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RESEARCH ARTICLE

AP-2 Adaptor Complex-Dependent Enhancement of HIV-1 Replication by Nef in the Absence of the Nef/AP-2 Targets SERINC5 and CD4

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ABSTRACT Human immunodeficiency virus type 1 (HIV-1) Nef hijacks the clathrin adaptor complex 2 (AP-2) to downregulate the viral receptor CD4 and the antiviral multipass transmembrane proteins SERINC3 and SERINC5, which inhibit the infectivity of progeny virions when incorporated. In Jurkat Tag T lymphoid cells lacking SERINC3 and SERINC5, Nef is no longer required for full progeny virus infectivity and for efficient viral replication. However, in MOLT-3 T lymphoid cells, HIV-1 replication remains highly dependent on Nef even in the absence of SERINC3 and SERINC5. Using a knockout (KO) approach, we now show that the Nef-mediated enhancement of HIV-1 replication in MOLT-3 cells does not depend on the Nef-interacting kinases LCK and PAK2. Furthermore, Nef substantially enhanced HIV-1 replication even in triple-KO MOLT-3 cells that simultaneously lacked the three Nef/AP-2 targets, SERINC3, SERINC5, and CD4, and were reconstituted with a Nef-resistant CD4 to permit HIV-1 entry. Nevertheless, the ability of Nef mutants to promote HIV-1 replication in the triple-KO cells correlated strictly with the ability to bind AP-2. In addition, knockdown and reconstitution experiments confirmed the involvement of AP-2. These observations raise the possibility that MOLT-3 cells express a novel antiviral factor that is downregulated by Nef in an AP-2-dependent manner.

IMPORTANCE The HIV-1 Nef protein hijacks a component of the cellular endocytic machinery called AP-2 to downregulate the viral receptor CD4 and the antiviral cellular membrane proteins SERINC3 and SERINC5. In the absence of Nef, SERINC3 and SERINC5 are taken up into viral particles, which reduces their infectivity. Surprisingly, in a T cell line called MOLT-3, Nef remains crucial for HIV-1 spreading in the absence of SERINC3 and SERINC5. We now show that this effect of Nef also does not depend on the cellular signaling molecules and Nef interaction partners LCK and PAK2. Nef was required for efficient HIV-1 spreading even in triple-knockout cells that completely lacked Nef/AP-2-sensitive CD4, in addition to the Nef/AP-2 targets SERINC3 and SERINC5. Nevertheless, our results indicate that the enhancement of HIV-1 spreading by Nef in the triple-knockout cells remained AP-2 dependent, which suggests the presence of an unknown antiviral factor that is sensitive to Nef/AP-2-mediated downregulation.

KEYWORDS AP-2, CD4, LCK, Nef, PAK2, SERINC5, human immunodeficiency virus, virus replication

Nef is a virulence factor of human immunodeficiency virus type 1 (HIV-1) and other primate lentiviruses that is required for high virus loads and for viral pathogenicity *in vivo* (1, 2). Although Nef is not an essential HIV-1 gene product, it can substantially enhance HIV-1 spreading among primary human $CD4^+$ T cells (3, 4). Notably, Nef proteins from all three HIV-1 groups and from several highly divergent simian immunodeficiency viruses (SIVs) efficiently promote HIV-1 replication in human peripheral blood mononuclear cells (PBMC),

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indicating that the enhancement of virus spreading is a highly conserved function of Nef (5). However, the underlying mechanism remains to be fully elucidated.

Among the many reported *in vitro* activities of Nef, one of the most conserved is the downregulation of the viral entry receptor CD4 from the surface of infected cells (6–8). This activity involves the formation of a ternary complex between Nef, the cytoplasmic domain of CD4, and the clathrin adaptor complex 2 (AP-2), which orchestrates the internalization of CD4 through clathrin-mediated endocytosis (9, 10). Additionally, Nef engages AP-2 to prevent the incorporation of the antiviral multipass transmembrane proteins SERINC3 and SERINC5 into progeny virions (11–15). Nef also co-opts the AP-1 clathrin adaptor complex to downregulate certain major histocompatibility complex class I (MHC-I) molecules (16).

The Nef-mediated downregulation of CD4 and MHC-I is thought to protect infected cells from different forms of cell-mediated cytotoxicity (17–19). However, it has also been shown that high levels of cell surface CD4 impair the release and infectivity of HIV-1 virions and that Nef counteracts these effects by downregulating CD4 (20, 21). Furthermore, it has been observed that the ability of Nef to downregulate CD4 strongly correlates with its ability to enhance HIV-1 replication in primary cells *in vitro* (22). Consistent with these *in vitro* results, the ability to downregulate CD4 correlated with the Nef-mediated enhancement of HIV-1 pathogenicity in humanized mouse models (23, 24). Together, these findings suggest that the downregulation of CD4 by Nef can directly promote HIV-1 replication.

The downregulation of SERINC5 by Nef largely accounts for its well-documented ability to enhance the specific infectivity of progeny virions (11, 12, 25). Although most HIV-1 Nef proteins additionally downregulate SERINC3, exogenous SERINC3 has little effect on HIV-1 progeny virus infectivity, whereas exogenous SERINC5 can lower it dramatically (26). Nevertheless, the downregulation of endogenous SERINC3 likely contributes to the effect of Nef on progeny virus infectivity (12). Furthermore, at least in Jurkat Tag cells, the downregulation of SERINC3 and SERINC5 accounts for the Nef-mediated enhancement of HIV-1 spreading (12). SERINC3 and SERINC5 have also been shown to inhibit HIV-1 by promoting innate immune signaling (27). Consistent with these observations, the selective disruption of the SERINC5 antagonism of a simian immunodeficiency virus (SIV) Nef protein impaired virus replication in primary $CD4^+$ T cells, attesting to the biological relevance of this function of Nef (28).

In addition to cellular trafficking machinery, Nef engages host proteins to modulate the activation state and the actin cytoskeleton of infected cells (29). In particular, it has long been known that Nef interacts with the SH3 domains of certain Src family tyrosine kinases through a conserved proline-rich motif (30, 31). In an early study, Nef interacted only with the Src kinases HCK and LYN, which are both expressed at rather low levels in $CD4^+$ T cells (30). However, inhibitors of Nef-dependent HCK activation have been shown to block HIV-1 replication in macrophages, which express substantial amounts of HCK (32). It subsequently emerged that Nef also interacts with the Src kinase LCK, which is highly expressed in primary $CD4^+$ T cells and plays a key role in T cell receptor signaling (33–35). Notably, Nef reroutes LCK from the plasma membrane to an intracellular compartment (36), and triggers LCK-dependent signaling at the trans-Golgi network (37), which has been suggested to facilitate HIV-1 replication (37).

Another well-documented interaction partner of Nef is the p21-activated kinase 2 (PAK2) (38, 39), a member of the highly conserved PAK family of serine/threonine protein kinases that regulate the cytoskeleton and function as effectors of the RHO family GTPases CDC42 and RAC (40). The ability of Nef to interact with PAK2 is generally conserved (41) as is the ability of Nef to trigger actin cytoskeleton remodeling in a PAK2-dependent manner (42). Furthermore, the ability of Nef to associate with activated PAK2 has been implicated in its ability to stimulate HIV-1 replication in freshly isolated primary T cells (43).

Nef enhances HIV-1 replication in some T cell lines, but the effects can be rather modest (44). However, we recently observed that HIV-1 replication in MOLT-3 T lymphoid

cells is highly dependent on Nef (26). Our results also indicated that unlike in Jurkat Tag cells, the ability of Nef to antagonize SERINCs did not account for its effect on HIV-1 replication in MOLT-3 cells or in primary cells (26). Moreover, Nef robustly promoted HIV-1 replication in MOLT-3 cells that predominantly expressed a Nef-resistant CD4 molecule, indicating that CD4 downregulation was not required (26). We now show that the Nef-interacting kinases LCK and PAK2 are also not required. Furthermore, Nef remained fully capable of promoting HIV-1 spreading even in triple-knockout (KO) MOLT-3 cells that simultaneously lacked the Nef/AP-2 complex targets SERINC3, SERINC5, and CD4 and expressed only a Nef-resistant CD4 to support HIV-1 entry. Nevertheless, our results indicate that AP-2 remained specifically required for the enhancement of HIV-1 replication by Nef in these cells. These observations point to the existence of an unknown antiviral factor that is counteracted by Nef in an AP-2-dependent manner.

