Small Molecule Inhibition of HIV-1–Induced MHC-I Down-Regulation Identifies a Temporally Regulated Switch in Nef Action

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HIV-1 Nef triggers down-regulation of cell-surface MHC-I by assembling a Src family kinase (SFK)-ZAP-70/Syk-PI3K cascade. Here, we report that chemical disruption of the Nef-SFK interaction with the small molecule inhibitor 2c blocks assembly of the multi-kinase complex and represses HIV-1–mediated MHC-I down-regulation in primary CD4+ T-cells. 2c did not interfere with the PACS-2–dependent trafficking of Nef required for the Nef-SFK interaction or the AP-1 and PACS-1–dependent sequestering of internalized MHC-I, suggesting the inhibitor specifically interfered with the Nef-SFK interaction required for triggering MHC-I down-regulation. Transport studies revealed Nef directs a highly regulated program to down-regulate MHC-I in primary CD4+ T-cells. During the first two days after infection, Nef assembles the 2c-sensitive multi-kinase complex to trigger down-regulation of cell-surface MHC-I. By three days postinfection Nef switches to a stoichiometric mode that prevents surface delivery of newly synthesized MHC-I. Pharmacologic inhibition of the multi-kinase cascade prevents the Nef-dependent block in MHC-I transport, suggesting the signaling and stoichiometric modes are causally linked. Together, these studies resolve the seemingly controversial models that describe Nef-induced MHC-I down-regulation and provide new insights into the mechanism of Nef action.

INTRODUCTION

After infection by HIV-1, the acute viremia induces an immune response that includes the development of anti-HIV CD8+ cytotoxic T lymphocytes (CTLs) (Gandhi and Walker, 2002). Though a significant number of the circulating CTL population is directed against HIV-1–infected cells, the virus escapes the adaptive immune response, establishing reservoirs in numerous cell types that can resist highly active antiretroviral therapy (HAART) (Stevenson, 2003). During disease progression, the HIV-1 viral load increases greatly, destroying most of the CD4+ lymphocytes, leaving patients increasingly susceptible to opportunistic infections (Douek et al., 2003).

The adaptive immune response requires members of the class I major histocompatibility complex (MHC-I) to present viral antigens on the surface of infected cells, which destroys the infected cell by the cytolitic, apoptosis-inducing actions of CTLs (Lieberman, 2003). Large DNA viruses, including herpesviruses and poxviruses, possess a large collection of immune evasive genes that are expressed in a coordinated manner to target nearly every step in the biosynthesis, assembly, transport, and cell-surface localization of MHC-I molecules (Yewdell and Hill, 2002; Peterlin and Trono, 2003). By contrast, HIV-1 relies on the single 27-kDa N-myristyoylated early gene product Nef to down-regulate MHC-I (Peterlin and Trono, 2003). Nef is required for the onset of AIDS and can affect cells in many ways, including alteration of T-cell activation and maturation, subversion of the apoptotic machinery, and the down-regulation of cell-surface molecules, notably CD4 and MHC-I (Fackler and Baur, 2002).

Down-regulation of CD4 through the clathrin/AP-2 pathway to lysosomes eliminates interference of the viral receptor with HIV-1 envelopment or release. Down-regula-
fected primary CD4+ T-cells binds represses MHC-I down-regulation in HIV-1–infected T-cells by interfering with formation of the multi-kinase complex. We further show that Nef-induced MHC-I down-regulation in primary CD4+ T-cells is manifest by the sequential action of the signaling mode, which lasts for more than two days after infection, followed by the stoichiometric mode by three days postinfection. Interference with the multi-kinase complex that triggers the signaling mode disrupts the subsequent stoichiometric block in MHC-I transport, suggesting the two modes are causally linked. These studies challenge the current dogma of Nef-mediated MHC-I down-regulation (Hansen and Bouvier, 2009) and suggest Nef orchestrates a highly regulated molecular program consisting of the sequential action of signaling followed by stoichiometric modes to evade immune surveillance.

**MATERIALS AND METHODS**

**Cells, Viruses, and Plasmids**

293T, A7, BSC-40, HeLa-CD4+, H9 CD4+ T-cells, and CEM T-cells were cultured as described (Hung et al., 2007). Peripheral blood was obtained from healthy HLA-A*0201+ volunteer by leukapheresis or venipuncture using protocols approved by the OHSU Institutional Review Board (protocols IRB00004039 and IRB00002251) or by the International Medical Center of Japan and the Kumamoto University Ethical Committee. Primary human CD4+ T-cells were isolated as described (Hung et al., 2007) and cultured in RPMI 1640 containing 10% FBS and supplemented with IL-2 (50 U/ml; Sigma) and 1 μg/ml PHA (Sigma, St. Louis, MO) before infection. HIV-1NL4-3, vaccinia virus (VV), and vaccinia recombinants expressing FLAG-tagged Nef (Nef/FLAG) were used as described (Blagoveshchenskaya et al., 2005). Adenoviruses expressing HA-tagged PACS-1 or PACS-2 were described previously (Blagoveshchenskaya et al., 2002; Simmen et al., 2005). Nef-eYFP, NefAAA-eYFP and pPten were previously described (Hung et al., 2007) and pnuGFP was obtained from Dharmacon (Boulder, CO).

**Inhibitors, siRNAs, qRT-PCR, and Kinase Assays**

PI-103 (Calbiochem, San Diego, CA) and 2c (2,4-dihydroxy-5-[1-methoxy-2-methylphenyl]benzene-1,3-diazyde) (Kyowa Hakko Kirin Co., Tokyo, Japan) were used as indicated. 2c toxicity was determined by MIT (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Control (nonspecific) siRNA and siRNAs specific for Hck, Lyn, Src, PACS-1, PACS-2, or the μLA subunit of AP-1 (Smartpool, Dharmacon) were nucleofected (Amaxa, Gaithersburg, MD) into cells according to manufacturer’s instructions. RNA was purified from H9 cells nucleoected with siRNAs as indicated in figure legends using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. cDNA was reverse transcribed from RNA using the random decamers from the RETROscript Kit (Ambion, Austin, TX) via manufacturers' instructions. Utilizing commercially-available Hck, Lyn, Src primers (Qiagen) and SYBR green qPCR reagent (SA-Biosciences, Frederick, MD), q-PCR was conducted on a StepOnePlus Real-time PCR system (Applied Biosciences, Foster City, CA). PI3K assays were performed as described (Emert-Sedlak et al., 2009).

