty in viral eradication by the immune system (Chun et al., 1998; Chun et al., 1997). **(2) Mutagenic escape variants**: reverse transcriptase (RT) lacks proofreading capability and has evolved the ability to "jump" (switch templates) during reverse transcription (Huber et al., 1989). As a consequence, HIV-1 is highly recombinogenic, leading to viral quasispecies that are inadequately recognized by neutralizing antibodies and cytotoxic T lymphocytes (CTLs) (Domingo et al., 2012). **(3) Concealment**: Conserved domains within HIV-1 envelope are typically hidden under variable loops, only being transiently exposed during viral entry. Thus, epitopes targeted by neutralizing antibodies are largely inaccessible (Johnson and Desrosiers, 2002). **(4) Cell tropism**: HIV-1 infection results in the rapid decline of $CD4^+$ T cells, the primary cellular target of the virus (Ho et al., 1995; Wei et al., 1995). Helper $CD4^+$ T cells indirectly assist in multiple aspects of viral clearance through the release of cytokines, stimulating robust humoral and cell-mediated immunity (O'Shea and Paul, 2010). Consequently, their elimination severely hampers immunity against any opportunistic

infections. **(5) Immune evasion through virally encoded factors**: HIV-1 possesses accessory proteins that serve to specifically impair innate and adaptive immunity as well as counteract intrinsic restriction factors. Therefore, from an evolutionary standpoint, the acquisition of accessory genes is perhaps one of the main factors that drive persistent HIV-1 pathogenesis. Hence, a continual understanding of the exact role and functions accessory proteins play in promoting viral persistence may help in the future development of new therapeutics to stem viral replication and dissemination.

4.2 CCR7: a new cellular target of the HIV-1 accessory protein Vpu

In this work (Chapter 2), we describe Vpu-mediated reduced cell surface expression of the chemokine receptor CCR7 on primary $CD4^+$ T cells. CCR7 is a chemokine homing receptor that mediates the coordinated recirculation of T cells (specifically naïve and central memory T (T_{CM}) cells) between the blood and secondary lymphoid organs (SLO) (Legler et al., 2014). We found Vpu to be both necessary and sufficient for this function.

Downregulation of CCR7, however, did not correlate with a concomitant decrease in total CCR7 levels, but rather a relocalization of the protein within a perinuclear compartment: the *trans*-Golgi Network (TGN). Consequently, we found that HIV-1-infected cells were impaired in their ability to migrate towards a CCL19-mediated gradient *in vitro*. The downregulation of CCR7 by Vpu may therefore be a way for the virus to hinder the generation of a proper immune response by impairing the ability of T cells to migrate into SLO. Conversely, CCR7 downregulation on T cells that become infected within lymphoid tissues may allow their egress into efferent lymph vessels,

promoting systemic viral spread. Regardless, important mechanistic and biological questions regarding this function of Vpu remain, which will be further discussed here.

4.2.1 What domains/residues/motifs within Vpu are important for downregulating CCR7?

Vpu is a Type I integral membrane protein that possesses an N-terminal transmembrane domain (TMD) as well as two cytoplasmic α-helices separated by a conserved region harboring a pair of constitutively phosphorylated serine residues (Dubé et al., 2010a). Vpu's serine residues are necessary to "mimick" a canonical phosphodegron motif that recruits an Skp1-Cullin1-F-box ($SCF^{\beta\text{-}TrCP}$) E3 ubiquitin ligase to degrade CD4 (Margottin et al., 1998). Randomization of Vpu's TMD (VpuRD), but not mutation of its serine residues, inhibits CCR7 downregulation (Ramirez et al., 2014). This suggests the TMD in Vpu is required to modulate CCR7, whereas recruitment of an $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex is likely not necessary. The latter was further supported by the ability of Vpu to downmodulate CCR7 despite pharmacological inhibition of Cullin-RING ligase (CRL) activity (Ramirez et al., 2015).

Besides Vpu's TMD and phosphoserine residues, recent reports have identified other functional regions, specifically within the cytoplasmic portion of Vpu, that are important for downregulating the restriction factor BST-2 and CD1d (Bächle et al., 2015; Kueck and Neil, 2012). First, this includes a Vpu motif ($E_{59}xxxL_{63}V_{64}$) that resembles an acidic-dileucine sorting signal ((D/E)xxxL(L/I/M)). These signals usually facilitate binding to Adaptor Protein (AP) complexes (Canagarajah et al., 2013). Accordingly, Vpu utilizes the ELV motif to interact with AP-1; sequestering BST-2 within a perinuclear compartment (TGN) and reducing its density on the cell surface (Jia et al., 2014).

