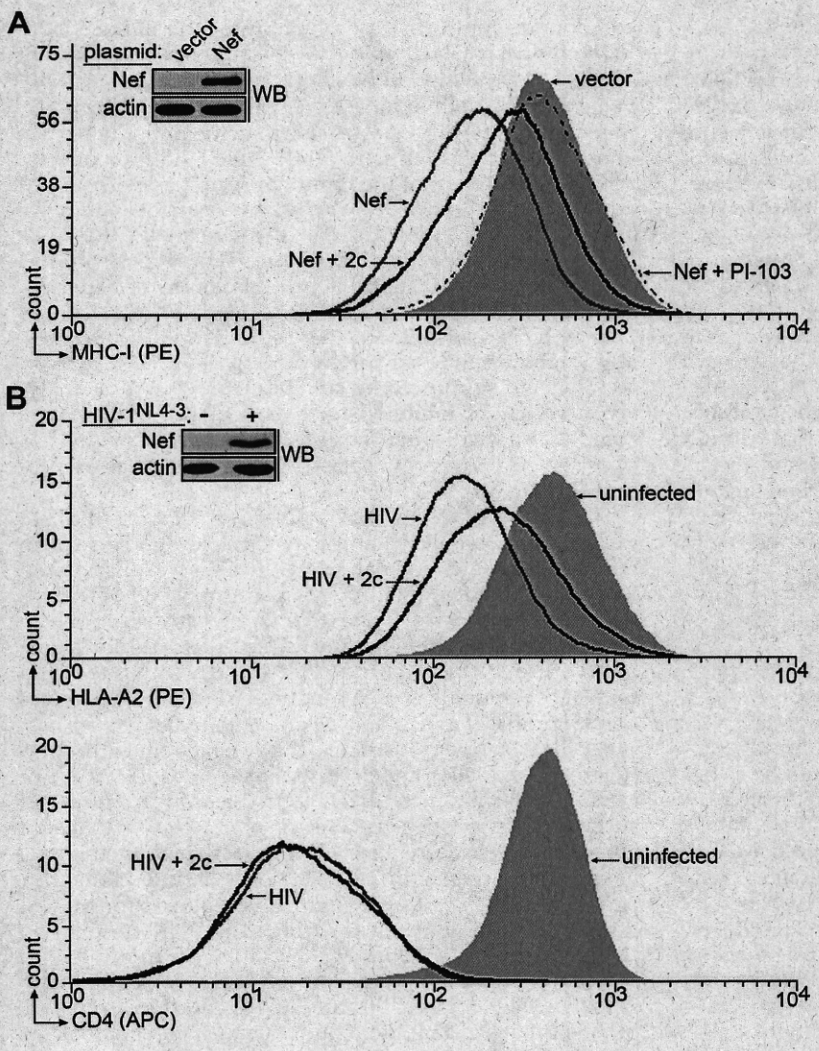

Figure 2. 2c represses HIV-1-induced down-regulation of MHC-I but not CD4. (A) H9 cells expressing eYFP (vector) or Nef-eYFP (Nef) for 24 h were treated or not with 20 μM 2c (Nef + 2c) or 5 μM of the class I PI3K inhibitor, PI-103 (Nef + PI-103) for another 16 h. At 40 h, cultures were analyzed by flow cytometry using W6/32. MFI: vector = 479, Nef = 208, Nef + 2c = 312, Nef + PI-103 = 444. Inset: Western blot showing expression of Nef-eYFP and actin. (B) Parallel cultures of primary $CD4^+$ T-cells were infected with HIV-1^{NL4-3} and treated or not with 20 μM 2c at days 5 and 7 postinfection. At 8 d postinfection the cells were stained with p24-FITC, BB7.2, and CD4-APC and then analyzed for down-regulation of HLA-A2 (top) and CD4 (bottom) by flow cytometry as described in Methods. MHC-I MFI: uninfected = 518, HIV = 193, HIV + 2c = 301. CD4 MFI: uninfected = 413, HIV = 25.1, HIV + 2c = 25.2. Inset: Western blot showing expression of Nef and actin.

