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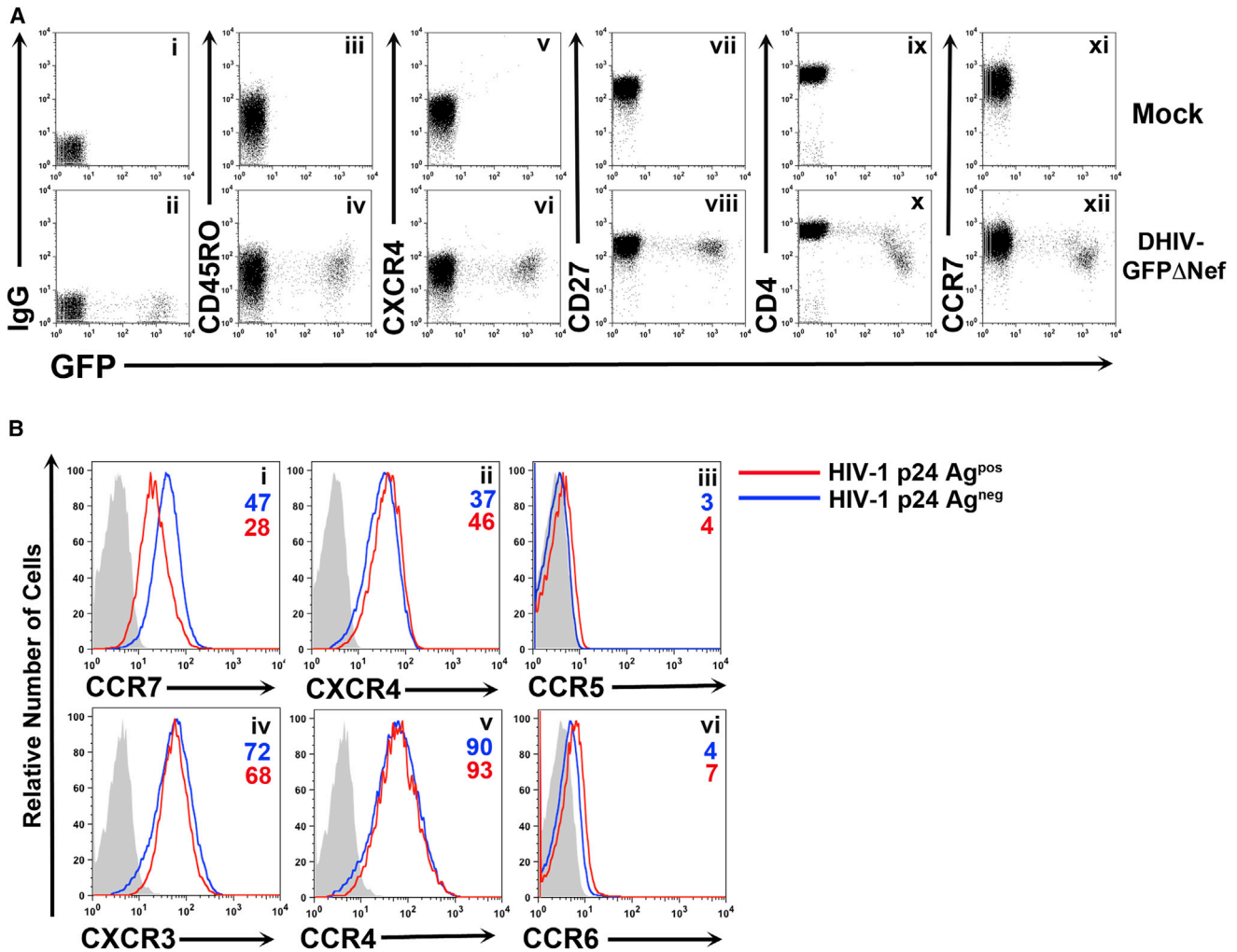
Figure 1. HIV-1 Downregulates the Chemokine Receptor CCR7 from the Surface of Infected Primary CD4+ T Cells