Mutation of this motif, however, only had a partial effect on impairing Vpu's ability to downregulate CCR7 (Figure 4.1). Thus, future studies should focus on depleting cellular pools of AP-1 to fully delineate the necessity of this co-factor in Vpu-mediated CCR7 downregulation.

Second, Bachle and colleagues utilized chimeras between active (Subtype B) and inactive (Subtype C) Vpus to identify a conserved C-terminal APW motif important for CD1d downmodulation (Bächle et al., 2015). It is tempting to speculate that the APW motif may also play a role in modulating CCR7, given that Vpu also mislocalizes, rather than degrades, CD1d (Moll et al., 2010). Moreover, whether the Vpu APW motif physically binds CD1d or is necessary to interact with some other cellular factor implicated in host vesicular trafficking has yet to be shown.

4.2.2 Does Vpu affect de novo synthesized or recycled CCR7 from reaching the PM?

Reports on CCR7 biology have shown that the chemokine receptor is relatively stable on the PM unless engagement with one of its ligands (CCL19 / CCL21) occurs (Otero et al., 2006). Consequently, we found that Vpu does not increase either the constitutive or CCL19-mediated internalization rate of CCR7, consistent with other targets of Vpu such as BST-2, NTB-A and CD1d (Dube et al., 2009; Mitchell et al., 2009; Moll et al., 2010; Ramirez et al., 2014; Shah et al., 2010). Nonetheless, future studies should examine in greater detail the internalization rate of CCR7 at longer time-points (our experiment was carried out in a time frame of 60 minutes) to evaluate: i.) The steady-state endocytosis of CCR7 and ii.) Although not likely, whether Vpu has any influence on increasing this rate.

Our studies also included the treatment of cells with cyclohexamide (CHX), a protein synthesis inhibitor, to determine whether Vpu degrades CCR7. We found no difference in the total levels of CCR7 within HIV-1 infected cells regardless of CHX treatment (Ramirez et al., 2014). While this suggests a mechanism distinct from degradation, it does not exclude the ability of Vpu to inhibit *de novo* synthesized CCR7 from reaching the PM. In other words, if in the presence of CHX, surface levels of CCR7 were restored (or partly restored) within Vpu-expressing cells, this would be consistent with Vpu blocking the anterograde trafficking of newly synthesized CCR7 molecules. On the other hand, if CHX treatment had no effect on Vpu downregulating CCR7, this may suggest that Vpu impairs the recycling of CCR7 back to the cell surface, a phenomenon that has been reported for both BST-2 and CD1d (Dubé et al., 2010b; Moll et al., 2010). Moreover, the ability to infect cells with an inducible HIV-1 provirus may provide another alternative for answering some of these mechanistic questions, whereby the ability to chronologically monitor CCR7 downregulation by Vpu could be studied in greater temporal and spatial details (Dube et al., 2011).

4.2.3 Does Vpu affect the glycosylation pattern of CCR7?

It was recently reported that Vpu affects the glycosylation pattern of NTB-A, where only the high mannose form is detectable within Vpu-expressing cells (Bolduan et al., 2013). Vpu does not degrade NTB-A (Shah et al., 2010), but sequesters the protein in a perinuclear compartment: the TGN (Bolduan et al., 2013). As post-translational processing occurs within the TGN, blocking the egress of NTB-A from the ER (Brefeldin A) or utilizing ER-trapped Vpu mutants (Skasko et al., 2011; Vigan and Neil, 2011) inhibited Vpu from affecting NTB-A's glycosylation pattern (Bolduan et al., 2013).

CCR7 contains one N-linked glycosylation site and, like NTB-A, is mislocalized within the TGN when Vpu is present (Bolduan et al., 2013; Ramirez et al., 2014). Thus, whether Vpu has any role in affecting glycosylation of CCR7, or conclusive evidence that downregulation of CCR7 by Vpu occurs via a post-ER mechanism, remains to be determined.