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-$Nef_{LL/AA}$ 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 Nef_{AXXA}-PI3K* 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 $EEEE_{65}$ and $PXXP_{75}$ sites, which trigger MHC-I internalization and subsequent M_{20}-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 M_{20}-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 signaling by dephosphorylating PIP_3. Thus, loss of PTEN results in constitutively elevated levels of PI3K/Akt signaling characteristic of many tumor cell lines. Although acute treatment of CEM or Jurkat cells with PI3K inhibitors

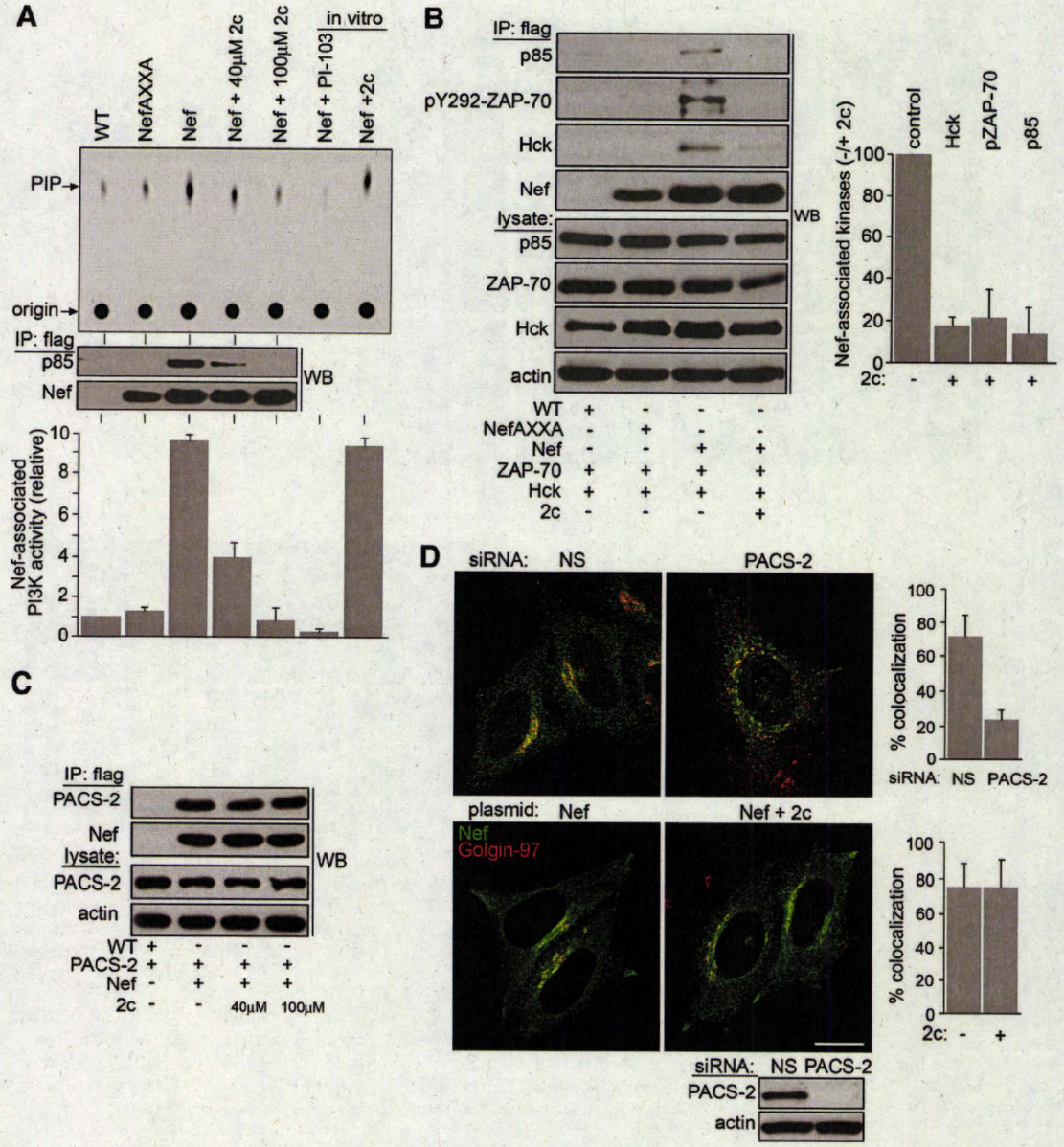

Figure 3. 2c blocks the ability of Nef to assemble the multi-kinase complex. (A) H9 cells were infected with VV:WT, VV:Nef_{AXXA}/f, or VV:Nef/f (moi = 10, 8 h) and treated with 40 or 100 μM 2c for 4 h (which showed no toxicity in this time frame, supplemental Figure S3, b and c). Nef/f was immunoprecipitated and Nef-associated class I PI3K p85 regulatory subunit was detected by Western blot, and associated PI3K activity was measured using an in vitro lipid kinase assay as described in Methods. As controls, 40 μM 2c and 1 μM PI-103 were incubated with the eluted fraction for 10 min before kinase assay (in vitro samples). Each assay was measured in triplicate and results are