**Flow Cytometry and Immunofluorescence Microscopy**

For flow cytometry, cells were processed as described (Hung et al., 2007; Atkins et al., 2008) and stained using the following antibodies: anti-MHC-I (W6/32), anti-HLA-A2 (BB7.2 BD, San Jose, CA), anti-CD4-APC (Biolegend, San Diego, CA), or anti-p24-PE (Virostat, Portland, ME) as indicated in legends. PE-conjugated donkey anti-mouse IgG (Jackson IR, West Grove, PA) was used to stain MHC-I– and HLA-A2-positive cells. Isootype-matched antibodies (Serotec, Raleigh, NC) were used as negative controls. Samples were processed on a FACScalibur (BD) as described (Atkins et al., 2008) and data analyzed using FCS express (De Novo Software, Los Angeles, CA). For immunofluorescence microscopy, cells were processed as indicated in legends and processed for immunofluorescence as described (Atkins et al., 2008). Confocal images were captured as described (Atkins et al., 2008). Immunofluorescence were acquired on a Leica SP5 confocal microscope and analyzed using Imaris 7.0. A mask for each field of cells was generated based on the fluorescence signal of Golgin-97 and the percent colocalization of Golgin-97 with Nef-eYFP was determined and presented as the mean ± SD from at least 20 cells per condition.

**Immunoprecipitation, Western Blot, and Antibody Uptake**

Cells infected with the indicated VV recombinants were harvested as described (Atkins et al., 2008). Where indicated, cells were treated with the...
corresponding concentration of 2c before harvest. Flag-tagged Nef constructs were immunoprecipitated with mAb M2-agarose (Sigma), and associated proteins were detected by Western blot. The following antibodies were obtained from the American Type Culture Collection (ATCC): rabbit anti-HA.11 (Covance, San Diego, CA); rabbit anti-Hck, anti-Lyn (Santa Cruz, Santa Cruz, CA); rabbit anti-PTEN, anti-p-PTEN, anti-2c, anti-phospho-2c (Upstate, Bed ford, MA); anti-phospho2c-ZAP-70 (BD); anti-Akt, anti-p-Akt, anti-His6, anti-PTEN, anti-cleaved caspase-3 (Cell Signaling, Danvers, MA); anti-PTEN (Chemicon, Bedford, MA); anti-Nef #2949 (obtained through NIH AIDS Research and Reference Reagent Program), anti-MHC-I K455 (provided by K. Traub, University of Pittsburgh), AP-1 (Sigma), anti-FACS-1 (703), and anti-FACS-2 (Atkins et al., 2008). Antibody uptake by mAb W6/32 was performed as described (Blaugoveschenskaya et al., 2002).

NMR Spectroscopy

The cDNA encoding consensus nef (obtained from the NIH AIDS Research and Reference Reagent Program) was His6-tagged, inserted into pET21 vector (EMD Chemicals, San Diego, CA), and protein was expressed in Rosetta 2 (DE3) E. coli, cultured in modified minimal medium using [14N]Cl as the sole nitrogen source, and induced with 0.4 mM IPTG at 18°C for 16 h. Soluble forms of His6-tagged Nef proteins were purified over a Ni+2-NTA column (GE Healthcare, Uppsala, Sweden) and subsequent gel-filtration on a Superdex200 26/60 column (GE Healthcare) equilibrated with 25 mM sodium phosphate buffer (pH 7.5), 150 mM NaCl, 1 mM DTT, and 0.02% sodium azide. Two-dimensional (2D) 1H-15N heteronuclear single quantum coherence (HSQC) experiments (Bodenhausen and Ruben, 1980) were performed at 27°C on a Bruker Avance 700 MHz spectrometer equipped with a 5-mm, triple resonance, and z-axis gradient cryoprobe. The 1H-15N HSQC spectrum of free Nef was obtained using a 80-μM uniform 15N-labeled Nef sample in 10 mM MOPS (pH 7.0), 100 mM NaCl, 5 mM DTT, and 0.02% sodium azide. A series of 1H-15N HSQC spectra were acquired to monitor chemical shift changes upon addition of aliquots of a 10-mM 2c stock solution, dissolved in DMSO, to the Nef sample. The Nef:2c molar ratios of the solutions were 1:0, 1:0.6, 1:1, 1:2, 1:3, 1:4, and 1:5. A control series of 1H-15N HSQC spectra were also obtained after adding the same amounts of DMSO without 2c to the free Nef sample. In this series, in contrast to the titration with the 2c solution, minimal spectral changes occurred (data not shown), confirming that the changes after addition of 2c were indeed caused by this organic molecule and not the solvent.

Transport Assay and Endo H Treatment

H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses for 24, 48, or 72 h. After infection, cells were subjected to pulse-chase/surface bionylation as described (Blaugoveschenskaya et al., 2002). Briefly, to IP with HC10, cells were lysed in m-RIPA [1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0)] and boiled for 1 h at 55°C to denature MHC-I proteins. To IP with HC10 M2c, cells were lysed in PBS (pH 7.2) containing 1% NP-40. Bound MHC-I proteins were eluted from protein A sepharose beads (Sigma) by boiling in TBS, 5% SDS, 2% NP-40, and 2% sodium deoxycholate. One-third of the eluate was used to assess total MHC-I while the rest was incubated with streptavidin agarose (Pierce, Rockford, IL) to capture bionylated MHC-I. For Endo H treatment, MHC-I was eluted by boiling in 10 mM Tris-HCl (pH 7.4) containing 1% SDS, precipitated with acetone and resuspended in glycoprotein denaturation buffer (NEB, Ipswich, MA) and digested with Endo H for 1 h at 37°C. Samples were separated by SDS-PAGE and processed using Amplify (GE Healthcare). Quantification was performed using NIH Image J.