4.2.4 Is downmodulation of CCR7 a conserved Vpu function?

The *vpu* open reading frame is expressed within the genomes of both pandemic (M) and nonpandemic (N, O and P) HIV-1 groups, as well as some related simian immunodeficiency virus (SIV) isolates: SIV_{cpz} (chimpanzee), SIV_{gor} (gorilla), SIV_{gsn} (greater spot-nosed monkey), SIV_{mon} (mona monkey), SIV_{mus} (mustached monkey) and SIV_{den} (Dent's mona monkey) (Kirchhoff, 2010). It is now widely accepted that HIV-1 M, N, O, and P are a direct result of zoonotic transmission from SIVs found within central chimpanzees and Western lowland gorillas (Sharp and Hahn, 2010). Remarkably, Group M Vpu proteins possess the sole ability to counteract BST-2, CD4, NTB-A and CD1d, whereas Groups N, O, and P are either suboptimal antagonists or lack at least one of these functions (Sauter et al., 2011; Sauter et al., 2009; Sauter et al., 2012; Yang et al., 2011). This has led to the speculation that the ability of Group M Vpu to evade intrinsic, innate, and adaptive antiviral mechanisms, particularly in the counteraction of BST-2, has been a major cause in contributing to the global AIDS pandemic (Kirchhoff, 2010; Sauter et al., 2009). Therefore, it would be interesting to determine if Vpu downmodulation of CCR7 is a unique attribute of pandemic Group M viruses, or whether this Vpu function is maintained between all HIV-1 Groups as well as *vpu*-encoding SIV strains. Moreover, whether non-*vpu* encoding SIV strains, as well as HIV-2, possess an inherent ability to

downregulate CCR7, remains to be determined. Importantly, we have found that Group M Vpu isolates obtained from a transmitted founder (TF) or chronic carrier (CC) virus (Parrish et al., 2013) preserve the ability to downregulate CCR7 within primary $CD4^+$ T cells, implying this Vpu function is not just associated with lab-adapted HIV-1 strains (NL4-3; Figure 4.2).

4.2.5 What is the biological significance of CCR7 downregulation by Vpu in vivo?

The relevance for HIV-1 downregulating CCR7 *in vivo* remains unknown. We determined that Vpu-expressing cells display altered migration patterns towards the CCR7 ligand CCL19 *in vitro* (Ramirez et al., 2014). This may reflect a strategy by the virus to disrupt coordinated lymphocyte trafficking into SLO to evade a proper anti-HIV immune response. The recent finding of downregulation of CD62L (our own unpublished results) by both Nef and Vpu (Vassena et al., 2015) further supports this, as CD62L is necessary (along with CCR7) for lymphocytes to traverse high endothelial venules (HEVs) and gain entry into SLO.

On the other hand, lymph nodes constitute one of the major sites of HIV-1 viral replication. Downregulation of CCR7 and CD62L on HIV-1-infected T cells may therefore favor exit from lymph nodes and entrance into efferent lymph vessels, promoting systemic viral infection. Indeed, to assess whether T cell trafficking had a role in the spread of HIV-1, Murooka and colleagues treated BLT (bone marrow/liver/thymus) humanized mice with a drug (FTY720: fingolimod) that retains lymphocytes within lymphatic tissues (Murooka et al., 2012). High-level viremia was prevented only when the drug was given at the time of initial infection, but not when

administered during a pre-established infection (Murooka et al., 2012). This latter finding was also observed when FTY720 was administered to simian-human immunodeficiency virus (SHIV)-infected macaques (Kersh et al., 2009). Taken together, these data suggest that T cell migration is important for promoting systemic viral dissemination.

Moreover, two phenotypes were observed among T cells infected with GFP-tagged HIV-1 in BLT mice: a reduction in cell motility (relative to uninfected GFP-expressing T cells) and the formation of Env-dependent multinucleated syncytia harboring long membrane tethers (Murooka et al., 2012). The authors concluded that decreased migratory potential might therefore serve to slow down HIV-1-infected cells long enough to mediate cell-cell contacts (i.e., virological synapses) for efficient intercellular viral transfer while still being motile enough to establish systemic viral dissemination (Murooka et al., 2012). Importantly, reduced cell motility was lost upon infection with an HIV-1 derived lentiviral vector lacking accessory proteins (Murooka et al., 2012). It is therefore tempting to speculate that Vpu and / or Nef may play some role in this phenotype, especially since variable decreased expression of CCR7 on HIV-infected T cells was observed though not further commented on.