presented as the mean ± SD. (B) Left: H9 cells were coinfected with VV:WT, VV:Nef_{AXXA}/f, or VV:Nef/f (moi = 6, 8 h) and VV:ZAP70 (moi = 4, 8 h). Nef/f was immunoprecipitated and coprecipitating Hck, phospho-ZAP-70 and p85 were detected by Western blot. Right: The amount of each Nef-associated kinase was quantified with Image J and presented numerically as the relative amount of Nef-associated kinase ± 20 μM 2c. Error bars represent the mean ± SD from 3 independent experiments. (C) H9 cells were coinfected with VV:Nef/f (moi = 6, 8 h) and VV:PACS-2 (moi = 4, 8 h) and treated with 40 or 100 μM 2c for 4 h before harvest. Nef/f was immunoprecipitated and coprecipitating PACS-2 was detected by Western blot. (D) Upper: HeLa cells expressing Nef-eYFP together with a control siRNA (NS) or PACS-2 siRNA (Western blot of siRNA knockdown shown at bottom). Lower: HeLa cells expressing Nef-eYFP were treated with 20 μM 2c for 16 h. Cells were stained with anti-Golgin 97 (red) and visualized by confocal microscopy (scale bar, 10 μm). Morphometric analysis was performed as described in Methods. Error bars are presented as the mean ± SD from at least 20 cells per condition and three independent experiments.

prevents new PIP_3 synthesis, the absence of PTEN results in persistently elevated levels of PIP_3 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