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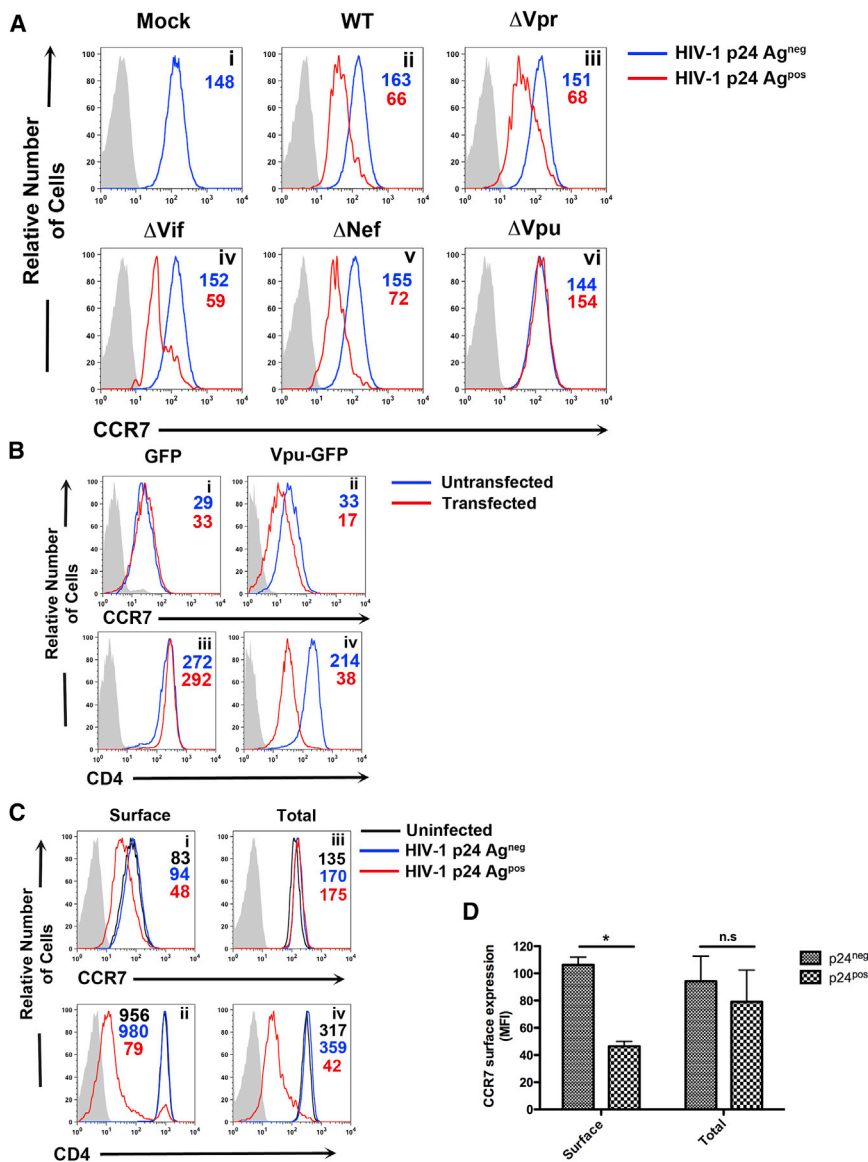
Figure 2. HIV-1 Vpu Is Necessary and Sufficient for Surface Downmodulation, but Not Degradation, of CCR7

(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.