4.3 HIV-1 Vpu: a versatile viral protein

In our second body of published work (Chapter 3), we established that Vpu is a multifunctional accessory factor capable of interfering with cellular targets via CRL-dependent and -independent mechanisms (Ramirez et al., 2015). All HIV accessory proteins can act as viral adaptors by usurping host cellular machinery, with subversion of the ubiquitin proteasome system (UPS) being a common tactic. CRLs represent one of the largest classes of E3 ubiquitin ligase complexes within the UPS, and as such regulate

numerous cellular processes (Bosu and Kipreos, 2008). CRLs may therefore serve as an "Achilles heel" that viruses can manipulate to facilitate evasion from host defense mechanisms.

For example, Simian Virus 5 (SV5), a member of the paramyxovirus virus family, encodes a V protein that uses a cullin4-containing (CRL4) complex to induce the degradation of STAT1/2 (signal transducer and activator of transcription) heterodimers, hindering induction of the host interferon response (Precious et al., 2005a; Precious et al., 2005b; Precious et al., 2007). The Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen (LANA) "mimicks" a component of the cullin5-containing (CRL5) complex to target the tumor suppressors von-Hippel Lindau (VHL) and p53 for degradation, providing a favorable environment for tumor progression in KSHV-infected cells (Cai et al., 2006). Vaccinia virus (VACV— the smallpox vaccine) requires Cullin3 and Rbx1 to replicate, though the exact mechanism and substrates (if any) remain unknown (Mercer et al., 2012). Furthermore, the HIV-1 Vif and HIV-2 Vpx proteins hijack CRL5 and CRL4 complexes to degrade the restriction factors APOBEC3G and SAMHD1, thereby facilitating efficient viral replication (Hrecka et al., 2011; Laguette et al., 2011; Yu, 2003).

HIV-1 Vpu relies on the $SCF^{\beta\text{-}TrCP}$ E3 Ubiquitin Ligase complex (also known as a cullin1-containing complex: CRL1) to induce poly-ubiquitination and subsequent proteasomal degradation of CD4 (Margottin et al., 1998; Willey et al., 1992). Vpu also utilizes a CRL1 complex to eventually degrade the restriction factor BST-2, though not in the same manner as CD4. Instead, Vpu targets BST-2 for endo-lysosomal degradation in an ESCRT-dependent manner (Caillet et al., 2011; Douglas et al., 2009; Iwabu et al.,

2009; Janvier et al., 2011; Mitchell et al., 2009). However, whether Vpu-mediated BST-2 degradation was a direct cause or consequence of BST-2 surface downregulation remained undetermined. Moreover, the requirement of the CRL1 complex in Vpu downregulation of BST-2 remained controversial (Goffinet et al., 2010; Mangeat et al., 2009; Tervo et al., 2011).

To clarify this dilemma in primary $CD4^+$ T cells, we therefore made use of a neddylation-activating enzyme (NAE) inhibitor (MLN4924) to effectively block CRL activity (Soucy et al., 2009). Neddylation is a necessary post-translational step that induces a conformational change within cullins, thereby activating them (Rabut and Peter, 2008). MLN4924 completely abrogated Vpu's ability to downregulate CD4 but not BST-2, CCR7, or NTB-A (Ramirez et al., 2015). Furthermore, primary Vpu isolates maintained the ability to down-regulate BST-2 in the presence of MLN4924, suggesting recruitment of CRL machinery had not been lost during adaptation to cell culture. Finally, siRNA depletion of cellular pools of cullin 1 partially restored CD4, but not BST-2 surface levels within Vpu-expressing cells (Ramirez et al., 2015). Taken together, these results suggest that BST-2 degradation is a consequence of BST-2 downregulation, at least within $CD4^+$ T cells.

Vpu therefore utilizes both CRL- and non-CRL-mechanisms to interfere with its cellular targets and is the only HIV accessory protein presently known to possess these dual attributes. Similar to HIV Nef, Vpu can alter the intracellular vesicular trafficking of host proteins, thereby reducing their expression at the cell surface. This versatility makes Vpu a truly adept protein at modifying the host environment in a manner conducive to promoting immune evasion. Consequently, further research into this accessory factor will

undoubtedly help in our understanding, and hopefully in better combating, HIV-1 pathogenesis.