RESULTS

Propagation of SERINC-resistant HIV-1 in primary cells remains Nef dependent. We previously reported that the SERINC antagonist glycoMA, when inserted into HIV-1_{NL4-3} in place of *nef*, enhanced HIV-1 propagation in Jurkat cells to a comparable extent as Nef itself. In marked contrast, glycoMA had no effect on the spreading of Nef^- HIV-1_{NL4-3} in MOLT-3 cells or in primary human PBMC. To confirm that glycoMA cannot substitute for Nef in primary human cells, we have now examined its ability to promote the spreading of HIV-1_{NL4-3} in $CD4^+$ T cells. Primary human $CD4^+$ T cells were infected immediately after purification by negative selection and stimulated with phytohemagglutinin (PHA) 3 days later, conditions under which robust effects of Nef on HIV-1 spreading have been observed. As shown in Fig. 1A, Nef was critical for the propagation of HIV-1_{NL4-3} in primary $CD4^+$ T cells under these conditions, and glycoMA did not rescue the replication defect of Nef^- HIV-1_{NL4-3}. Since glycoMA potently counteracts both SERINC3 and SERINC5, these results implied that the considerable replication defect of Nef^- HIV-1_{NL4-3} in primary $CD4^+$ T cells was not solely due to an inability to counteract SERINCs.

To further examine this issue, we used a variant of HIV-1_{NL4-3} termed HIV-$1_{NL-JRFL}$, which has the *env* gene replaced with that of the primary HIV-1 strain JRFL. Importantly, unlike the Env protein of HIV-1_{NL4-3}, which is highly SERINC5 sensitive, Env_{JRFL} is largely resistant to endogenous levels of SERINC5. Nevertheless, the propagation of HIV-$1_{NL-JRFL}$ in primary $CD4^+$ T cells that were infected prior to stimulation was similarly dependent on Nef as the propagation of HIV-1_{NL4-3}, and neither virus was rescued by glycoMA (Fig. 1A). In a separate experiment, we observed that Nef also substantially enhanced the spread of HIV-$1_{NL-JRFL}$ in primary human PBMC that were infected after PHA stimulation (Fig. 1B). Taken together, these observations provide evidence for a SERINC-independent restriction of Nef^- HIV-1 in primary $CD4^+$ T cells.

Nef strongly enhances HIV-1 replication in cells lacking LCK. Like in primary $CD4^+$ T cells, the replication of SERINC-resistant HIV-1 in MOLT-3 T lymphoid cells depends on Nef (45). Indeed, MOLT-3 cells severely restrict the replication of Nef^- HIV-1 even in the absence of SERINC3 and SERINC5 (45). Therefore, we used MOLT-3 cells as a model system to examine the involvement of other host factors. One factor that has been implicated in the enhancement of HIV-1 replication by Nef in T cells is the lymphocyte-specific tyrosine kinase LCK (37, 46). Several studies indicate that Nef physically interacts with LCK and affects LCK-mediated signaling (33–35). However, whether Nef has a positive or negative effect on LCK has been controversial. There is evidence that Nef activates LCK and that this ultimately leads to the induction of HIV-1 transcription and replication (47). In contrast, other studies conclude that Nef impairs the kinase activity of LCK (33, 34), which could conceivably support virus replication by inhibiting activation-induced cell death (29). It has also been proposed that Nef does not affect the overall activity of LCK but rather triggers the relocalization of active LCK from the plasma membrane to intracellular membranes (37).

Using CRISPR/Cas9-mediated gene editing, we obtained two MOLT-3-derived clones that completely lack LCK (Fig. 2A) but exhibit CD4 surface levels comparable to

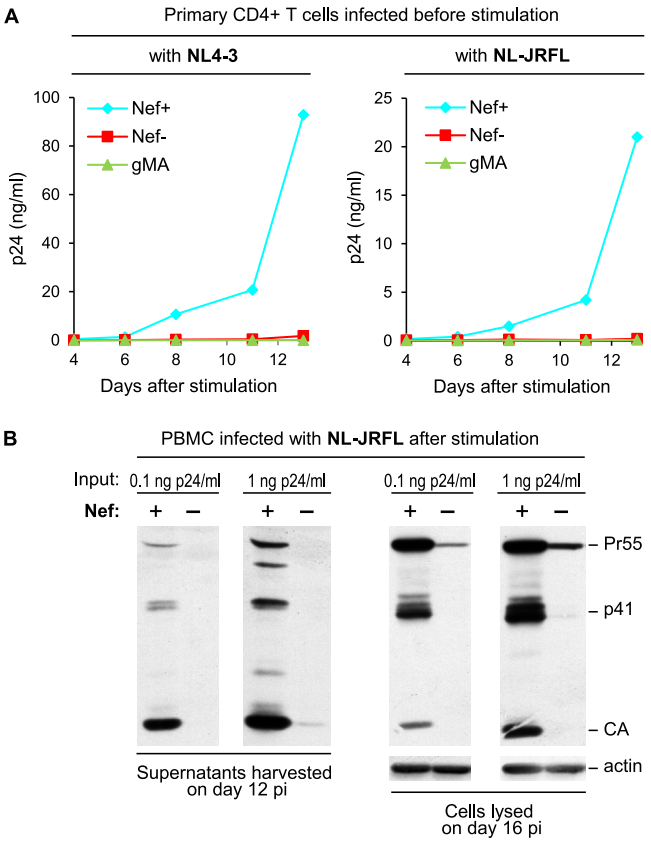

FIG 1 Propagation of SERINC-resistant HIV-1 in primary cells remains Nef-dependent. (A) Virus growth curves showing that Nef enhances the replication of both SERINC-sensitive (NL4-3) and SERINC-resistant (NL-JRFL) HIV-1 strains in primary human $CD4^+$ T cells infected prior to stimulation. In contrast, the potent SERINC antagonist glycoMA (gMA) does not enhance HIV-1 replication in this context. $CD4^+$ T cells were enriched by negative selection, infected with HIV-1_{NL4-3} or HIV-$1_{NL-JRFL}$ (at 1 ng p24/mL), and stimulated with PHA on day 3 after infection. HIV-1 replication was examined by monitoring p24 accumulation in the culture supernatants over time. (B) Nef enhances the spreading of the SERINC-resistant NL-JRFL strain in prestimulated human PBMC. After stimulation with PHA, PBMC were infected with 0.1 or 1 ng p24/mL, and culture supernatants and cell lysates were examined by Western blotting with anti-CA on day 12 and day 16 postinfection, respectively.

those on the parental MOLT-3 cells (Fig. 2B), even though LCK is normally tightly associated with the CD4 cytoplasmic domain and can affect the internalization rate of CD4 (48). In both LCK KO clones, the ability of HIV-1_{NL4-3} to replicate remained as dependent on Nef as in parental (LCK^+) MOLT-3 cells, as determined by measuring Gag expression in the infected cells by Western blotting (Fig. 2C) and by monitoring virus release over time in two independent experiments (Fig. 2D; see also Fig. S1 in the supplemental material). These results demonstrate that LCK is dispensable for the marked enhancement of HIV-1 replication by Nef in MOLT-3 cells.

Nef can fully support HIV-1 replication in the absence of all group I PAKs. Early studies have revealed that a Nef-associated kinase (NAK) is a member of the p21-activated kinase (PAK) family of serine/threonine kinases (38, 49, 50). NAK was later identified as PAK2 (39, 51), and several lines of evidence indicate that the Nef-PAK2 interaction plays an important role in HIV-1 replication by affecting the apoptosis of infected cells (52), their activation state (43, 53), or their ability to transmit HIV-1 via cell-cell contacts (54).

To examine whether PAK2 is strictly necessary for the ability of Nef to enhance HIV-1 replication, we used the CRISPR/Cas9 approach to obtain PAK2 KO MOLT-3 cells. While PAK2 was readily detectable by Western blotting in the parental MOLT-3 cells, PAK2 could not be detected in the PAK2 KO MOLT-3 cells (Fig. 3A). However, the levels of surface CD4 on the parental and PAK2 KO MOLT-3 cells were comparable (Fig. 3B).