niscent 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
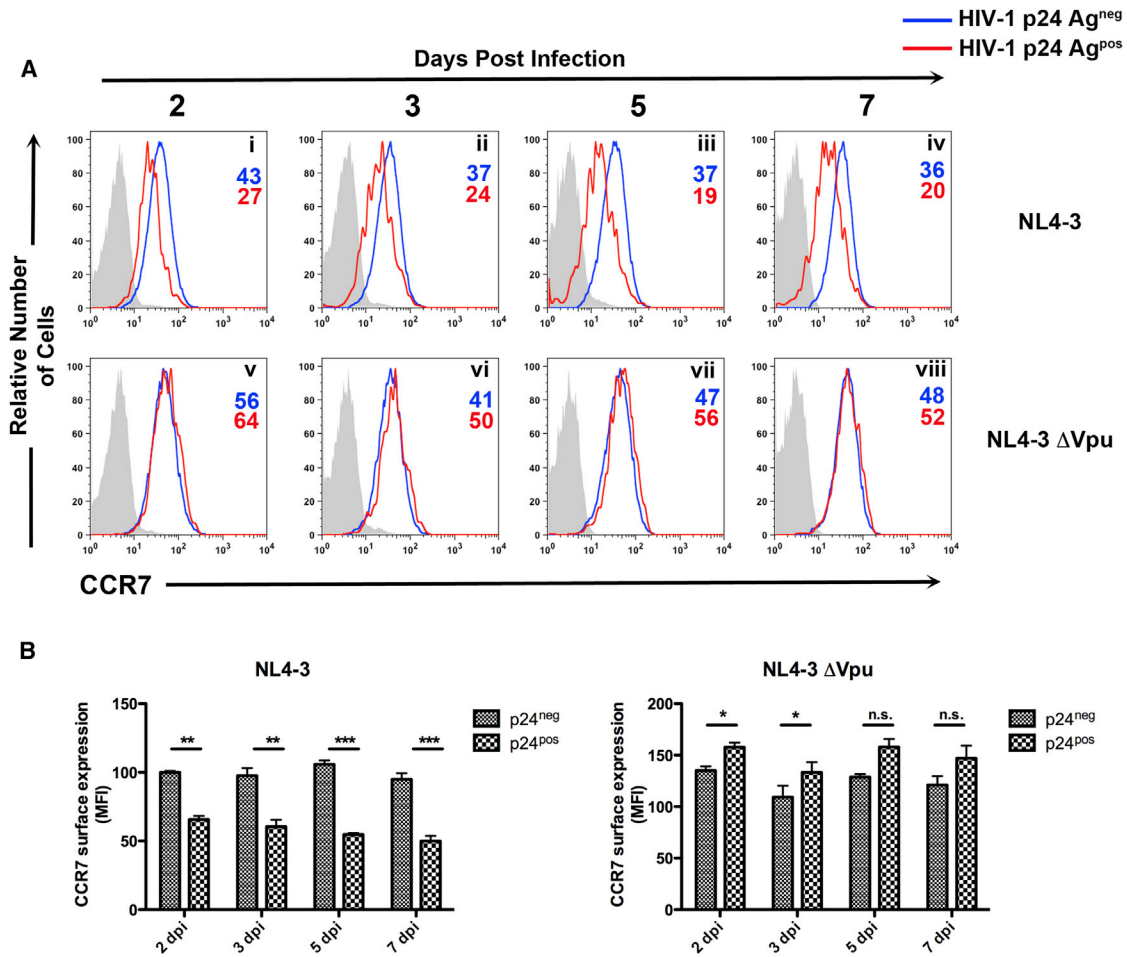
Therefore, to determine whether Vpu colocalizes and/or sequesters CCR7, we transfected HeLa cells with a CCR7 fusion construct bearing a C-terminal mCherry tag (CCR7-mCherry) along with Vpu-GFP. In the absence of Vpu-GFP, CCR7-mCherry localized both at the cell surface and intracellularly (Figure 4A, 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 remi-

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 (Evrard-Todeschi et al., 2006; Wu et al., 2003). To address whether Vpu interaction with β-TrCP is required for CCR7 downregula-