Protein Interaction Assays

Plasmids expressing GST, GST-MHC-I CDNeff (provided by J. Guatelli, UCSF), His6-Hck-OR GST-Nef (strain 1A4-3), were transformed in BL21 E. coli and cultures were induced with 1 mM IPTG (Calbiochem) for 4 h at 37°C. Bacterial pellets were resuspended in lysis buffer [50 mM Tris (pH 7.6), 1.5 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 0.1 mM DTT, 10 mM MgCl2] containing protease inhibitors (0.5 mM PMSF and 0.1 μM each of aprotinin, E-64, and leupeptin), lysed using a French Press (Aminco, Rockville, MD) and incubated with GST-sepharose (GE Healthcare). For the interaction with GST-MHC-I CDNefeff, A7 cells were lysed in 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 5 mM EDTA, 150 mM NaCl, 10 mM MgCl2 with protease inhibitors (0.5 mM PMSF and 0.1 μM each of aprotinin, E-64, and leupeptin). A7 lysates were added to GST-Sepharose bound to the proteins of interest overnight at 4°C. The bound proteins were washed with 20 μl of binding buffer for one h, washed three times in lysis buffer and once in 50 mM Tris (pH 8.0), and resuspended in SDS-PAGE sample buffer. For the His6-Hck interaction with GST-Nef, the proteins were mixed at a 2:1 ratio (His6-Hck:Net) for 30 min at 4°C in binding buffer [0.1% NP-40, 0.1 mM EDTA, 20 mM Tris (pH 7.5), 150 mM NaCl] containing the indicated concentrations of 2c. After incubation, GST-Sepharose was added and subsequently washed three times in binding buffer and resuspended in SDS-PAGE sample buffer.

RESULTS

HIV-1 Nef Uses a Subset of SFKs to Trigger MHC-I Down-Regulation

Formation of the SFK-ZAP70/Syk-P13K multi-kinase complex is initiated by the PXPPxxG-dependent binding of Nef to the SH3 domain of a Golgi region-localized SFK, directly activating the kinase (Hung et al., 2007). Of the seven known SFKs only a subset—Hck, Lyn, and Src—can be directly activated after Nef binding and are found in the Golgi region, suggesting one or more of these kinases mediate MHC-I down-regulation (Matsuda et al., 2006; Trible et al., 2006; Hiyoshi et al., 2008; Pulvirenti et al., 2008). To test this, H9 CD4+ T-cells treated with siRNA specific for Hck, Lyn, or Src (Figure 1A) were infected with recombinant viruses expressing Nef and the extent of MHC-I down-regulation was determined. Nef-induced MHC-I down-regulation was slightly repressed by siRNA knockdown of Hck alone but blocked by knockdown of Hck, Lyn, and Src together. The data suggest that Hck, Lyn, and Src function redundantly in Nef-induced MHC-I down-regulation.

Small Molecule Inhibition of the Nef-SFK Interaction

The determination that Nef assembles a multi-kinase complex to trigger MHC-I down-regulation suggests this pathway can be selectively targeted pharmacologically. Indeed, isofom-specific class I PI3K inhibitors repress Nef-induced MHC-I down-regulation in primary CD4+ T-cells (Hung et al., 2007; Atkins et al., 2008). An alternative approach would be to block protein-protein interactions required for complex assembly without affecting cellular enzyme activity directly. The characterized binding of Nef to SFK SH3 domains represents an ideal target for this type of inhibitor (Arold et al., 1997). One candidate compound, 2c (supplemental Figure S2), is a derivative of the compound UCSi15A that disrupts the PXPP-dependent binding of Sam68 to Src (Oneyama et al., 2003). To test whether 2c disrupts Nef-SFK binding, we treated H9 CD4+ T-cells coexpressing flag-tagged Nef (Net/f) and Hck with increasing concentrations of 2c. Hck was used because it binds Nef with higher affinity than other SFKs and Nef can assemble Hck into the multi-kinase complex (Hung et al., 2007; Atkins et al., 2008). Coimmunoprecipitation analysis showed 2c repressed the interaction between Nef and Hck in a dose-dependent manner as well as repressed the interaction with Lyn or Src (Figure 1B and supplemental Figure S3). Next, we incubated GST-Nef and His6-Hck with increasing concentrations of 2c and found that 2c disrupted Nef-Hck binding in a dose-dependent manner (Figure 1C). To determine whether the ability of 2c to inhibit Nef-SFK binding also blocked Nef-induced SFK activation, we incubated recombinant, purified Nef, and Hck with increasing concentrations of 2c and measured the resulting Nef-induced Hck activity using a FRET-based in vitro kinase assay. Under these assay conditions, activation of Hck is dependent upon Nef (Emert-Sedlak et al., 2009). As shown in Figure 1D, 25 μM 2c substantially repressed Nef-dependent Hck activity without affecting Hck in the absence of Nef. High 2c concentrations (≥100 μM), however, directly inhibited Hck activity. These findings suggest 2c has a bi-modal effect on Hck activity; at low concentrations 2c disrupts Nef-induced kinase activity, whereas high concentrations of 2c inhibit Hck directly.