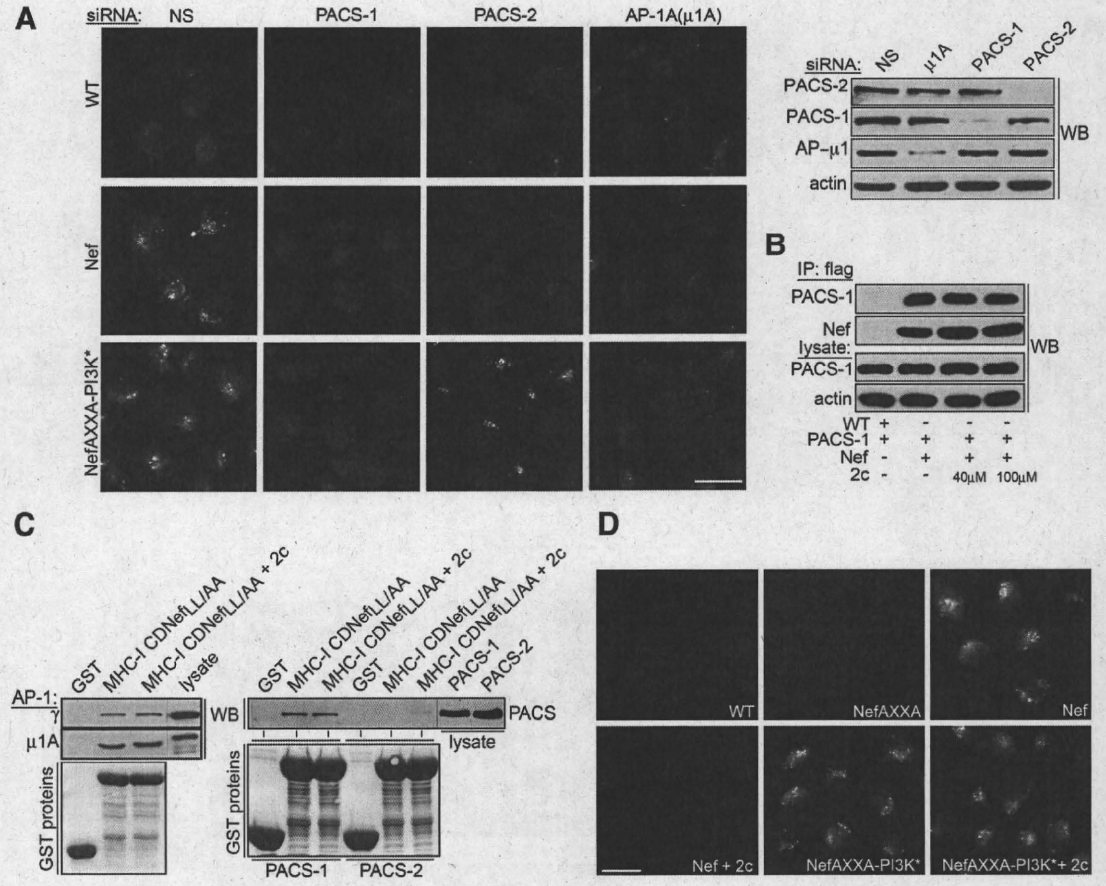

Figure 4. PACS-1 and AP-1 are required downstream of the 2c-sensitive multi-kinase complex. (A) Left: H9 cells were nucleofected with pmaxGFP together with a control siRNA (NS) or siRNAs specific for PACS-1, PACS-2, or μ1A. After 48 h, cells were infected with VV:WT, VV:Nef, or VV:Nef_{AXXA}-P13K* (moi = 10, 5 h). Cells were incubated with W6/32 (3 μg/ml) for 30 min, chased for an additional 30 min, then incubated with 0.5% acetic acid (pH 3.0) in 0.5M NaCl to remove surface antibody, fixed, and processed for immunofluorescence microscopy. Scale bar, 10 μm. Right: A portion of the cells from left were analyzed by Western blot for extent of siRNA knockdown. (B) H9 cells were coinfected with VV:Nef/f (moi = 6, 8 h) and VV:PACS-1 (moi = 4, 8 h) and treated with 40 or 100 μM 2c for 4 h before harvest. Nef/f was immunoprecipitated, and coprecipitating PACS-1 was analyzed by western blot. (C) Left: GST-MHC-I $CDNef_{LL/AA}$ or GST was mixed with A7 cell lysate, treated with 20 μM 2c, and captured g or 1μA subunits of AP-1 detected by Western blot. Right: Lysates from A7 cells expressing HA-tagged PACS-1 or PACS-2 were incubated with GST-MHC-I $CDNef_{LL/AA}$ or GST, treated with 20 μM 2c, and bound PACS proteins detected by Western blot. (D) H9 cells were treated or not with 20 μM 2c for 18 h and then infected with VV:WT, VV:Nef, VV:Nef_{AXXA}, or VV:Nef_{AXXA}-P13K* (moi = 10, 5 h). Cells were incubated with W6/32 (3 μg/ml) for 30 min and then chased for an additional 30 min and processed for immunofluorescence microscopy as described in the legend to panel A. Scale bar, 10 μm.

(phosphorylated) Akt (Figure 5A), suggesting that PTEN-replete CEM cells may be rescued in their ability to regulate PIP_3 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 $HIV\text{-}1^{NL4\text{-}3}$ that either lack or express Nef were subjected to pulse-chase/surface biotinylation to monitor

