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

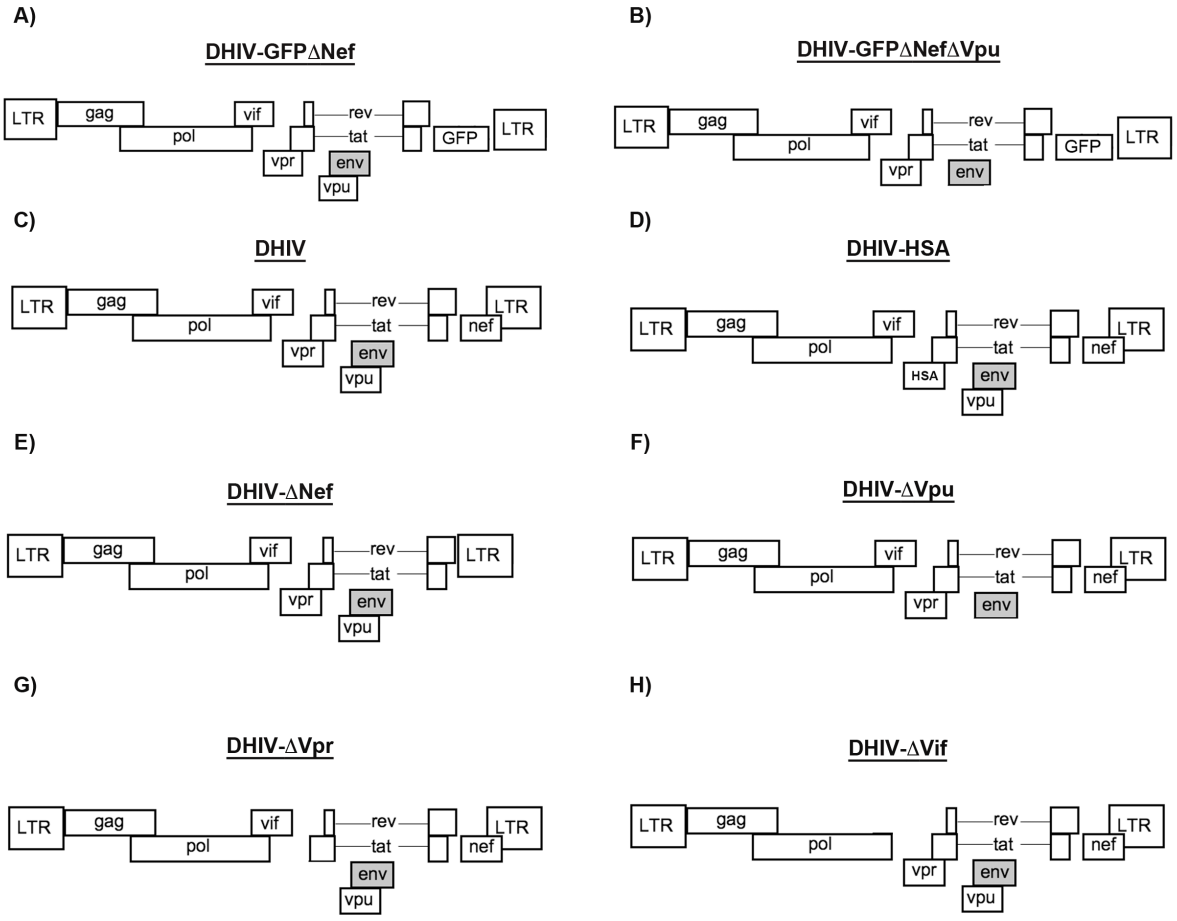

Figure S1: DHIV Constructs. (Related to Figure 1) The HIV-1_{NL4-3} sequence was cut between two BglII sites to efficiently delete envelope/gp120 (gray box) but maintain in-frame Tat, Rev and RRE ORFs. This mutant lentiviral vector was thus termed "defective" HIV, or DHIV. All constructs, with the exception 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