tion, 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,
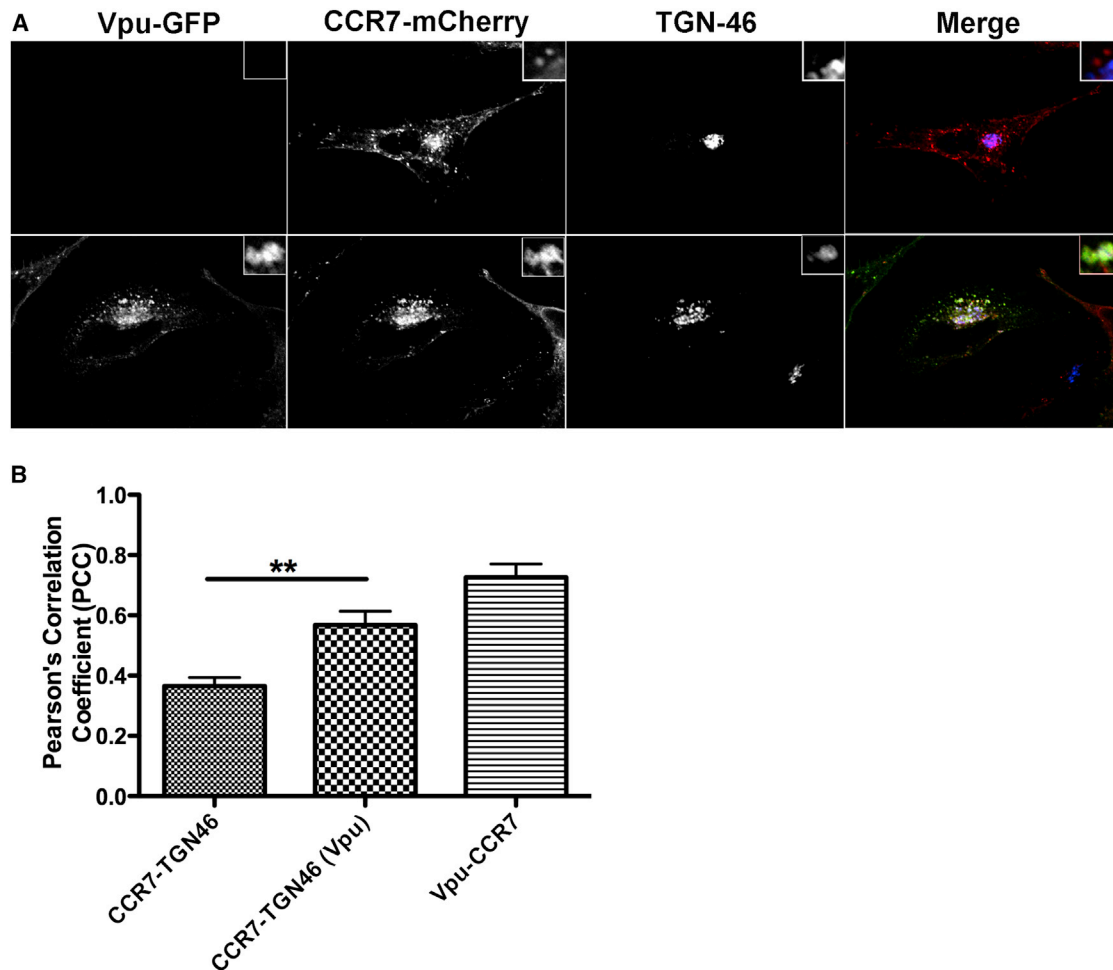
Figure 4. Vpu Colocalizes with CCR7 within the TGN