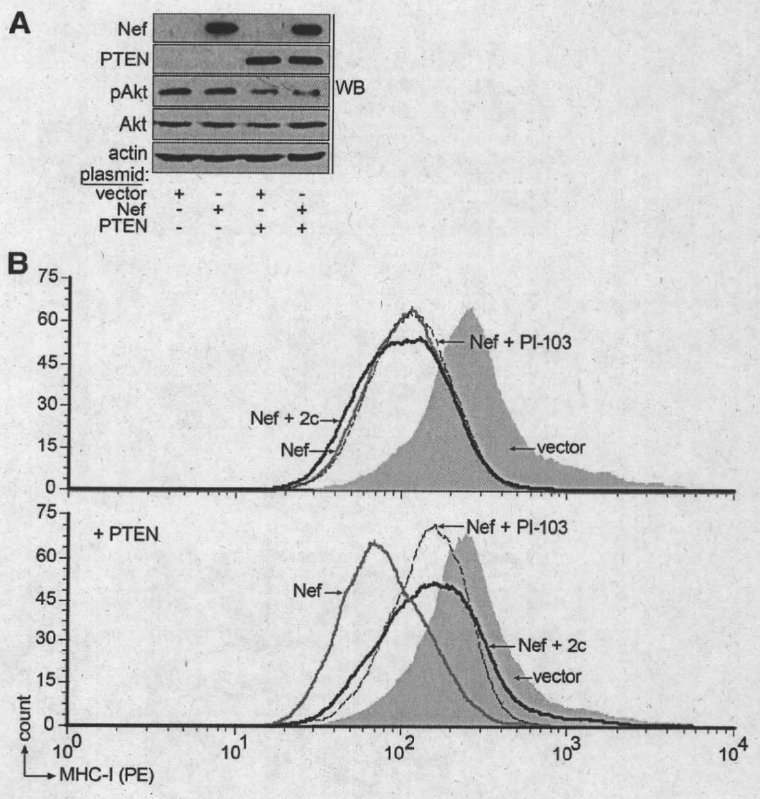

Figure 5. CEM cells do not model primary CD4$^+$ T-cells in Nef action. (A) Western blot analysis of PI3K-dysregulated CEM cells rescued or not for PTEN. (B) Top: CEM cells expressing eYFP (vector) or Nef-eYFP (Nef) were treated or not with 20 μM 2c (Nef + 2c) for 16 h before analysis or 1 μM PI-103 (Nef + PI-103) for 3 h before analysis in media containing 0.5 mM ß-mercaptoethanol. Cells were analyzed by flow cytometry using W6/32 as described in Methods. MFI: vector = 425, Nef = 125, Nef + 2c = 123, Nef + PI-103 = 128. Bottom: CEM cells expressing PTEN with either eYFP (vector) or Nef-eYFP (Nef) were processed as described above. MFI: vector = 384, Nef = 94.6, Nef + 2c = 232, Nef + PI-103 = 162.

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$^+$ 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$^-$ or Nef$^+$ pseudotyped HIV-1 for 24 or 72 h were pulse-labeled with [^{35}S]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 followed by 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