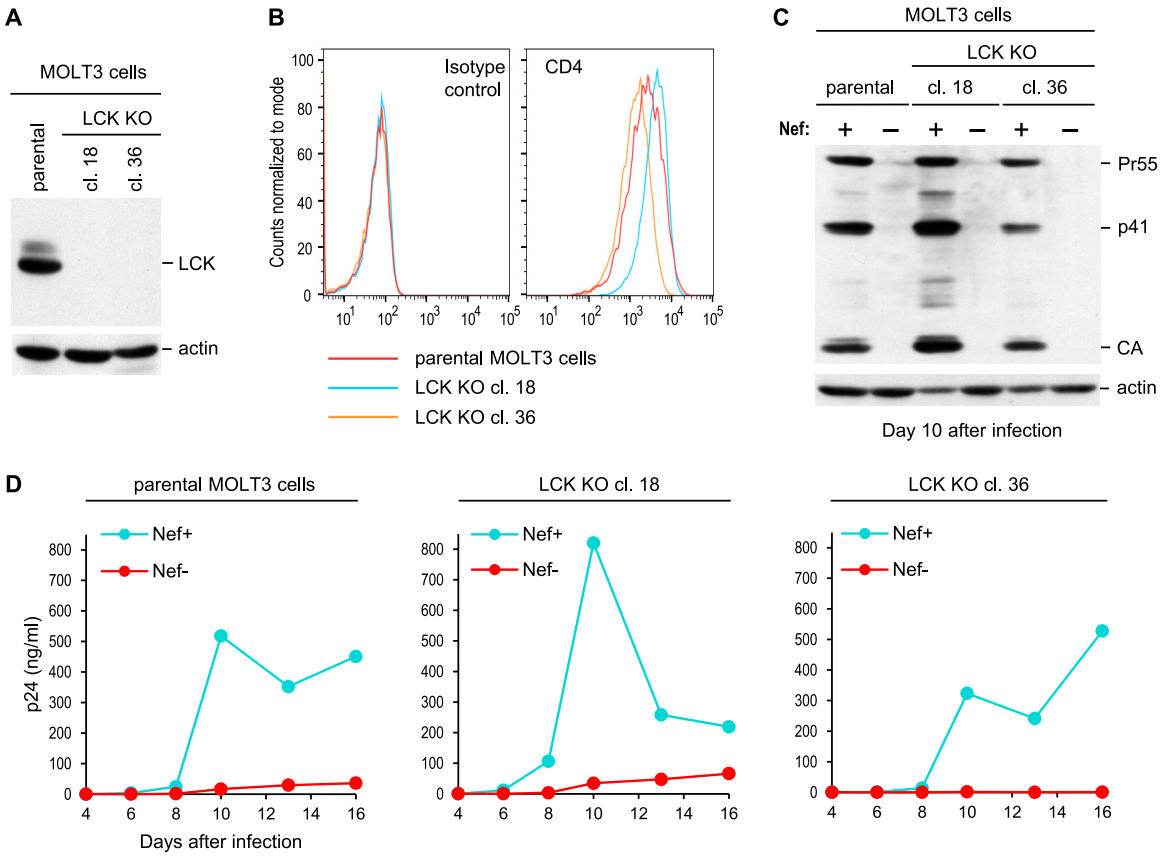

FIG 2 Nef strongly enhances HIV-1 replication in MOLT-3 cells lacking LCK. (A) LCK expression in parental MOLT-3 cells and in LCK KO clones analyzed by Western blotting. (B) CD4 surface levels on the same cells analyzed by flow cytometry. (C) Western blots showing the effects of Nef on HIV-1 replication in parental MOLT-3 cells and in LCK KO clones. The cells were infected with equal amounts (0.2 ng p24/mL) of Nef^+ or Nef^- $HIV\text{-}1_{NL4\text{-}3}$, and cell lysates were examined with anti-CA and anti-actin 10 days after infection. (D) Virus replication in the same cultures monitored by measuring p24 accumulation in the supernatants over time.

Overall, the cells lacking PAK2 were somewhat less permissive for HIV-1 replication than the parental cells (Fig. 3C and D). However, in two independent experiments, Nef remained clearly capable of enhancing HIV-1 replication in the absence of PAK2 (Fig. 3C and D; see also Fig. S2 in the supplemental material).

Although most studies suggest that NAK is PAK2, it has also been reported that Nef can bind to and activate PAK1 (55), which together with PAK2 and PAK3 belongs to the group I PAKs (40). Furthermore, it has been shown that the depletion of PAK1, and to a lesser extent of PAK3, blocks HIV-1 infection in Jurkat cells (56). We, therefore, examined the possibility that, in the absence of PAK2, Nef can use other group I PAKs to enhance HIV-1 replication.

As shown in Fig. 4A, MOLT-3 cells express both PAK1 and PAK2 at the mRNA level but lack mRNA encoding PAK3. Thus, to obtain cells that lack all group I PAKs, PAK2 KO MOLT-3 cells were subjected to a second round of gene editing with a single guide RNA (sgRNA) targeting PAK1. Two clones that lack both PAK1 and PAK2 (Fig. 4B) but exhibit surface CD4 levels comparable to the parental cells (Fig. 4C) were infected with Nef^+ or Nef^- $HIV\text{-}1_{NL4\text{-}3}$, and virus replication was examined by monitoring Gag expression levels (Fig. 4D) and the release of p24 antigen (Fig. 4E). Although both double-KO clones proved to be less permissive than the parental cells, Nef remained fully capable of enhancing HIV-1 replication in both clones (Fig. 4D and E). Furthermore, the enhancement of HIV-1 replication by Nef in the double-KO clones was reproducible in a repeat experiment (Fig. S2). We conclude that, at least in MOLT-3 cells, group I PAKs are not required for this activity of Nef.

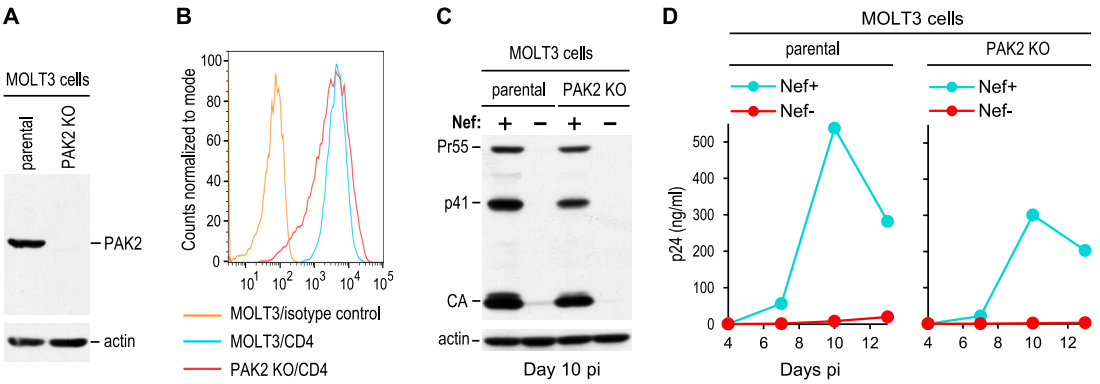

FIG 3 Nef can strongly enhance HIV-1 replication in cells lacking PAK2. (A) PAK2 expression in parental and in PAK2 KO MOLT-3 cells analyzed by Western blotting. (B) CD4 surface levels on the same cells analyzed by flow cytometry. (C) Western blots showing that the effects of Nef on HIV-1 replication in parental and in PAK2 KO MOLT-3 cells are comparable. The cells were infected with 0.2 ng p24/mL of Nef^+ or Nef^- HIV-1_{NL4-3}, and cell lysates were analyzed as in Fig. 2C. (D) Virus replication monitored in parallel by measuring p24 accumulation in the supernatants.

Nef requirement in cells that lack the Nef/AP-2 targets SERINC3, SERINC5, and CD4. We previously showed that Nef profoundly enhanced HIV-1 replication in MOLT-3 cells that ectopically expressed an excess amount of a Nef-resistant CD4 molecule (45). However, because of the presence of both Nef-resistant ectopic CD4 and of endogenous CD4 in these cells, we could not strictly rule out that the downregulation of the Nef-sensitive endogenous CD4 played a role in the enhancement of HIV-1 replication.