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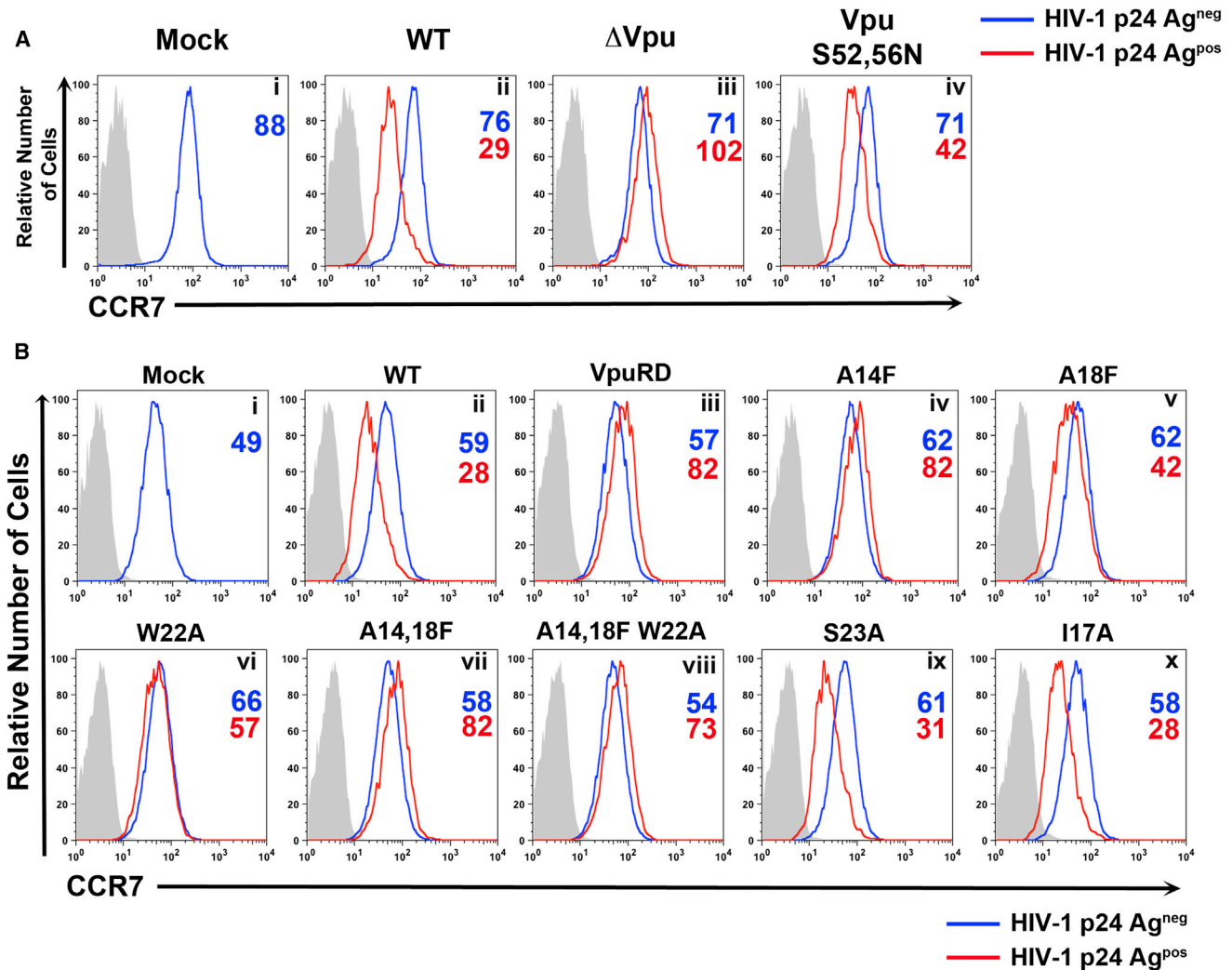
Figure 5. CCR7 Surface Downregulation Requires Vpu's TMD, but Not its Conserved Serines

(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 iden-

tify 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
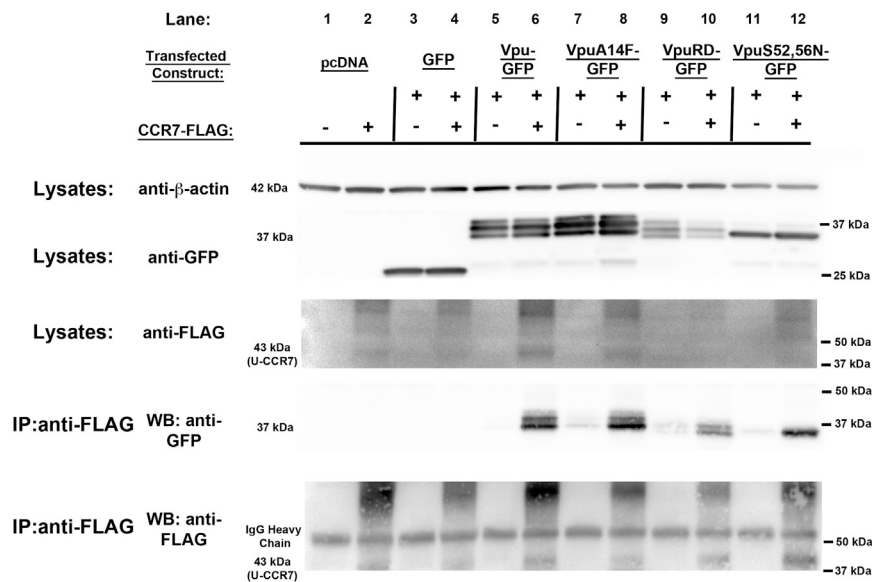
Figure 6. CCR7 Interacts with Vpu