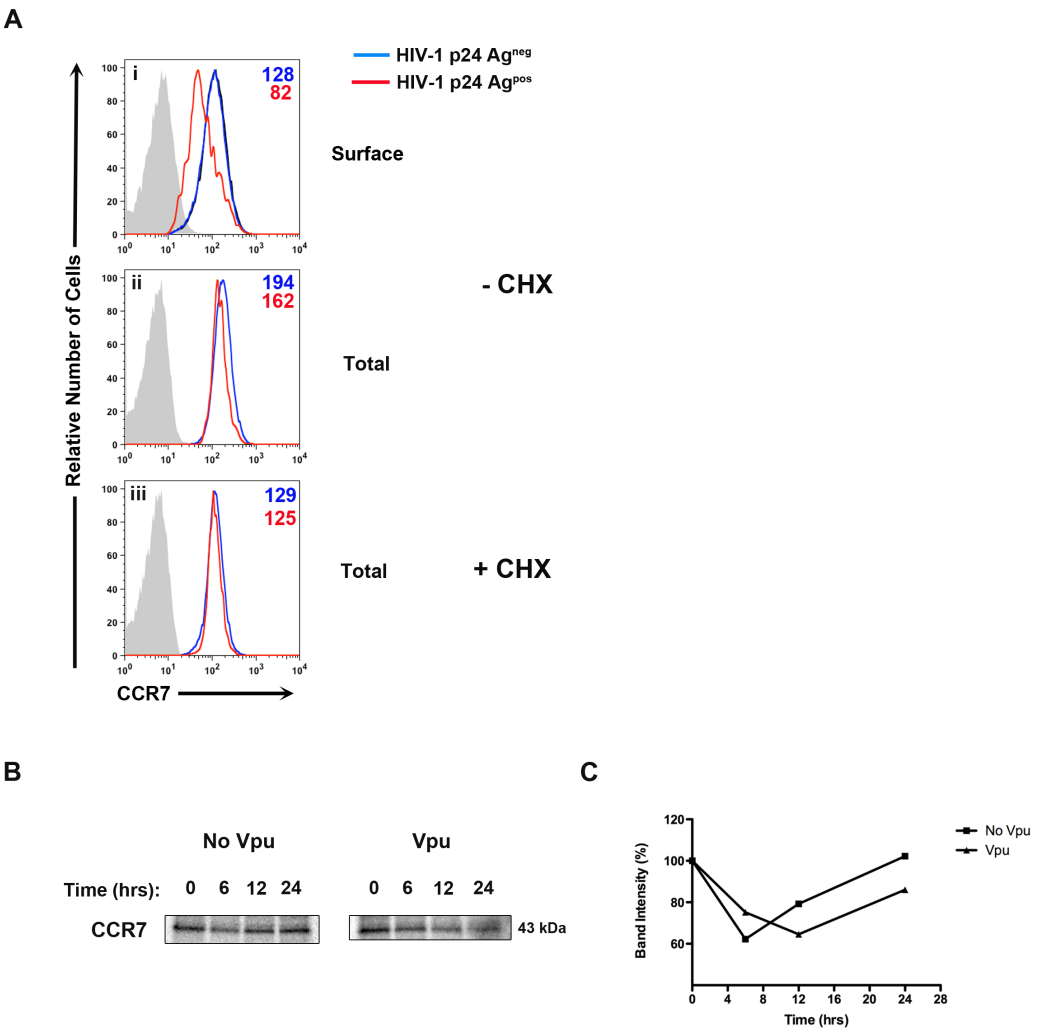

Figure S2: Vpu does not degrade CCR7. (Related to Figure 2) A.) Activated $CD4^+$ T cells were infected with DHIV. Two days later, cells were incubated in either the absence or presence of cyclohexamide (10 µg/ml) to block new protein synthesis. Twenty-four hours later, cells were either surface stained 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

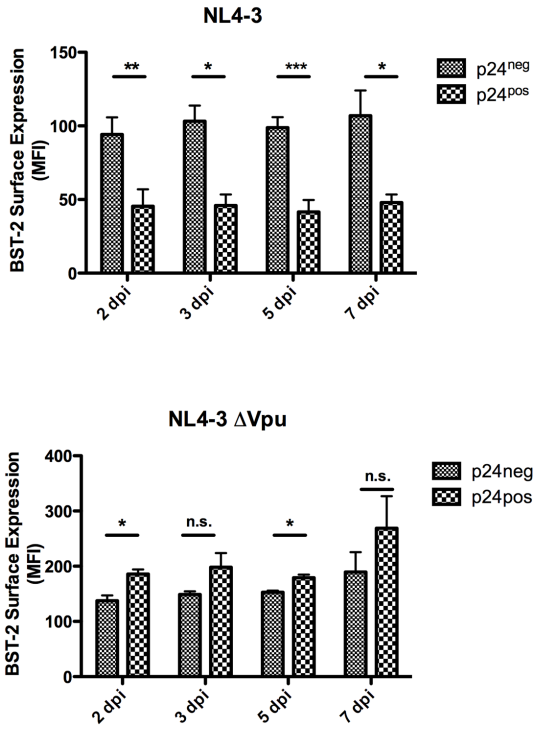

Figure S3: BST-2 is downregulated from the surface of HIV-1_{NL4-3} infected cells. (Related to Figure 3) Primary $CD4^+$ T cells were infected with either HIV-1_{NL4-3} or HIV-$1_{NL4-3}\Delta$Vpu at an MOI=.1. At two, three, five and seven days post infection, cells were surface stained for BST-2,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