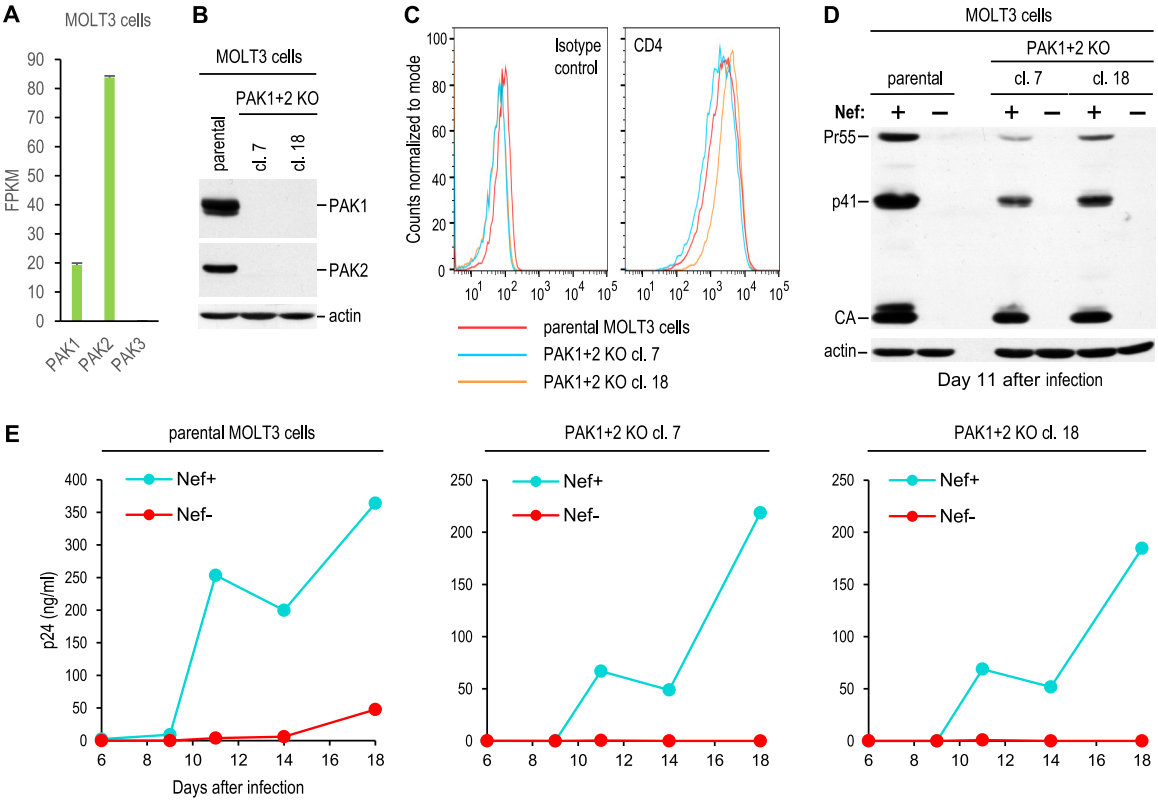

FIG 4 Nef can fully support HIV-1 replication in the absence of all group I PAKs. (A) Expression of group I PAK mRNAs in MOLT-3 cells quantified by transcriptome sequencing (RNA-seq) as fragments per kilobase of transcript per million mapped reads (FPKM). (B) Expression of PAK1 and PAK2 in parental MOLT-3 cells and in PAK1/PAK2 double-KO clones analyzed by Western blotting. (C) CD4 surface levels on the same cells analyzed by flow cytometry. (D and E) Replication of Nef^+ and Nef^- HIV-1_{NL4-3} in the same cells after infection with 0.2 ng/mL p24, monitored by Western blotting of cell lysates with anti-CA (D) and by measuring p24 accumulation in the supernatants (E).

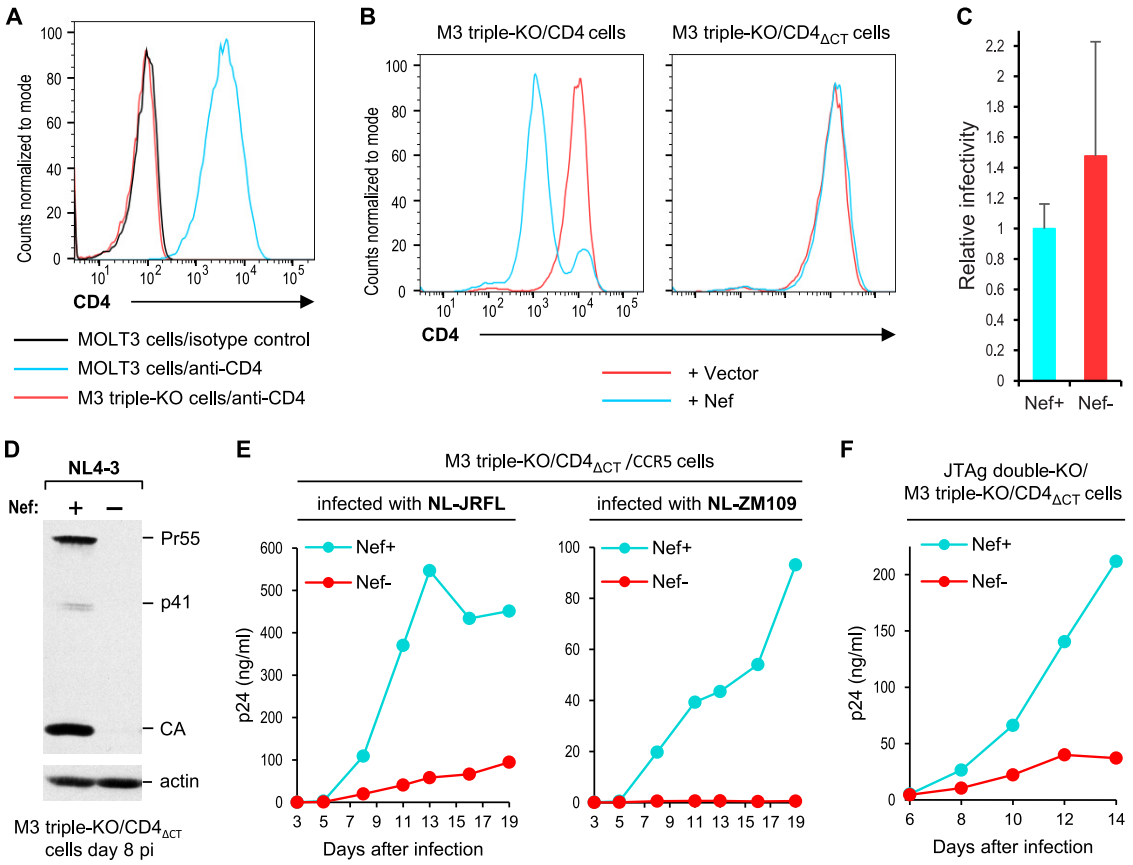

FIG 5 Nef remains required for efficient HIV-1 replication in MOLT-3 cells lacking SERINCs and Nef-sensitive CD4. (A) CD4 surface levels on parental MOLT-3 cells and on M3 triple-KO (SERINC3/SERINC5/CD4 KO) cells analyzed by flow cytometry. (B) CD4 surface levels on M3-triple-KO/CD4 and M3 triple-KO/$CD4_{\Delta CT}$ cells stably transduced with empty MSCVpuro (vector) or with a version expressing Nef_{LAI}. (C) Relative infectivities of Nef^+ and Nef^- HIV-1_{NL4-3} virions produced in M3 triple-KO/$CD4_{\Delta CT}$ cells measured using MOLT-3/ZsGreen reporter cells. Data are mean of three experiments with SD. (D) Replication of Nef^+ and Nef^- HIV-1_{NL4-3} in M3 triple-KO/$CD4_{\Delta CT}$ cells. The cells were infected with 0.2 ng p24/mL, and cell lysates were examined by Western blotting with anti-CA and anti-actin on day 8 postinfection (p.i.). (E) Replication of Nef^+ and Nef^- versions of the R5-tropic NL-JRFL and NL-ZM109 viruses in M3 triple-KO/$CD4_{\Delta CT}$/CCR5 cells infected with 0.2 ng p24/mL, monitored by measuring p24 accumulation in the supernatants. (F) Replication of Nef^+ and Nef^- HIV-1_{NL4-3} in heterokaryons formed between M3 triple-KO/$CD4_{\Delta CT}$(GFP_{8-11}) cells and JTAg double-KO(GFP_{1-7}) cells transiently expressing the HN and F proteins of NDV. GFP-positive cells were sorted by FACS and infected with 5 ng p24/mL. Virus replication was monitored by measuring p24 accumulation in the supernatants.