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 regard-

less 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.
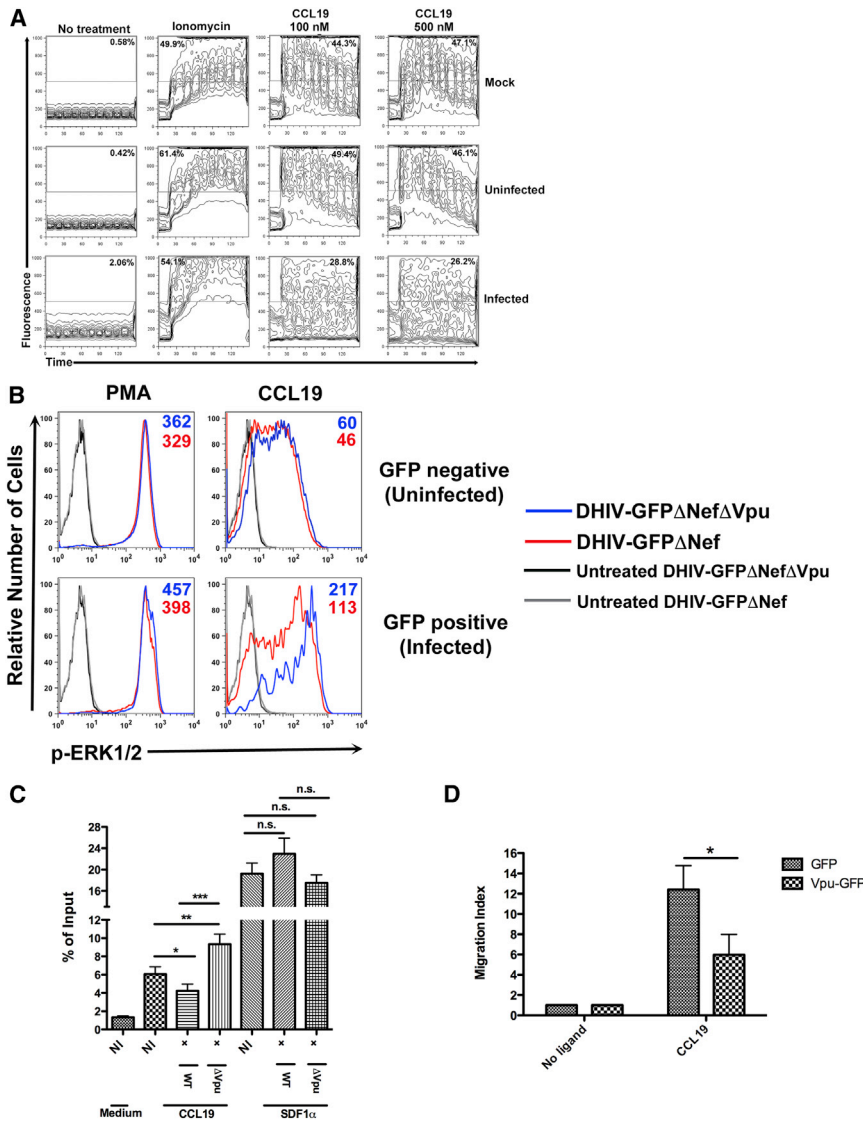
Finally, to also determine whether Vpu was sufficient to induce an impaired chemotactic response, CCRF-CEM cells were nucleofected with expression plasmids encoding GFP or Vpu-GFP. Twenty-four hours later, the cells were subjected to in vitro transmigration assays. As shown in Figure 7D, Vpu-GFP-expressing 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.

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

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