The ability of 2c to selectively inhibit Nef-induced Hck activation suggested 2c may bind Nef directly, thereby affecting Hck activity. Accordingly, 1H-15N HSQC NMR analysis of the interaction of 2c with recombinant Nef in solution revealed 2c...
Figure 1. 2c interferes with Nef-SFK binding. (A) H9 CD4+ T-cells were nucleofected (Amaxa) on days 1 and 3 with pmaxGFP and nonspecific siRNA or siRNAs that target Hck, Lyn, or Src alone or in combination. On day 4 cells were infected with VV:WT (gray filled) or VV:Nef (lines, unfilled) (moi = 10, 8 h), and analyzed by flow cytometry using mAb W6/32 as described in experimental procedures. MFI: NS siRNA (WT = 167, Nef = 99.5); Nef + Hck siRNA = 120; + Lyn siRNA = 103; + Src siRNA = 117; + Hck, Lyn, Src = 169. MFI, mean fluorescence intensity. (B) H9 cells were coinfected with VV:WT, VV:Nef, or VV:Hck and treated with 10, 20, or 50 μM 2c for 4 h before harvest. Nef proteins were immunoprecipitated, and coprecipitating Hck was detected by Western blot. (C) GST-Nef was incubated with His6-Hck and treated with increasing concentrations of 2c. GST-Nef was captured, and bound His6-Hck was quantified using NIH Image J. Accordingly, nonspecific binding of GST to His6-Hck was subtracted and values were normalized to the binding of GST-Nef to His6-Hck in the absence of 2c. Each condition was assayed in triplicate, and results are presented as the mean ± SD. (D) Hck alone (gray bars) or Hck plus Nef (black bars) were treated with increasing concentrations of 2c. The resulting Hck enzyme activity was measured using a fluorometric assay and expressed relative to 100% control activity. Each condition was assayed in quadruplicate, and results are presented as the mean ± SD. (E) Superposition of 1H-15N HSQC spectra of Nef in the absence (blue) and presence (red) of 2c. The 1H-15N HSQC spectra were recorded on 80 μM uniform 15N-labeled Nef samples at 27°C in the absence (blue) and presence (red) of 1 mM 2c (1:12.5 M ratio of Nef to 2c). All assignable amide resonances (J. Jung, I.-J.L. Byeon, J. Ahn, and A.M. Gronenborn, unpublished data) that exhibit chemical shift changes >0.06 ppm are labeled with residue name and number. The resonances of Y135, V146, and F185 are labeled only on the free Nef spectrum because the identification of the bound resonances was not straightforward due to very large shift changes or severe line broadening beyond detection. The resonances of M79 (Δδ = 0.041ppm) and T80 (Δδ = 0.025ppm), which are located immediately after the P72XXP75 motif, are also labeled. Concentrations of 2c as low as 48 μM, representing a 1:0.6 M ratio of Nef...
induces a number of chemical shift changes in amide resonances of Nef (Figure 1E). These include relatively small chemical shift changes (0.025–0.063 ppm) at V24, M90, and T90 located within the Nef polyproline helix that binds the RT loop of SH3 domains on SFKs (Lee et al., 1995), suggesting 2c may influence the conformation of the polyproline helix. The presence of resonance overlap in the NMR spectra precluded more detailed analysis involving the disordered regions of Nef such as the N-terminal region preceding the polyproline helix and the internal loop consisting of residues E49–K70. More pronounced chemical shift changes (>0.1 ppm) were observed for the resonances of Y135, K144, V146, K184 (R184 in Figure 1F [PDB ID:2NEF]), I185, and L189 which are clustered on the opposite side of the SH3 domain binding site. Other residues in this region such as V146 A190, F191, M192, and E197, and T198 also undergo substantial (>0.06 ppm) changes. These chemical shift perturbations caused by the addition of 2c imply that 2c could bind to the cleft formed by the central β-sheet and the C-terminal α-helices of Nef, which may provide a good binding pocket (Figure 1F). Together, these findings suggest 2c may interfere with Nef-induced SFK activation most likely by allosteric inhibition of SFK binding or possibly by directly influencing the binding mode of the polyproline helix to SFKS or both.

2c Represses the Ability of HIV-1 to Down-Regulate MHC-I

The 2c concentration range that selectively inhibited Nef-dependent SFK activation showed no measurable cell toxicity up to 60 µM for two days, suggesting 2c could be tested in culture (Figure S4, panel A). Accordingly, we treated H9 cells expressing Nef-eYFP with 2c or the class I PI3K inhibitor PI-103, which specifically blocks Nef-induced MHC-I down-regulation (Hung et al., 2007), and measured cellular surface MHC-I (Figure 2A). In agreement with earlier studies (Hung et al., 2007; Atkins et al., 2008), Nef-eYFP induced an ~twofold down-regulation of MHC-I that was blocked by PI-103. Similarly, 2c partially blocked MHC-I down-regulation. Next, we asked whether 2c could interfere with HIV-1-induced MHC-I down-regulation in primary CD4+ T-cells. We infected primary CD4+ T-cells with HIV-1NL4-3 and treated the cultures with vehicle or 2c. At 8 d postinfection, the cells were analyzed for cell-surface HLA-A2 and CD4 by flow cytometry (Figure 2B). We found that 2c partially blocked down-regulation of MHC-I but had no effect on down-regulation of CD4, which is mediated by a class I PI3K-independent pathway (Hung et al., 2007).

2c Disrupts Formation of the Multi-Kinase Complex

The ability of 2c to disrupt Nef-SFK binding as well as HIV-1–induced MHC-I down-regulation suggested this compound would disrupt assembly of the SFK-ZAP-70-PI3K multi-kinase complex. Accordingly, we found that 2c blocked the ability of Nef to recruit class I PI3K activity using an in vitro lipid kinase assay (Figure 3A). Moreover, this result was not due to cell toxicity or induction of apoptosis nor was it due to nonspecific inhibition of class I PI3K as treatment of the immunoprecipitate with 40 µM 2c had no effect on enzyme activity (supplemental Figure S4, B and C and Figure 3A). The titration analysis of 2c on Nef-induced Hck activation (Figure 1D) suggests 40 µM 2c blocked Nef-induced PI3K stimulation by selective inhibition of Nef-induced SFK activation, whereas 100 µM 2c may block Nef-induced PI3K stimulation by additionally inhibiting SFK activation directly. The PI3K activity results were supported by coimmunoprecipitation analysis in which 2c inhibited formation of the multi-kinase complex by disrupting the interaction of Nef with Hck, phospho-ZAP-70 and the class I PI3K p85 regulatory subunit (Figure 3B).

The ability of 2c to disrupt Nef-SFK binding did not exclude the possibility that it may affect additional steps in the MHC-I down-regulation pathway. We first asked whether 2c interfered with steps upstream of Nef-SFK binding. Accordingly, we found that 2c had no effect on the Nef-PACS-2 interaction nor the PACS-2–dependent trafficking of Nef-eYFP to the paranuclear region, which is required for Nef-SFK binding and Nef-induced MHC-I down-regulation (Figure 3, C and D and Atkins et al., 2008).

Because Nef M20 mediates the interaction with AP-1 (Roeth et al., 2004) and is essential for sequestration of MHC-I molecules after their internalization from the cell surface (Blagoveshchenskaya et al., 2002), we tested whether AP-1 was required in the last stage of this signaling pathway. Accordingly, H9 cells knocked down for AP-1, PACS-2, or PACS-1 which is required for MHC-I down-regulation but not for triggering PI3K stimulation (Piguet et al., 2000; Atkins et al., 2008), were infected with viruses expressing Nef or the NefAAAXX–PI3K* chimera, which rescues the inability of NefAAAXX to down-regulate MHC-I by overriding the requirement for assembly of the SFK-ZAP-70-PI3K complex (supplemental Figure S5 and Blagoveshchenskaya et al., 2002; Hung et al., 2007). The ability of Nef or NefAAAXX–PI3K* to down-regulate MHC-I in cells knocked down for expression of PACS-2, PACS-1, or AP-1A was assessed by antibody uptake to discern the importance of each protein in steps upstream or downstream of PI3K stimulation (Figure 4A). Consistent with our determination that Nef requires PACS-2 upstream of PI3K (Atkins et al., 2008), siRNA knockdown of PACS-2 blocked MHC-I uptake by Nef but not by NefAAAXX–PI3K*. By contrast, siRNA knockdown of AP-1A or PACS-1 blocked MHC-I uptake induced by Nef and NefAAAXX–PI3K*, demonstrating AP-1 and PACS-1 act downstream of PI3K stimulation.