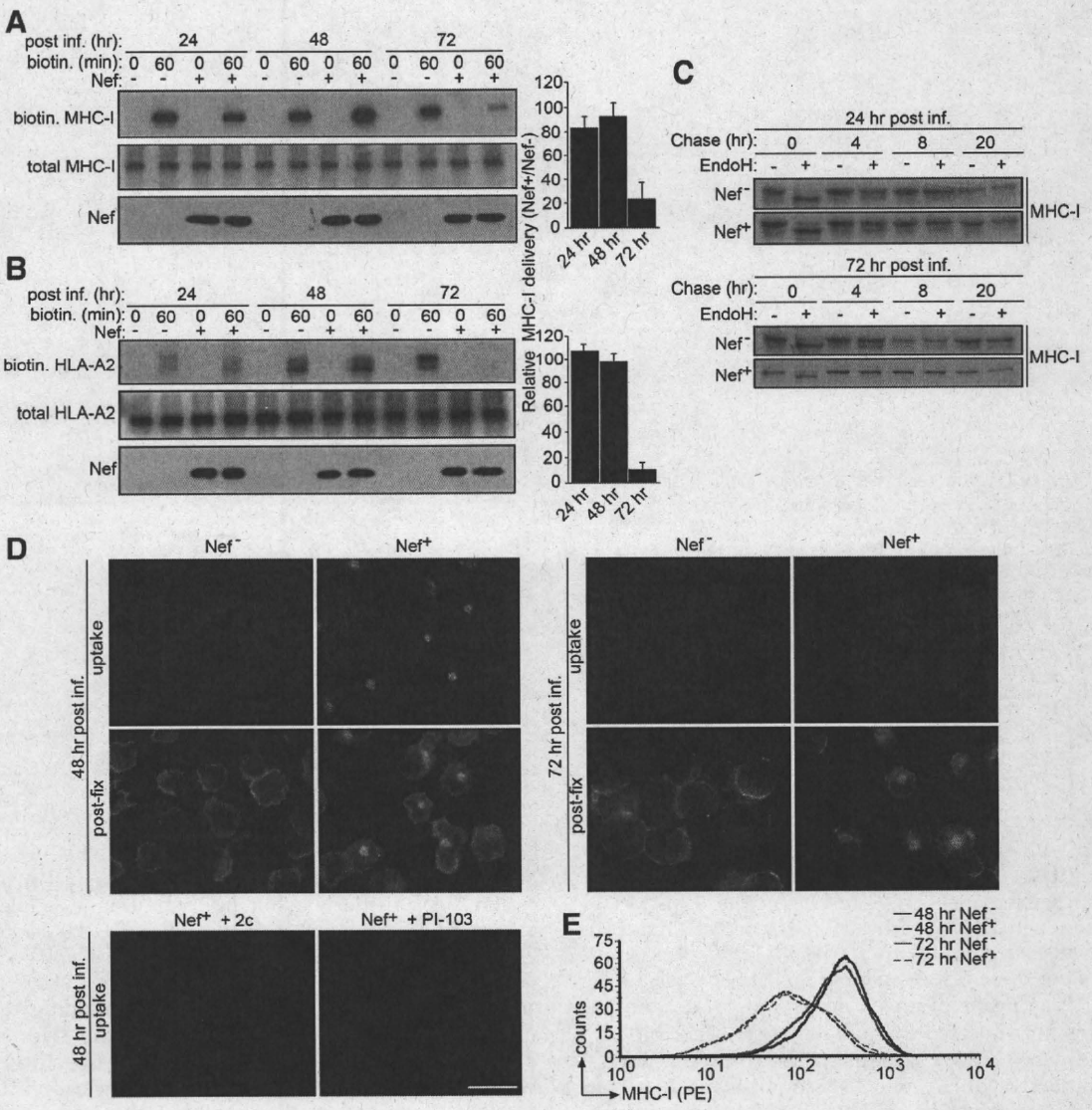

Figure 6. Nef-induced MHC-I down-regulation switches from a signaling to a stoichiometric mechanism. (A) H9 cells infected with Nef$^-$ or Nef$^+$ pseudotyped HIV-1^{NL4-3} viruses (moi = 2.3) for 24, 48, or 72 h and subjected to pulse-chase/surface biotinylation using HC10 associated MHC-I and quantified 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-1^{NL4-3} 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-1^{NL4-3} 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-1^{NL4-3} 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.

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-1 and then treated with 2c or PI-103 at 24 or 48 h postinfection. At 72 h postinfection, the level of cell-surface MHC-I was analyzed by flow cytometry, demonstrating that addition of 2c or PI-103 for 24 or 48 h repressed the ability of Nef to down-regulate MHC-I (supplemental Figure S7). Next, the cells were subjected to the pulse-chase/surface biotinylation assay. In agreement with the results in Figure 6 and the flow cytometry results, Nef expressed for 72 h repressed delivery of newly synthesized MHC-I to the cell surface (Figure 7). By contrast, treatment of the infected cells with either 2c or PI-103 for as little as 24 h before the pulse-chase/surface biotinylation prevented Nef from blocking cell-surface delivery of MHC-I (Figure 7, A and B, respectively). Together, these results suggest that sustained PI3K signaling driven by the multi-kinase complex is required for Nef-induced MHC-I down-regulation to switch from a signaling to a stoichiometric mode.