Figure S4

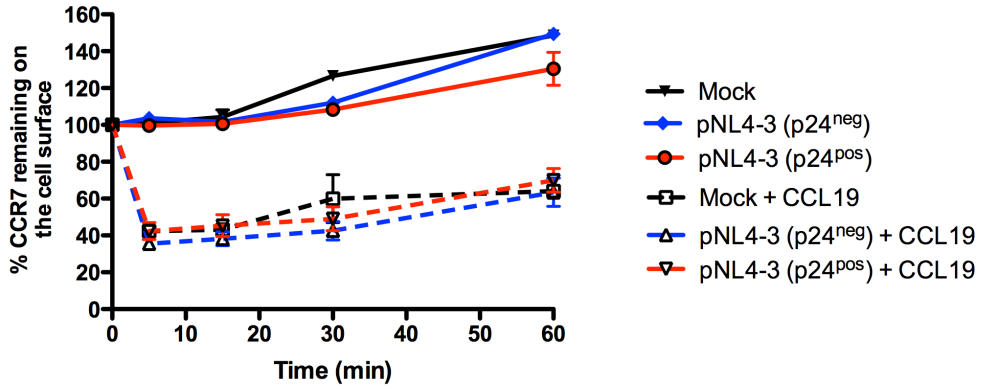

Figure S4: Vpu does not increase the endocytosis rate of CCR7. (Related to Figure 4) Primary $CD4^+$ T cells were infected with HIV-1_{NL4-3} at an MOI=.1. Seven days post infection, cells were or were not treated with CCL19 (1µg/ml) for 10 minutes at 37°C. Next, cells were stained with an antibody against 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

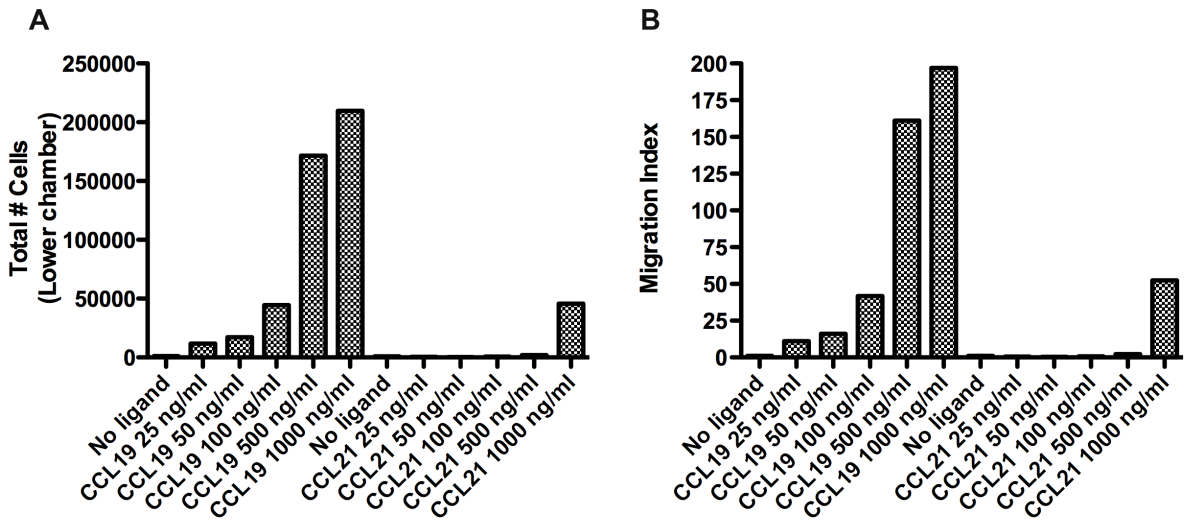

Figure S5: *In vitro* cultured T_{CM} do not respond to CCL21 as efficiently as they do to CCL19. (Related to Figure 7) 4 x 10^5 activated primary $CD4^+$ T cells were incubated for 1 hour at 37°C in transwell chambers in chemotaxis medium (RPMI 1640 + .5% BSA). Either the number of total cells present 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 and confirmed by sequencing. 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}ΔVpu 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}ΔVpu 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 co-transfected 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 x 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 x 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 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.