To test potential effects of 2c on the later stages of this pathway, we asked whether 2c interfered with the interaction between Nef and PACS-1 or AP-1. We determined that 2c had no effect on the Nef-PACS-1 interaction (Figure 4B). Additionally, the interaction of AP-1 with Nef M20 and the MHC-I cytosolic domain can be recapitulated using the chimeric protein GST-MHC-I CD-NefLL/AA to capture AP-1 from cytosol preparations (Noviello et al., 2008). This chimera consists of Nef with an LL165→AA substitution, which blocks the LL165→AA-dependent binding to adaptors, fused to the MHC-I cytosolic domain. We found that 2c did not interfere with the ability of GST-MHC-I CD-NefLL/AA to capture AP-1 (Figure 4C, left).
ingly, we found that GST-MHC-I CD-Nef LL/AA captured PACS-1 but not PACS-2, consistent with our determination that Nef requires PACS-1 subsequent to PI3K stimulation to down-regulate MHC-I (Figure 4C, right). Moreover, similar to our findings with AP-1, 2c failed to disrupt the interaction of GST-MHC-I CD-NefLL/AA with PACS-1. To further evaluate whether 2c disrupts MHC-I down-regulation downstream of PI3K stimulation, we repeated the antibody uptake experiment (Figure 4D). We found that 2c blocked the ability of Nef but not NefAXXAxPI3K* to down-regulate MHC-I, indicating 2c specifically acts upstream of PI3K stimulation.

PTEN-Null CEM Cells Fail to Phenocopy Nef Action in Primary CD4+ or H9 Cells

Our results using primary CD4+ T-cells and H9 cells suggest 2c disrupts HIV-1-mediated MHC-I down-regulation by interfering with the ability of Nef to assemble the SFK-ZAP-70/Syk-PI3K complex. This signaling pathway explains the importance of the EEEE65 and PXXP 75 sites, which trigger MHC-I internalization and subsequent M20-mediated sequestering of internalized MHC-I molecules (Blagoveshchenskaya et al., 2002; Hung et al., 2007). However, an alternate model of MHC-I down-regulation, which largely relies on CEM T-cells stably expressing MHC-I, envisions a PI3K-independent pathway based largely on the M20-mediated stoichiometric block of newly synthesized MHC-I molecules en route to the cell surface (Kasper and Collins, 2003). We therefore transfected CEM cells with plasmids expressing PTEN alone or together with Nef (Figure 5A), and tested whether 2c or PI-103 would repress MHC-I down-regulation. Because transfected PTEN can be inhibited by oxidation in leukemic cells, these experiments were conducted in 0.5 mM β-mercaptoethanol (Silva et al., 2008). In contrast to H9 cells or primary CD4+ T-cells, both compounds failed to repress MHC-I down-regulation in CEM cells (Figure 5B, top), suggesting Nef may down-regulate MHC-I in CEM cells by a mechanism different from it uses in H9 or primary CD4+ T-cells. Alternatively, the disparate findings may have resulted from the dysregulated PI3K signaling inherent to CEM cells, which would override the requirement for the multi-kinase complex (Astoul et al., 2001). Indeed, many leukemic cell lines such as CEM and Jurkat lack the tumor suppressor PTEN, which is a lipid phosphatase that attenuates PI3K/Akt signaling characteristic of many tumor cell lines. Although acute treatment of CEM or Jurkat cells with PI3K inhibitors...
prevents new PIP3 synthesis, the absence of PTEN results in persistently elevated levels of PIP3 that mediate PI3K-stimulated pathways even in the presence of PI3K inhibitors, thereby conferring resistance to the effect of multi-kinase complex inhibition (Hung et al., 2007). We therefore expressed PTEN alone or PTEN together with Nef (Figure 5A) and determined that PTEN alone had little effect on the cell-surface levels of endogenous MHC-I, which is expected because this enzyme is normally expressed in CD4+ T-cells (Figure 5B, bottom). Reexpression of PTEN, however, repressed the constitutively elevated PI3K/Akt signaling present in CEM cells as determined by a decrease in active PIP3.
Akt (Figure 5A), suggesting that PTEN-replete CEM cells may be rescued in their ability to regulate PIP3 levels and would thus be responsive to treatment with PI3K inhibitors. Accordingly, reexpression of PTEN rescued the ability of PI-103 or 2c to repress MHC-I down-regulation in CEM cells, similar to that observed in primary CD4+/H9 cells or H9 cells (Figure 5B bottom and see Figure 2). These results demonstrate that an intact PI3K regulatory network is required to study PI3K-dependent steps in signaling pathways, including Nef-induced MHC-I down-regulation.

**Nef Down-Regulates MHC-I by a PI3K-Triggered Endocytic Pathway Followed by a Transport Block**

Whereas aberrant phosphoinositide metabolism in CEM cells can explain the confusion underlying the requirement by Nef for the multi-kinase complex to down-regulate MHC-I in primary CD4+ T-cells or H9 cells (Figure 5B bottom and see Figure 2), these results demonstrate that an intact PI3K regulatory network is required to study PI3K-dependent steps in signaling pathways, including Nef-induced MHC-I down-regulation.

(prophosphorylated) Akt (Figure 5A), suggesting that PTEN-replete CEM cells may be rescued in their ability to regulate PIP3 levels and would thus be responsive to treatment with PI3K inhibitors. Accordingly, reexpression of PTEN rescued the ability of PI-103 or 2c to repress MHC-I down-regulation in CEM cells, similar to that observed in primary CD4+ T-cells or H9 cells (Figure 5B bottom and see Figure 2). These results demonstrate that an intact PI3K regulatory network is required to study PI3K-dependent steps in signaling pathways, including Nef-induced MHC-I down-regulation.