To address this possibility, we first knocked out endogenous CD4 and then ectopically expressed a Nef-resistant CD4 in the KO cells. As parental cells for the CD4 knockout, we used previously generated double-KO MOLT-3 cells lacking SERINC3 and SERINC5, in which HIV-1 replication remains highly dependent on Nef (45). In addition to SERINC3 and SERINC5, the resulting MOLT-3 (M3) triple-KO cells completely lack CD4 (Fig. 5A).

CD4 expression was restored after the stable transduction of M3 triple-KO cells with retroviral vectors encoding either wild-type (WT) CD4 (M3 triple-KO/CD4 cells) or the $CD4_{\Delta CT}$ molecule (M3 triple-KO/$CD4_{\Delta CT}$ cells) (Fig. 5B). The $CD4_{\Delta CT}$ molecule lacks the 25 C-terminal residues of the cytoplasmic domain, which are essential for the downregulation of CD4 by Nef (45). To formally demonstrate that the truncated CD4 on the surface of M3 triple-KO/$CD4_{\Delta CT}$ cells is resistant to Nef, the M3 triple-KO/CD4 and M3 triple-KO/$CD4_{\Delta CT}$ cells were both stably transduced with a retroviral vector encoding Nef or the empty vector. As expected, Nef clearly downregulated wild-type CD4 from the surface of M3 triple-KO/CD4 cells (Fig. 5B). In contrast, the truncated CD4 expressed by the M3 triple-KO/$CD4_{\Delta CT}$ cells was completely resistant to Nef-mediated downregulation (Fig. 5B). Notably, Nef did not enhance the specific infectivity of HIV-1_{NL4-3} virions produced in M3 triple-KO/$CD4_{\Delta CT}$ cells (Fig. 5C), as quantified using MOLT-3/ZsGreen indicator cells (57).

Nevertheless, the ability of HIV-1_{NL4-3} to spread in M3 triple-KO/$CD4_{\Delta CT}$ cells after infection at a low multiplicity was highly dependent on Nef (Fig. 5D).

To determine whether the Nef-dependency of the X4-tropic HIV-1_{NL4-3} in M3 triple-KO/$CD4_{\Delta CT}$ cells is shared by R5-tropic HIV-1 viruses, we stably expressed CCR5 in these cells. The resulting M3 triple-KO/$CD4_{\Delta CT}$/CCR5 cells were infected with the R5-tropic NL-JRFL and NL-ZM109 variants of HIV-1_{NL4-3}, which encode the Env proteins of primary subtype B and subtype C HIV-1 strains, respectively (26). As shown in Fig. 5E, NL-JRFL and NL-ZM109 both required Nef to spread efficiently in M3 triple-KO/$CD4_{\Delta CT}$/CCR5 cells. Taken together, these results demonstrate that Nef can profoundly enhance HIV-1 replication in the combined absence of SERINC3, SERINC5, and CD4.

To determine whether the MOLT-3 phenotype is dominant, M3 triple-KO/$CD4_{\Delta CT}$ cells were fused with JTAg double-KO cells lacking SERINC3 and SERINC5, in which Nef is not required for efficient HIV-1 replication (12). To facilitate the isolation of heterokaryons, we stably expressed one component of a split green fluorescent protein (GFP) system (GFP^{1-7}) in the JTAg double-KO cells, and the other component (GFP^{8-11}) in the M3 triple-KO/$CD4_{\Delta CT}$ cells. As expected, GFP expression could not be detected when JTAg double-KO(GFP^{1-7}) cells were mixed at a 1:1 ratio with M3 triple-KO/$CD4_{\Delta CT}$(GFP^{8-11}) cells (see Fig. S3 in the supplemental material). However, GFP expression became apparent when JTAg double-KO(GFP^{1-7}) cells that had been transiently transfected with the fusogenic Newcastle disease virus (NDV) HN and F glycoproteins were mixed with M3 triple-KO/$CD4_{\Delta CT}$(GFP^{8-11}) cells (Fig. S3). GFP-positive cells were isolated by fluorescent-activated cell sorting (FACS) and infected with Nef+ or Nef$^-$ HIV-1_{NL4-3}. As shown in Fig. 5F, virus replication was considerably more robust in the presence of Nef, consistent with the possibility that Nef counteracted an unidentified inhibitory factor.

Effect of Nef on HIV-1 replication in the triple-KO cells correlates with AP-2 binding. HIV-1 Nef is thought to engage the AP-2 clathrin adaptor complex through conserved dileucine and diacidic motifs to accelerate the endocytosis of CD4 (9, 58–61) and to downregulate SERINC3 and SERINC5 (11, 13–15). For instance, a mutation in Nef that targets the conserved dileucine motif (LL164,165AA) abolishes binding to AP-2 and other clathrin adaptor complexes and impairs the abilities of Nef to downregulate CD4 and to counteract SERINC5 (11, 61–63). Interestingly, the LL164,165AA mutation also markedly and reproducibly reduced the ability of Nef to enhance HIV-1 replication in M3 triple-KO/$CD4_{\Delta CT}$ cells, which lack these Nef/AP-2 targets (see Fig. S4 in the supplemental material). We therefore examined the effects of additional mutations that impair the Nef/AP-2 interaction on HIV-1 replication in M3 triple-KO/$CD4_{\Delta CT}$ cells.

Nef residues W57 and L58 have been implicated in the direct binding of Nef to the cytoplasmic domain of CD4 (64), and consistent with this notion, the WL57,58AA mutation disrupts the ability of Nef to downregulate CD4 while preserving the ability to downregulate MHC-I (23, 65). However, a recent crystal structure of a CD4-Nef-AP-2 complex suggests that the WL57,58AA mutation interferes with CD4 binding indirectly by affecting the positioning of the N-terminal loop of Nef at the interface between the AP-2 α subunit and the Nef core (10). In M3 triple-KO/$CD4_{\Delta CT}$ cells, the WL57,58AA mutation substantially impaired the replication of HIV-1_{NL4-3} (Fig. 6A and D; see also Fig. S5 in the supplemental material), even though the truncated CD4 expressed on these cells lacks all of the determinants involved in the interaction with Nef (10).

A crystal structure of Nef bound to the α and $\sigma 2$ subunits of AP-2 indicates that a salt bridge between R134 and D175 stabilizes the conformation of the Nef C-terminal loop, which is directly involved in AP-2 binding (66). Consistent with this model, an R134E mutation eliminated the ability of Nef to bind AP-2 and to downregulate CD4 (66). Interestingly, the R134E mutation also abrogated the ability of Nef to enhance HIV-1 replication in M3 triple-KO/$CD4_{\Delta CT}$ cells (Fig. 6B and D; see also Fig. S5).

In addition to the critical dileucine and diacidic motifs, the C-terminal loop of Nef contains other determinants that are involved in the association with AP-2 (66, 67). In particular, mutations in a hydrophobic region downstream of the dileucine motif impaired both AP-2 binding and CD4 downregulation (67). For instance, the LH170,171AA mutation within this region impaired AP-2 binding and abrogated CD4 downregulation by