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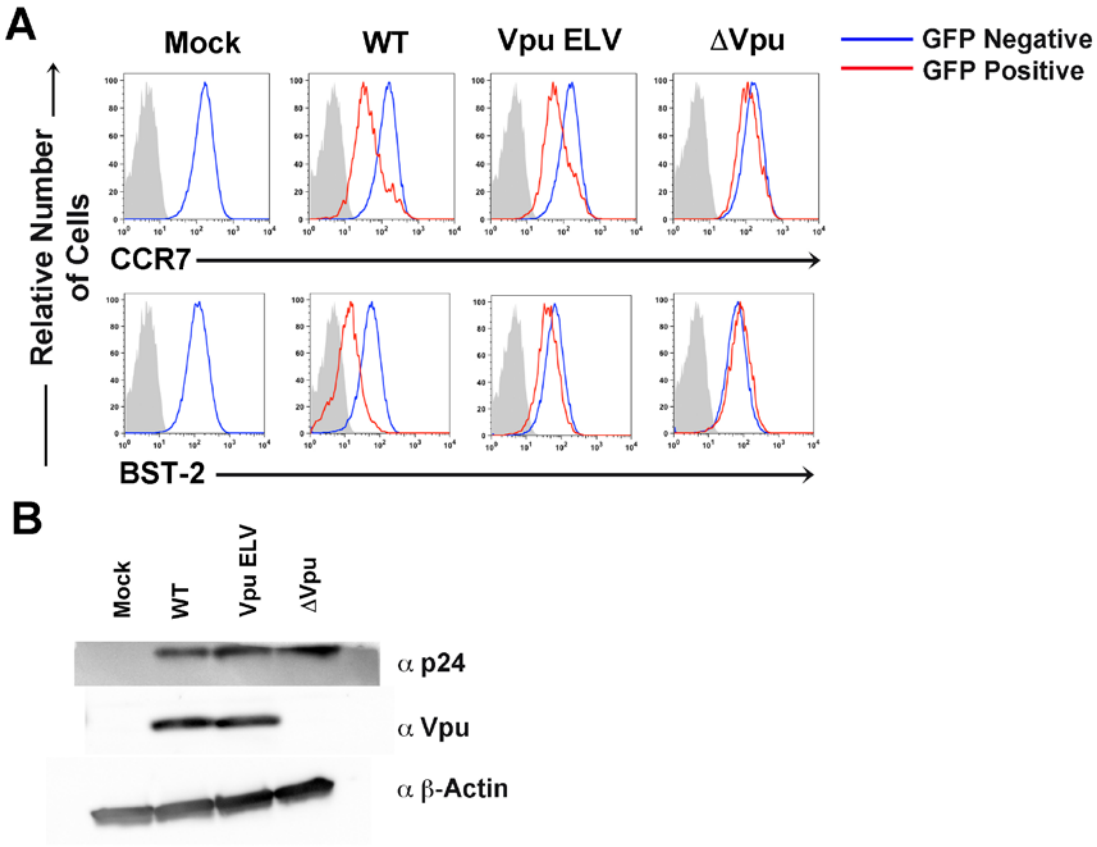

Figure 4.1: The Vpu ELV motif plays a minor role in CCR7 downregulation.
All lentiviral constructs were derived from the HIV-1_{NL4-3} sequence and possess a frame shift mutation within envelope/gp120 but maintain in-frame Tat, Rev, and RRE ORFs. These were thus termed "defective" HIV, or DHIV. All vectors used in this study also harbored GFP in place of Nef (termed DHIV-GFP). Terminology is as follows – WT: DHIV-GFP, Vpu ELV: a virus where the $E_{59}L_{63}V_{64}$ motif within Vpu was mutated to alanines, ΔVpu: a virus lacking Vpu. **A.)** Primary $CD4^+$ T cells were either mock infected or infected with the indicated viruses at an MOI=.5. 72 hrs post-infection, cells were surface stained for CCR7 or BST-2 and analyzed by flow cytometry. Histograms represent a comparison between GFP-negative (uninfected) and GFP-positive (infected) cells. The gray histogram represents an IgG matched isotype control. One representative experiment out of two is shown. **B.)** An aliquot of cells from A were lysed, subjected to western blot and probed for p24 and Vpu. β-actin was used as a loading control.