**Nef Down-Regulates MHC-I by a PI3K-Triggered Endocytic Pathway Followed by a Transport Block**

Whereas aberrant phosphoinositide metabolism in CEM cells can explain the confusion underlying the requirement by Nef for the multi-kinase complex to down-regulate MHC-I (Kasper and Collins, 2003; Schaefer et al., 2008), this defect did not readily explain why some studies found that Nef blocks delivery of newly synthesized MHC-I molecules en route to the cell surface—the stoichiometric model—whereas other studies found Nef relies on its ability to assemble the multi-kinase complex to internalize and sequester MHC-I molecules following their delivery to the cell surface—the signaling model. Although these disparate findings were originally attributed to uncharacterized differences in Golgi-to-cell-surface transport in T-cells versus HeLa cells (Kasper and Collins, 2003), closer inspection of the experimental paradigm revealed that the signaling model assessed MHC-I transport at 7–44 h postinfection while the stoichiometric model assessed ER-to-cell surface transport of MHC-I at longer postinfection times (Blagoveshchenskaya et al., 2002; Kasper et al., 2005; Hung et al., 2007). We therefore conducted a time course to measure the ability of Nef to impede cell surface delivery of newly synthesized endogenous MHC-I molecules (Figure 6A). Parallel cultures of H9 cells infected for 24, 48, or 72 h with pseudotyped HIV-1N134-3 that either lack or express Nef were subjected to pulse-chase/surface biotinylation to monitor...
delivery of MHC-I to the cell surface. At 24 and 48 h postinfection, Nef had no measurable effect on the transport of newly synthesized MHC-I to the cell-surface. By 72 h postinfection, however, Nef markedly repressed MHC-I transport. To test whether these results were specific to H9 cells or the use of HC10, which recognizes denatured HLA-B and C heavy chains, we repeated the pulse-chase/surface biotinylation in primary CD4+ T-cells using the conformation-dependent antibody, BB7.2, which recognizes HLA-A2. In agreement with our findings in H9 cells, Nef had no appreciable effect on cell-surface delivery of HLA-A2 for the first 48 h postinfection in primary CD4+/H11001 T-cells. Again, similar to H9 cells, at 72 h postinfection Nef markedly repressed HLA-A2 transport to the cell surface (Figure 6B).

To determine whether the switch in Nef-induced MHC-I down-regulation was coupled with a change in MHC-I stability, H9 cells infected with Nef/H11002 or Nef/H11001 pseudotyped HIV-1 for 24 or 72 h were pulse-labeled with [35S]Met/Cys for 15 min and chased for up to 20 h. Immunoprecipitation of endogenous MHC-I with HC10 showed that Nef had no obvious effect on MHC-I stability at 24 or 72 h postinfection (Figure S6). To assess whether Nef altered the rate of MHC-I transport, the immunoprecipitates were subjected to endoglycosidase H (Endo H) digestion, which demonstrated MHC-I molecules became Endo H resistant by 4 h postinfection irrespective of Nef expression or the time postinfection (Figure 6C). These findings suggest Nef does not impede MHC-I transport from early secretory pathway compartments nor does it markedly affect the stability of endogenous MHC-I molecules.

The inability of Nef to block ER-to-cell surface transport of MHC-I molecules for at least 48 h postinfection suggested that for the first two days Nef may down-regulate MHC-I by triggering the multi-kinase-dependent internalization and sequestering of MHC-I molecules from the cell surface. We tested this possibility using antibody uptake. H9 cells were infected with Nef+ or Nef− pseudotyped HIV-1 for 48 or 72 h and then incubated with anti–MHC-I (W6/32) in the absence or presence of 2c or PI-103. The cells were then fixed, permeabilized, and stained with a secondary antibody to detect internalized MHC-I (W6/32) and with antibody K455 to detect steady-state MHC-I (Figure 6D). In agreement with the biotinylation analysis, at 48 h postinfection Nef induced a marked increase in MHC-I internalization that overlapped with the MHC-I post-fix staining pattern. Treatment of the cells with 2c or PI-103 blocked antibody uptake, suggesting multi-kinase complex formation was required to down-regulate MHC-I at these time points. By contrast, at 72 h postinfection Nef failed to induce W6/32 uptake despite down-regulating MHC-I as determined by the K455 post-fix staining pattern. Analysis by flow cytometry revealed that Nef reduced cell surface levels of MHC-I to a similar extent at 48 h or 72 h postinfection (Figure 6E). Together, these experiments suggest that Nef-induced MHC-I down-regulation is manifest for two days by a Nef-assembled PI3K signaling pathway that sequesters MHC-I endocytosed from the cell-surface following a switch in Nef action at day three to a stoichiometric mechanism that prevents ER to cell surface transport of newly synthesized MHC-I.

The Signaling Mode Is Required for the Switch to the Stoichiometric Mode

The switch in Nef-induced MHC-I down-regulation from a signaling-based pathway to a stoichiometric mechanism did not appear to result from use of tumor cell lines, differences in antibodies used to immunoprecipitate MHC-I, or levels of Nef expression (see Figure 6). We therefore asked whether...
the conversion of Nef-induced MHC-I down-regulation from a signaling- to a stoichiometric-mode depended upon the activity of the multi-kinase complex. To test this possibility, replicate plates of H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses (moi = 2.3) for 24, 48, or 72 h and subjected to pulse-chase/surface biotinylation using HC10 associated MHC-I and quantified as described in Methods. Error bars represent the mean ± SD from 3 independent experiments. Cell viability was greater than 95% at each time point as measured by trypan blue exclusion and greater than 90% of the cells were infected with each virus as determined by GFP staining. (B) Primary CD4+ T-cells were processed and quantified as in panel A except using BB7.2 to IP native MHC-I. (C) H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses for 24 or 72 h, MHC-I was immunoprecipitated with mAb HC10 as in A and then digested or not with Endo H as described in Methods. (D) H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses for 48 or 72 h and then treated or not with PI-103 (5 µM) or 2c (20 µM) for 16 h. Cells were incubated with W6/32 (3 µg/ml) for 30 min and then chased and processed for immunofluorescence microscopy as described in the legend to Figure 4A. Post-fix. Total MHC-I was detected post-fixation by staining the cells with K455. Scale bar, 10 µm. (E) H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses for 48 or 72 h and analyzed for down-regulation of MHC-I as described in Methods. MFI: 48 h Nef− = 341, 48 h Nef+ = 110, 72 h Nef− = 302, 72 h Nef+ = 125.