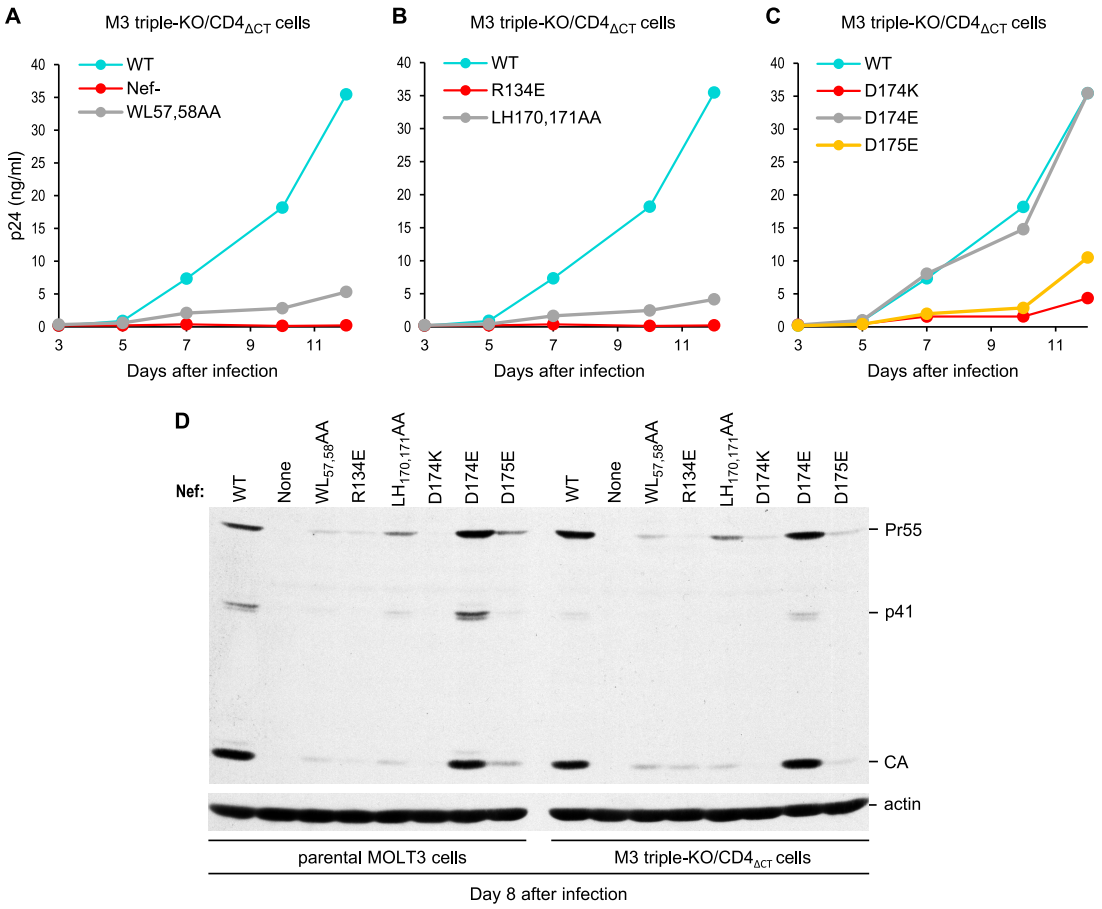

FIG 6 Replication of Nef mutants in M3 triple-KO/CD4$_{\Delta CT}$ cells lacking SERINCs and Nef-sensitive CD4. (A) Effect of a mutation (WL57,58AA) reported to disrupt binding of Nef to the cytoplasmic domain of CD4. (B) Effects of mutations that disrupt AP-2 binding. (C) Effects of mutations in a conserved diacidic motif that is specifically required for binding to AP-2 but not AP-1 or AP-3. Of note, AP-2 binding is unaffected by the conservative D174E substitution but is impaired by the equally conservative D175E substitution (61). (D) Side-by-side comparison of the abilities of the Nef mutants to spread in parental MOLT-3 cells and in M3 triple-KO/CD4$_{\Delta CT}$ cells. The cells were infected with WT (Nef^{+}) HIV-1$_{NL4-3}$ or with the indicated Nef mutants (0.2 ng p24/mL), and virus replication was monitored by measuring p24 accumulation in the supernatants (A to C) or by Western blotting of cell lysates with anti-CA (D). The virus replication curves are all from the same experiment.

Nef (67). The LH170,171AA mutation also clearly impaired the replication of HIV-1$_{NL4-3}$ in M3 triple-KO/CD4$_{\Delta CT}$ cells (Fig. 6B and D; see also Fig. S5).

We previously reported that a point mutation (D174K) in the conserved diacidic motif of Nef substantially impaired its ability to enhance HIV-1 replication in wild-type MOLT-3 cells (45) and now find that the D174K mutation has a comparable inhibitory effect on HIV-1 replication in M3 triple-KO/CD4$_{\Delta CT}$ cells (Fig. 6C and D; see also Fig. S5). In contrast, HIV-1$_{NL4-3}$ with a conservative substitution at the same position of Nef (D174E) replicated with wild-type kinetics in M3 triple-KO/CD4$_{\Delta CT}$ cells (Fig. 6C and D; see also Fig. S5). Of note, the D174E mutation also did not affect the binding of Nef to clathrin adaptor complexes (61). In contrast, a conservative substitution at position 175 of Nef (D175E) reduced binding to AP-2 without affecting binding to AP-1 or AP-3 (61). Interestingly, the D175E mutation clearly delayed HIV-1 replication in M3 triple-KO/CD4$_{\Delta CT}$ cells (Fig. 6C and D; see also Fig. S5). Thus, the effects of point mutations in the diacidic motif on HIV-1 replication correlated with their effects on AP-2 binding.

To examine whether the presence of SERINCs and of Nef-sensitive CD4 affected the phenotypes of these Nef mutants, we performed a side-by-side comparison of their abilities to spread in parental MOLT-3 cells and in M3 triple-KO/CD4$_{\Delta CT}$ cells. As shown in Fig. 6D, each mutant behaved similarly in both cellular contexts, consistent with the

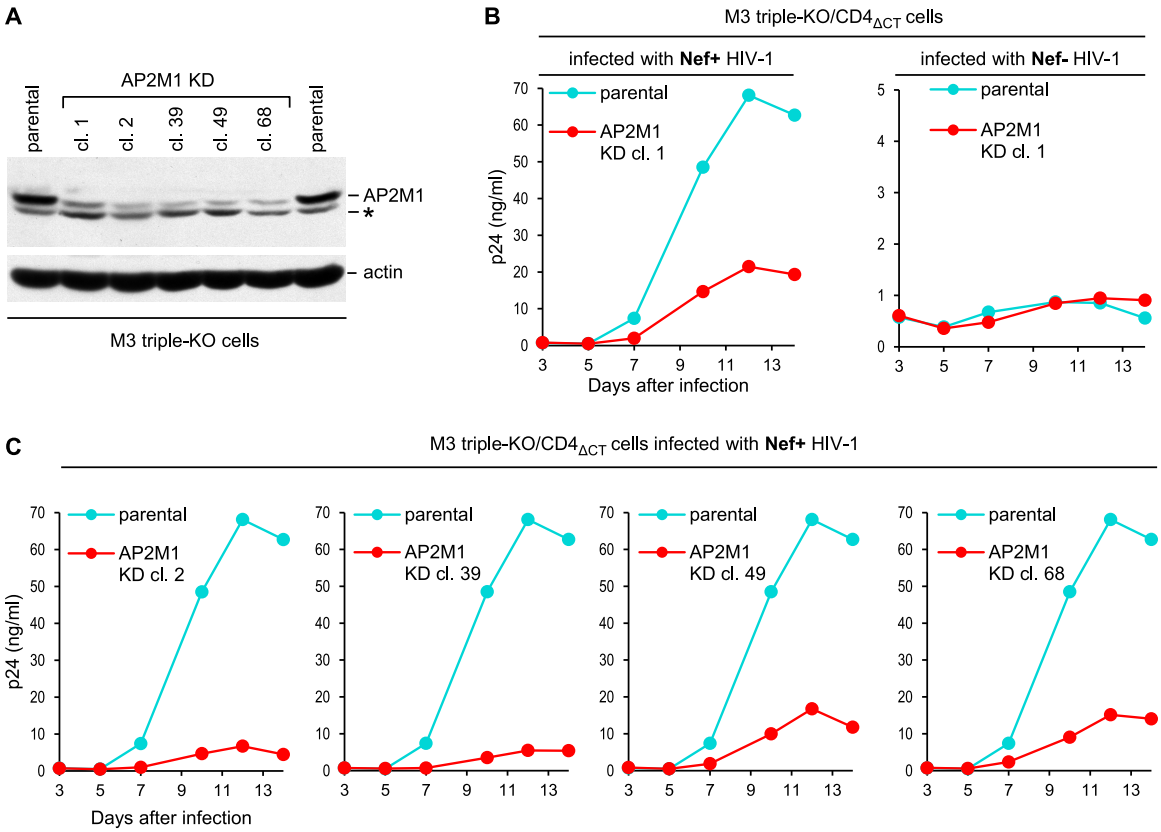

FIG 7 Knockdown of AP-2 subunit AP2M1 impairs WT HIV-1 replication in cells lacking SERINCs and Nef-sensitive CD4. (A) AP2M1 expression in parental M3 triple-KO (SERINC3/SERINC5/CD4 KO) cells and in M3 triple-KO-derived AP2M1 knockdown (KD) clones analyzed by Western blotting. The asterisk indicates a nonspecific band. (B and C) Replication of Nef$^+$ (B and C) and Nef$^-$ (B) HIV-1_{NL4-3} in parental M3 triple-KO cells and in M3 triple-KO-derived AP2M1 KD clones expressing the Nef-resistant $CD4_{\Delta CT}$ molecule. The cells were infected with 0.2 ng p24/mL, and virus replication was monitored by measuring p24 accumulation in the supernatants.

proposal that the requirement for Nef in MOLT-3 cells is primarily determined by an unknown restriction factor (45).