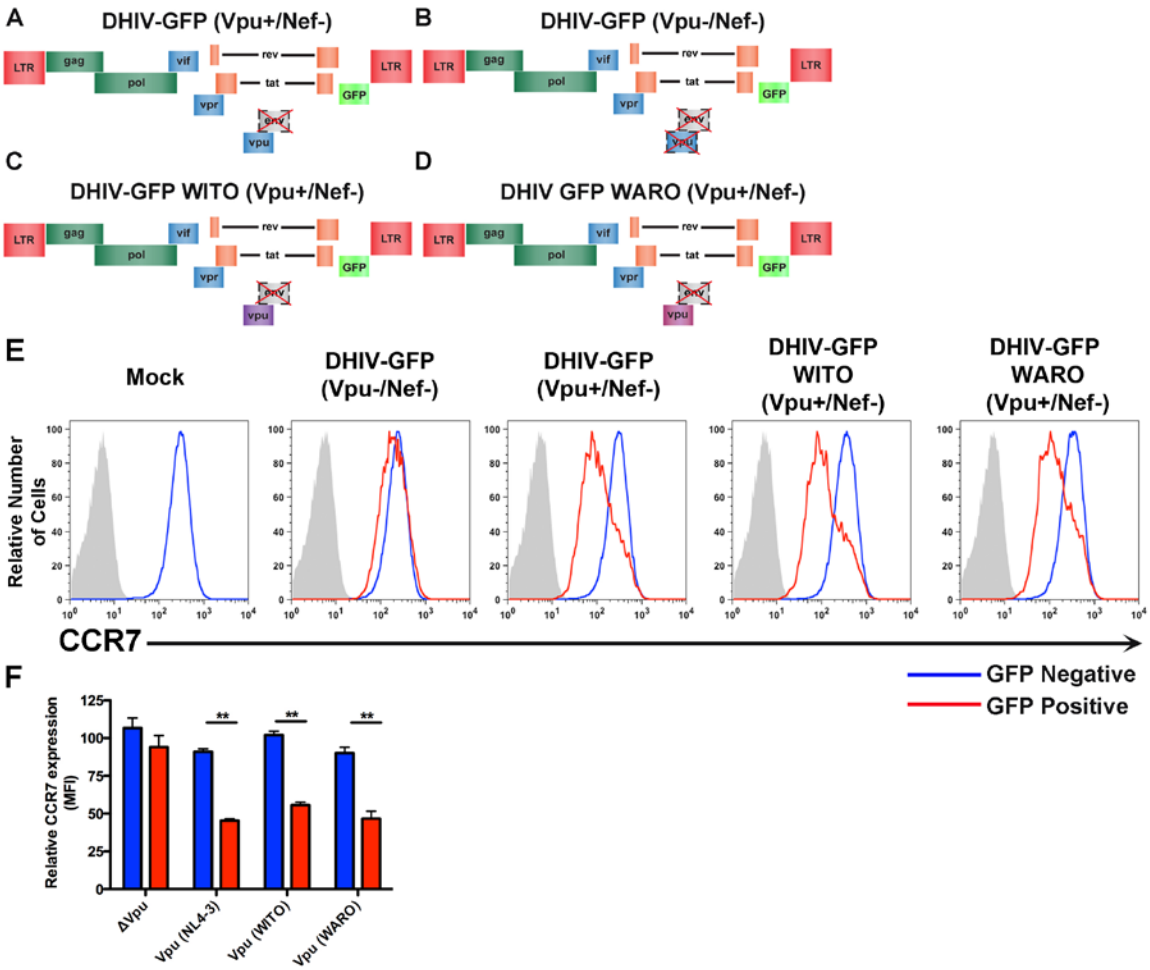

Figure 4.2: Primary Vpu isolates maintain the ability to downregulate CCR7
Lentiviral constructs used were derived from DHIV (as discussed in Figure 4.1) and are as follows: i.) GFP in place of Nef or ii.) Replacement of NL4-3 Vpu with primary Vpu isolates from either a transmitted founder (T/F; WITO) or chronic carrier (CC; WARO) virus. **A.)** DHIV-GFP (Vpu+/Nef -). **B.)** DHIV-GFP (Vpu-/Nef-). **C.)** DHIV-GFP WITO (Vpu+/Nef-). **D.)** DHIV-GFP WARO (Vpu-/Nef-). **E.)** Primary $CD4^+$ T cells were either mock infected or infected at an MOI=1 with the indicated viruses. Cells were subsequently stained to assess surface levels of CCR7 72 hrs post-infection between uninfected (GFP negative: blue line) and infected (GFP positive: red line) populations. A matched IgG isotype control is also shown (Gray histogram). **F.)** Relative mean fluorescence intensity (MFI) values for CCR7 surface levels from **(E)**. Data were normalized by setting the MFI values from uninfected (mock) cells to 100% and are depicted graphically as +/- SEM. A pairwise Student's t test between GFP negative and GFP positive cells was used to determine statistical significance: **p<.01.