Figure 6. Nef-induced MHC-I down-regulation switches from a signaling- to a stoichiometric mechanism. (A) H9 cells infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses (moi = 2.3) for 24, 48, or 72 h and subjected to pulse-chase/surface biotinylation using HC10 associated MHC-I and quantified as described in Methods. Error bars represent the mean ± SD from 3 independent experiments. Cell viability was greater than 95% at each time point as measured by trypan blue exclusion and greater than 90% of the cells were infected with each virus as determined by GFP staining. (B) Primary CD4+ T-cells were processed and quantified as in panel A except using BB7.2 to IP native MHC-I. (C) H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses for 24 or 72 h, MHC-I was immunoprecipitated with mAb HC10 as in A and then digested or not with Endo H as described in Methods. (D) H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses for 48 or 72 h and then treated or not with PI-103 (5 µM) or 2c (20 µM) for 16 h. Cells were incubated with W6/32 (3 µg/ml) for 30 min and then chased and processed for immunofluorescence microscopy as described in the legend to Figure 4A. Post-fix. Total MHC-I was detected post-fixation by staining the cells with K455. Scale bar, 10 µm. (E) H9 cells were infected with Nef− or Nef+ pseudotyped HIV-1NL4-3 viruses for 48 or 72 h and analyzed for down-regulation of MHC-I as described in Methods. MFI: 48 h Nef− = 341, 48 h Nef+ = 110, 72 h Nef− = 302, 72 h Nef+ = 125.
DISCUSSION

We report that Nef directs a highly regulated program to down-regulate MHC-I consisting of the sequential use of the signaling and stoichiometric modes of action. During the first two days after infection, Nef uses the signaling mode to down-regulate MHC-I. This mode requires the binding of Nef to a Golgi region-localized SFK that can directly activate to assemble a multi-kinase complex that triggers down-regulation of cell-surface MHC-I in CD4^+ T-cells. Using the small molecule inhibitor 2c to disrupt the Nef-SFK interaction, we repressed HIV-1 mediated down-regulation of cell-surface HLA-A2 in primary CD4^+ T-cells. This 2c-sensitive signaling pathway is present in primary CD4^+ T-cells and in H9 cells, which are replete for PTEN and are sensitive to inhibition of the multi-kinase complex. CEM cells, however, lack PTEN and thus fail to phenocopy the MHC-I down-regulation pathway used in primary CD4^+ T-cells. By three days postinfection, Nef switches to the stoichiometric mode that prevents delivery of newly synthesized MHC-I to the cell surface. Interference with formation of the multi-kinase complex disrupts the temporally controlled block in MHC-I transport, suggesting the Nef-directed signaling and stoichiometric modes are causally linked.

A systematic analysis of the steps underlying MHC-I down-regulation suggests 2c selectively blocks Nef action early in the down-regulation pathway at the binding of Nef to SFKs, notably Hck, Lyn, or Src (Figure 1 and supplemental Figure S3). However, the ability of 2c to directly inhibit Hck (or potentially other kinases upstream of class I PI3K), albeit at higher concentrations, may also contribute to the efficacy of 2c. Our NMR studies show that 2c affects the conformation of the N-terminal proline helix that binds the RT loop of SH3 domains on SFKs, especially V_{146}(Figure 1). Furthermore, the NMR data identify a potential 2c binding pocket, opposite the SH3 domain binding site, in which 2c induces significant changes of the amide resonances surrounding V_{146}, which has been previously identified as essential for Nef-Hck binding (Saksela et al., 1995). Interestingly, 2c induces a change in the resonance at K_{144}, which must be ubiquitylated for Nef to down-regulate CD4 (Jin et al., 2008). The lack of effect of 2c on HIV-1–induced CD4 down-regulation (Figure 2), however, suggests that ubiquitylation of Nef K_{144} is unaffected by 2c. Together, these studies suggest 2c may be a weak competitive inhibitor of Nef-SFK binding or may induce an allosteric change in Nef that indirectly represses binding to SFKs, explaining why micromolar concentrations of 2c are required to inhibit MHC-I down-regulation. In support of an allosteric mechanism, Nef alleles from long-term nonprogressors that fail to activate Hck exhibit mutations at a distance from the Hck SH3 docking site on Nef (Plosky et al., 2005; Hiyoshi et al., 2008; Emert-Sedlak et al., 2009; Hassan et al., 2009).

The signaling mode requires the PACS-2-dependent, Nef-assembled SFK-ZAP-70/Syk-PI3K multi-kinase complex to trigger increased internalization of cell-surface MHC-I molecules through an ARF6-regulated endocytic pathway (Blagoveshchenskaya et al., 2002; Hung et al., 2007; Atkins et al., 2008; Chaudhry et al., 2008). The internalized molecules...
are then sequestered into paranuclear compartments by a Nef M20, AP-1, and PACS-1-dependent process (Figure 4 and Blagoveshchenskaya et al., 2002; Chaudhry et al., 2008). Thus, AP-1 is required for both the signaling and stoichiometric modes of MHC-I down-regulation, but whether PACS-1 is also required in the stoichiometric mode or whether PACS-1 and AP-1 mediate common or separate sorting steps required for sequestering internalized MHC-I molecules into TGN/endosomal compartments remains to be determined. Nonetheless, these findings suggest the PACS proteins mediate distinct steps within the signaling pathway—the trigger phase (PACS-2) and the sequestering phase (PACS-1). By three days postinfection, Nef switches to a stoichiometric mode of down-regulation that prevents delivery of newly synthesized MHC-I molecules to the cell surface. The ability of 2c or PI-103 to prevent conversion from the signaling to stoichiometric mode suggests that signaling events directed by one or more of the kinases that form the multi-kinase complex may either result in post-translational modification of MHC-I, or may alter the activity of the membrane trafficking machinery that mediates the switch from the signaling to the stoichiometric mode. The precise mechanism controlling the switch between the signaling and stoichiometric modes warrants further investigation.