Effect of Nef on HIV-1 replication in the triple-KO cells depends on AP-2. The results described above specifically implicated AP-2 in the ability of Nef to enhance HIV-1 replication in MOLT-3 cells lacking the Nef/AP-2 targets SERINC3, SERINC5, and CD4. To confirm the role of AP-2, we subjected M3 triple-KO cells to a further round of CRISPR/Cas9-mediated gene editing with an sgRNA targeting AP2M1, the μ2 subunit of AP-2. Although this approach did not yield any cells that completely lacked AP2M1, we obtained several clones that expressed substantially reduced amounts (Fig. 7A). Of note, cells that completely lacked AP2M1 also could not be obtained in a previous study that used the same sgRNA, probably because a small amount of AP-2 is essential for viability (68).

To allow HIV-1 entry, the M3 triple-KO cell-derived AP2M1 knockdown (KD) clones, which lack endogenous CD4, were reconstituted with the Nef-resistant $CD4_{\Delta CT}$ molecule. As shown in Fig. 7B, WT (Nef$^+$) HIV-1_{NL4-3} spread less efficiently in the AP2M1 KD clone 1 than in the parental cells, whereas Nef-deficient HIV-1_{NL4-3} failed to spread even in the parental cells. To confirm that the enhancement of HIV-1 spreading by Nef depended on AP-2, we monitored the replication of WT (Nef$^+$) HIV-1_{NL4-3} in the remaining four AP2M1 KD clones and observed that it was impaired in each case (Fig. 7C).

To exclude off-target effects, the parental M3 triple-KO/$CD4_{\Delta CT}$ cells and two KD clones with relatively low residual AP2M1 expression levels (clones 2 and 39) were stably transduced with a retroviral vector encoding AP2M1. Western blotting showed that while AP2M1 levels in the parental cells were only minimally affected, AP2M1 expression

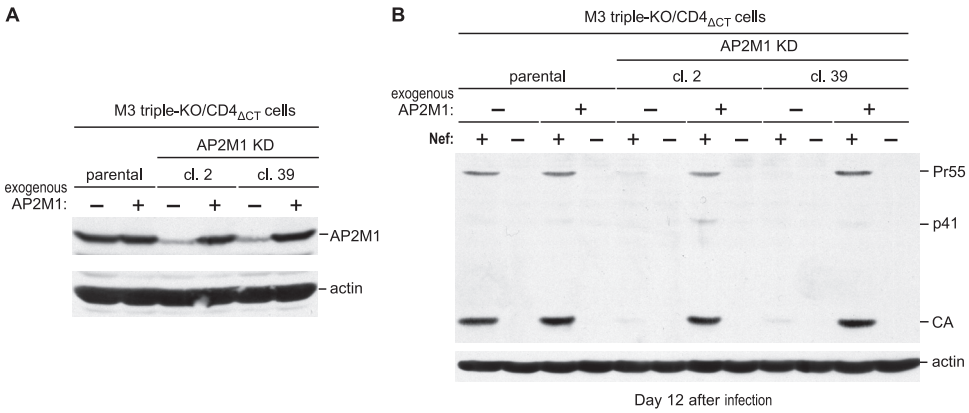

FIG 8 Restoration of AP2M1 expression rescues WT HIV-1 replication in cells lacking SERINCs and Nef-sensitive CD4. (A) Western blots showing AP2M1 expression in parental M3 triple-KO/CD4$_{\Delta CT}$ cells and in M3 triple-KO-derived AP2M1 KD/CD4$_{\Delta CT}$ clones stably transduced with the empty pCX4pur retroviral vector or with a version expressing AP2M1. (B) Replication of WT (Nef$^+$) and Nef$^-$ HIV-1$_{NL4-3}$ in the same cells. The cells were infected with 0.2 ng p24/mL, and cell lysates were examined with anti-CA and anti-actin 12 days after infection.

in both KD clones was fully restored (Fig. 8A). Importantly, while the ectopic expression of AP2M1 in the parental cells had no discernible effect on HIV-1 replication, it restored WT (Nef$^+$) HIV-1$_{NL4-3}$ replication in both AP2M1 KD clones to levels comparable to those in the parental cells (Fig. 8B).

Because Nef-deficient HIV-1$_{NL4-3}$ did not replicate to detectable levels in any of the cultures analyzed (Fig. 8B), we conducted another experiment in which infections with Nef-deficient HIV-1$_{NL4-3}$ were started with a 10-fold higher amount of input virus. Even under these conditions, only a slight increase in extracellular p24 antigen concentrations over a period of 3 weeks could be detected after infection of AP2M1 clone 2 cells (see Fig. S6 in the supplemental material). While virus production also remained modest after infection of AP2M1 clone 39 cells, a clear rise in extracellular p24 levels nevertheless became evident after an initial lag period (Fig. S6). Notably, extracellular p24 levels after infection with Nef-deficient HIV-1$_{NL4-3}$ were comparable in the presence and absence of ectopically expressed AP2M1 (Fig. S6). Taken together, these results confirm that AP-2 is crucial for the efficient replication of Nef$^+$ HIV-1 in MOLT-3 cells that lack the Nef/AP-2 targets SERINC3, SERINC5, and CD4.

DISCUSSION

HIV-1 and simian immunodeficiency virus Nef proteins enhance virus replication in primary CD4$^+$ T cells, particularly if these are infected before stimulation, but the molecular basis for this biological activity is incompletely understood. In Jurkat E6.1 T lymphoid cells, Nef appears to enhance HIV-1 replication primarily by counteracting SERINC5 because the unrelated SERINC antagonists Nef and glycoGag supported HIV-1 replication in these cells to a similar extent (45). Consistent with this observation, Nef is critical for HIV-1 propagation in Jurkat-derived JTAg cells but not in double-KO JTAg cells lacking SERINC3 and SERINC5 (12). However, the potent SERINC5 antagonist glycoGag was unable to substitute for Nef in supporting HIV-1 replication in MOLT-3 T lymphoid cells (45). Furthermore, HIV-1 replication remained highly dependent on Nef in MOLT-3 cells lacking SERINC3 and SERINC5 (45). Together, these observations suggested that MOLT-3 cells express another factor that is affected by Nef but not glycoGag. Since MOLT-3 cells resemble primary human PBMC in this regard (45), we have now used MOLT-3 cells as a model system to explore the possible involvement of other host factors through CRISPR/Cas9-mediated gene editing.

One highly conserved activity of lentiviral Nef proteins is the relocalization of the T cell receptor-proximal kinase LCK to an intracellular compartment (69). Nef inhibits the targeting of LCK to the immunological synapse, possibly to limit T cell signaling to

prevent activation-induced cell death (36). Instead, the Nef-induced retargeting of LCK triggers intracellular signaling to facilitate HIV-1 replication in primary human T lymphocytes (37). Furthermore, the association of Nef and LCK was found to be important for the enhancement of HIV-1 replication by Nef in a T cell line (46). In contrast, we now find that LCK is entirely dispensable for the stimulation of HIV-1 replication by Nef in MOLT-3 cells. Since HIV-1 replication in these cells is highly dependent on Nef (45), we conclude that Nef can profoundly enhance HIV-1 spreading in an LCK-independent manner.

The ability of Nef to interact with PAK2 is generally conserved among the different HIV-1 groups, indicating that the interaction is of biological significance (41). It has been reported that Nef-associated PAK induces antiapoptotic signaling in T cells and, thus, supports virus replication by decreasing HIV-1-induced cell death (52). Furthermore, it has been proposed that Nef enhances virus replication by engaging PAK2 to enhance the responsiveness of infected T cells to activating stimuli (43). In addition, Nef reduces the motility of infected T cells in a PAK2-dependent manner (42, 70, 71), possibly to facilitate the formation of stable virological synapses and thus the cell-to-cell transmission of HIV-1 (54). PAK2 also appears essential for the association of Nef with components of the exocyst complex (72), which has been suggested to contribute to the intercellular spread of HIV-1 via tunneling nanotubes (73). However, it is evident from the results of the present study that Nef can substantially enhance HIV-1 spreading in some other way because we find that the effect of Nef on HIV-1 spreading in MOLT-3 cells does not depend on PAK2 or any other group I PAK.