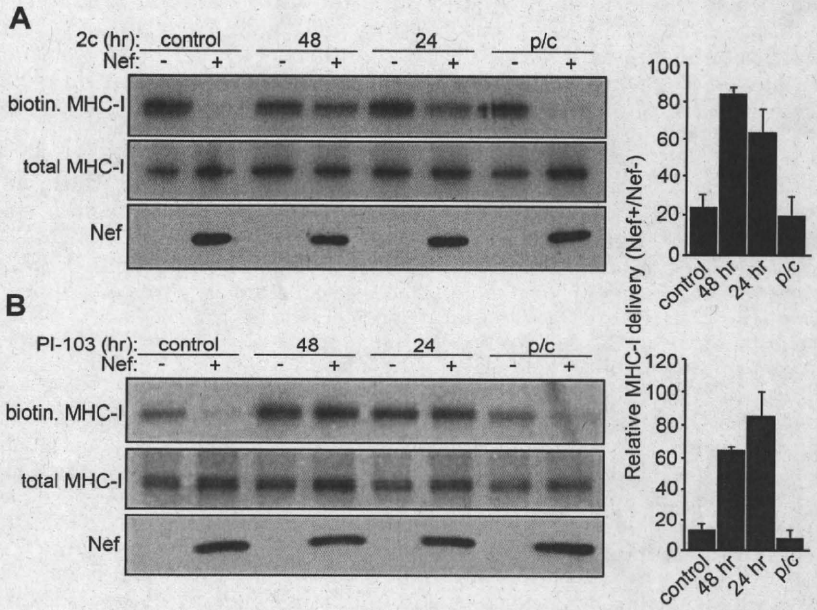

Figure 7. The PI3K signaling pathway is required for stoichiometric inhibition of MHC-I. (A) H9 cells were infected with Nef$^-$ or Nef$^+$ pseudotyped HIV-1^{NL4-3} viruses for a total of 72 h and either left untreated (control) or pretreated with 20 μM 2c for 48 h (2c 48 h) or 24 h (2c 24 h) before analysis at 72 h postinfection. The cells were then subjected to pulse-chase/surface biotinylation as described in Methods. The amount of HC10 associated MHC-I delivered to the cell surface was quantified as described in Methods. Error bars are presented as the mean ± SD from three independent experiments. (B) H9 cells were infected as above and left untreated (control) or pretreated with 5 μM PI-103 for 48 h (PI-103 48 h) or 24 h (PI-103 24 h) before analysis at 72 h postinfection and then processed as in panel A. The amount of MHC-I delivered to the cell surface was quantified as described in Methods. Error bars are presented as the mean ± SD from three independent experiments. (p/c), cells treated with 20 μM 2c (A) or 10 μM PI-103 (B) during the pulse-chase only.

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 it 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 polyproline helix that binds the RT loop of SH3 domains on SFKs, especially V_{74}(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 (Trible *et al.*, 2007). The ability of 2c to only partially inhibit MHC-I down-regulation in plasmid transfected cells may reflect a lower efficacy of this inhibitor compared with the class I PI3K inhibitor PI-103, which completely inhibits MHC-I down-regulation irrespective of the vector used (compare Figures 2 and S7). However, 2c can completely inhibit Nef-mediated MHC-I down-regulation in pseudovirus-infected cells, suggesting length of treatment, as well as the extent and duration of Nef expression, may influence the efficacy of this compound. The dialdehyde moiety in 2c may also be subject to chemical inactivation by reactive oxygen species characteristic of HIV-1 infection, precluding maximal efficacy of this inhibitor (Figure 2 and supplemental Figure S2 and Peterhans, 1997). Nonetheless, the findings reported here, together with the determination that 2c represses the Nef-Hck-dependent down-regulation of macrophage colony-stimulating factor (M-CSF) receptor and that diphenylfuropyriminde compounds that selectively block Nef-induced Hck activation also inhibit Nef-dependent HIV-1 replication, suggest that the future generation of potent and selective drug-like molecules that disrupt Nef-SFK binding may represent an attractive approach to the generation of novel HIV-1 therapeutics (Suzu *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 M_{20}-, 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 $AXXA_{75}$ mutation nonspecifically disrupts MHC-I down-regulation in PTEN-null cells (Swann *et al.*, 2001; Casartelli *et al.*, 2006) conflicts with the ability of Nef_{AXXA}-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 have also 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 Planelles, 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-$Nef_{LL/AA}$ to bind AP-1 μ1 subunit in vitro was used to assert that Nef does not require PACS-1 to down-regulate MHC-I in vivo (Noviello *et al.*, 2008; Singh *et al.*, 2009). However, GST-MHC-I CD-$Nef_{LL/AA}$ 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 monocyte lineages, of which macrophages produce a low but persistent level of virus 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

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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