The relative contributions of the signaling and stoichiometric modes to Nef-induced MHC-I down-regulation have been controversial, and discrepancies may have arisen from different experimental designs, choice of cell lines, and interpretation of negative results. For example, the signaling mode was initially dismissed as a result of differences in the efficiency by which Nef is able to impede cell surface delivery of MHC-I in T-cells versus HeLa cells (Kasper and Collins, 2003; Kasper et al., 2005). By contrast, we determined using parallel experiments that these differences instead result from the time postinfection at which MHC-I transport is analyzed. During early times postinfection and continuing for 48 h, Nef has no effect on the ER to cell surface transport of endogenous MHC-I molecules in HeLa, H9, and primary CD4+ T-cells whereas by 72 h Nef can block MHC-I transport (Figures 6 and 7 and Blagoveshchenskaya et al., 2002; Hung et al., 2007). This bimodal mechanism of Nef-mediated MHC-I down-regulation does not appear to result from differences in Nef expression or in the antibodies used, suggesting the modes of Nef action may be temporally regulated. Second, the failure of PI3K inhibitors to block MHC-I down-regulation in PTEN-deficient Jurkat, CEM, or U373 cells together with the confusion regarding regulation of PTEN activity in leukemic cell lines was used to assert that Nef mediates MHC-I down-regulation by a PI3K-independent mechanism (Kasper and Collins, 2003; Larsen et al., 2004; Schaefer et al., 2008). However, reexpression of PTEN in CEM or U373 cells restored sensitivity of Nef-mediated MHC-I down-regulation to 2c or PI3K inhibitors, whereas siRNA knockdown of PTEN in H9 T-cells rendered Nef-mediated MHC-I down-regulation resistant to PI3K inhibitors (Figure 5 and Hung et al., 2007). Therefore, the determination that the mechanism of Nef-induced MHC-I down-regulation in primary CD4+ T-cells is phenocopied by H9 cells but not CEM cells underscores the importance of choice of cell lines used to model Nef action (Figure 5). Thus, the ability of PTEN-deficient CEM and U373 tumor cells to override the requirement for the SFK-ZAP-70/Syk-PI3K multi-kinase complex in triggering Nef action likely explains the confusion in the literature regarding the importance of Nef sites in MHC-I down-regulation (Kasper and Collins, 2003; Larsen et al., 2004; Casartelli et al., 2006; Noviello et al., 2008; Schaefer et al., 2008). For example, the assertion that the AXXA75 mutation nonspecifically disrupts MHC-I down-regulation in PTEN-null cells (Swann et al., 2001; Casartelli et al., 2006) conflicts with the ability of NefAA75-PI3K to rescue MHC-I down-regulation in H9 cells (Figure 4) and with the pharamacologic repression of MHC-I down-regulation by treatment of primary CD4+ T-cells with PI-103, which inhibits class I PI3K, or with 2c or D1, which block the Nef-SFK interaction (Betzi et al., 2007; Hung et al., 2007; Hiyoshi et al., 2008; Emert-Sedlak et al., 2009 and Figures 1 and 2). Reliance on PTEN-deficient cell lines may not only have caused confusion in understanding the mechanism of MHC-I down-regulation but may also have contributed to conflicting findings in HIV-1 research ranging from the signaling pathways that reactivate latent HIV-1 to the secretion of HIV-1 Tat (Bosque and Plantelles, 2009; Rayne et al., 2010). Third, the failure of a dominant negative dynamin mutant, which interferes with clathrin-dependent endocytosis, to block MHC-I down-regulation was used to suggest that Nef does not direct MHC-I endocytosis (Swann et al., 2001). However, MHC-I is internalized by a clathrin/dynamin/AP-2-independent, ARF6-dependent pathway—both basally and in response to Nef (Le Gall et al., 2000; Blagoveshchenskaya et al., 2002; Caplan et al., 2002; Naslavsky et al., 2003; Hung et al., 2007; Chaudhry et al., 2008). Fourth, the ability of GST-MHC-I CD-NefLL/AA to bind AP-1 µ1 subunit in vitro was used to assert that Nef does not require PTEN-1 to down-regulate MHC-I in vivo (Noviello et al., 2008; Singh et al., 2009). However, GST-MHC-I CD-NefLL/AA can also interact with PACS-1 (Figure 4), contradicting the assumption by Guatelli et al. that this bacterial fusion protein interacts exclusively with AP-1 (Singh et al., 2009). Instead, these protein capture data are consistent with the determination in vivo that PACS-1 mediates Nef-induced MHC-I down-regulation subsequent to PI3K stimulation (Figure 4 and Atkins et al., 2008; see also Youker et al., 2009 for review). Lastly, whereas Nef can induce rapid degradation of overexpressed MHC-I (Kasper and Collins, 2003; Roeth et al., 2004; Kasper et al., 2005; Schaefer et al., 2008), we observed no marked effect on the stability or ER-to-Golgi trafficking of endogenous MHC-I (Figure 6 and supplemental Figure S6 and Blagoveshchenskaya et al., 2002; Hung et al., 2007). Thus, the extent to which overexpressed HLA-A2.1 is physiologically relevant to the mechanism underlying Nef-induced MHC-I down-regulation remains unclear.

The determination that Nef down-regulates MHC-I by the sequential use of the signaling mode followed by the stoichiometric mode raises the possibility that HIV-1 may adapt immune evasive strategies specific to the host cell activation state or reservoir type. Because the lifespan of activated CD4+ T-cells infected with HIV-1 is less than two days (Stevenson, 2003), and Nef uses the signaling mode to down-regulate MHC-I in CD4+ T-cells for two days before converting to the stoichiometric mode (Figures 6 and 7), the physiological relevance of the stoichiometric pathway in activated CD4+ T-cells remains uncertain. However, Nef also assembles the multi-kinase complex in cells of monocytic lineages, of which macrophages produce a low but persistent level of viruses that can last the duration of the cell’s natural lifespan (Hung et al., 2007; Alexaki et al., 2008). Future studies on the immune evasive program directed by HIV-1 Nef will require membrane trafficking experiments in relevant cell lines to determine the relative contributions of the signaling and stoichiometric modes of Nef action. The physiological significance of Nef’s bimodal MHC-I down-regulation pathway in viral reservoirs can then be correlated

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to disease progression. Finally, the ability of 2c and other small molecules to repress multiple actions of Nef suggests the multi-kinase complex may be an attractive approach for HIV-1 therapeutics.

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