Consistent with the observation that CD4 on the surface of cells infected with HIV-1 can reduce the infectivity of progeny virions (21), it has been noted that the ability of Nef to enhance HIV-1 replication in primary T cells or in *ex vivo* lymphoid tissue correlates with its ability to downregulate CD4 (22, 74). Furthermore, among several Nef activities, the ability to downregulate CD4 correlated most closely with the enhancement of HIV-1 pathogenicity in humanized mouse models (23, 24). In agreement with these reports, we observed previously that mutations that impaired the ability of Nef to downregulate CD4 also impaired its ability to promote HIV-1 replication in MOLT-3 cells (45). Conversely, we observed that Nef remained fully capable of enhancing HIV-1 replication in MOLT-3 cells overexpressing a Nef-resistant CD4 molecule (45). However, since these cells also expressed Nef-sensitive CD4 endogenously, we could not strictly rule out that the downregulation of the endogenous CD4 accounted for the effect of Nef on HIV-1 spreading (45). In the present study, we observed that Nef substantially enhanced HIV-1 replication even in triple-KO MOLT-3 cells that simultaneously lacked the three Nef targets SERINC3, SERINC5, and CD4 and expressed exclusively a Nef-resistant CD4 to allow HIV-1 entry. We conclude that the downregulation of CD4 is dispensable for the pronounced effect of Nef on HIV-1 spreading in MOLT-3 cells. Furthermore, SERINCs and CD4 do not act in a redundant manner to restrict the propagation of Nef-deficient HIV-1 in these cells.

Nef engages the AP-2 adaptor complex to downregulate both CD4 and SERINCs (9, 11, 13, 14, 58–60) and the AP-1 adaptor complex to decrease MHC-I surface levels (19, 75, 76). Notably, HIV-1 replication in MOLT-3 cells is markedly impaired by the LL164,164AA mutation in a conserved dileucine motif of Nef that disrupts binding to AP-2 and other clathrin adaptor complexes (45, 77, 78) and abrogates the ability of Nef to downregulate both CD4 and SERINC5 (11, 62). Similarly, HIV-1 replication in MOLT-3 cells is impaired by the DD174,175AA mutation in a conserved diacidic motif of Nef that specifically prevents Nef from binding to AP-2 but not AP-1 hemicomplexes and disrupts its ability to downregulate CD4 (23, 45, 77). Together, these observations implicated AP-2 in the enhancement of HIV-1 replication in MOLT-3 cells. We now find that various point mutations in Nef that have been reported to compromise its ability to interact with AP-2 impair HIV-1 replication even in MOLT-3 cells that lack the three Nef/AP-2 targets SERINC3, SERINC5, and CD4. Instead of endogenous CD4, these cells expressed a CD4 that lacks cytoplasmic tail sequences required for the downregulation of CD4 by Nef (Fig. 5) but not for its ability

to support HIV-1 entry (79). In particular, the phenotypes resulting from conservative substitutions within the D174/175 diacidic motif of Nef appear noteworthy. On the one hand, the D174E mutation, which does not affect AP-2 binding (61), had no effect on the ability of Nef to enhance HIV-1 replication. On the other hand, the equally conservative D175E substitution, which selectively impairs binding to AP-2 but not AP-1 or AP-3 (61), compromised HIV-1 replication both in the presence or absence of SERINC3, SERINC5, and Nef-sensitive CD4. Thus, the effects of mutations in the diacidic motif on HIV-1 replication correlated remarkably well with their effects on AP-2 binding.

CRISPR/Cas9-mediated gene editing of the AP2M1 subunit of AP-2 has been used previously to study protein trafficking in HeLa cells (68). Although a complete knockout could not be achieved, presumably because AP-2 is essential for cell viability, the approach yielded cells with substantially reduced AP2M1 expression and marked defects in transferrin receptor endocytosis (68). Based on these observations, we used the CISPR/Cas9 approach to target AP2M1 in MOLT-3 cells that already lacked SERINC3, SERINC5, and Nef-sensitive CD4 and were able to obtain several clones with clearly reduced AP2M1 expression levels. Interestingly, the ability of Nef$^+$ HIV-1 to replicate was compromised in all clones. The spreading of Nef$^+$ HIV-1 could be fully rescued through the ectopic expression of AP2M1, confirming that AP-2 plays an important role in this model system.

Taken together, our observations imply that the potent effect of Nef on HIV-1 replication in MOLT-3 cells depends on AP-2 even in the absence of several known Nef targets that have the potential to restrict HIV-1 and are downregulated in an AP-2-dependent manner. They thus suggest that Nef additionally engages AP-2 to control the trafficking of an unknown factor that affects HIV-1 spreading.

MATERIALS AND METHODS

HIV-1 proviral constructs. NL4-3/Nefstop is a *nef*-deficient variant of the full-length, X4-tropic HIV-1 molecular clone pNL4-3 (GenBank accession number M19921) that has *nef* codons 31 to 33 replaced by three consecutive premature termination codons (12). The full-length HIV-1 proviral constructs NL-JRFL and NL-ZM109 (both Nef$^+$) are R5-tropic variants of pNL4-3 that have been described (26, 45). Full-length variants of pNL4-3 with point mutations in *nef* were obtained by inserting mutant *nef* sequences generated by a PCR-based approach between unique XhoI and NcoI sites of pNL4-3.

Retroviral vectors. The retroviral vectors pCXbsrCD4 and pCXbsrCD4$_{\Delta CT}$ encode full-length human CD4 and a version that lacks most of the cytoplasmic domain, respectively (45). To obtain a retroviral vector expressing HIV-1 Nef, the *nef* gene of HIV-1$_{LAI}$ (GenBank accession number K02013) was inserted between the unique BglII and EcoRI sites of MSCVpuro (Clontech). The retroviral vector pCX4pur-synCCR5 encodes codon-optimized human CCR5 (45). The coding sequence for human AP2M1 (GenBank accession number BC004996) was cloned into the retroviral vector pCX4pur (80). The retroviral vector pCX4pur-Rluc8-GFP$^{1-7}$ encodes Renilla luciferase variant 8 (Rluc8) residues 1 to 155 fused to GFP residues 2 to 156. The retroviral vector pCX4pur-Rluc8-GFP$^{8-11}$ encodes GFP residues 157 to 230 fused to Rluc8 residues 156 to 311. The Rluc8-GFP coding sequences were amplified from phRL-CMV-based plasmids (81) and cloned into pCX4pur.

KO cells. MOLT-3 cells were obtained from the ATCC. To obtain MOLT-3 cells lacking LCK, preassembled ribonucleotide complexes consisting of purified Cas9 (Synthego) and chemically modified synthetic sgRNA (target sequence: 5′-GCTCCGCGTCCTTGCGGCTC-3′) (Synthego) were delivered into MOLT-3 cells via nucleofection using the Cell Line Nucleofector kit V with the Nucleofector II device (Lonza). Clones obtained by limiting dilution were screened by Western blotting with anti-LCK antibody 3A5 (Santa Cruz Biotechnology; sc-433). Protein loading was assessed with anti-actin antibody (Santa Cruz Biotechnology; sc-47778).

MOLT-3 cells lacking PAK2 were obtained by transiently transfecting a plasmid expressing an sgRNA targeting PAK2 (target sequence, 5′-GATTTCGTATGATCCGGTCG-3′) by nucleofection, along with a plasmid expressing Cas9. Clones obtained by limiting dilution were screened by Western blotting with anti-PAK2 (Cell Signaling Technology; 2608) and anti-actin antibodies. To obtain MOLT-3 cells lacking all group I PAKs, Cas9/sgRNA complexes targeting PAK1 (target sequence, 5′-AGGCACCGTGTACACAGCAA-3′) were delivered into PAK2 KO MOLT-3 cells by nucleofection. Clones obtained by limiting dilution were screened by Western blotting with anti-PAK1 (Cell Signaling Technology; 2602) and anti-actin antibodies.

MOLT-3 double-KO cells lacking SERINC3 and SERINC5 (MOLT-3 S3/5 double-KO cells) have been described (26). To obtain MOLT-3 cells lacking SERINC3, SERINC5, and CD4 (M3 triple-KO cells), Cas9/sgRNA complexes targeting CD4 (target sequence, 5′-GAGGTGCAATTGCTAGTGTT-3′) were delivered into MOLT-3 S3/5 double-KO cells by nucleofection. Clones obtained by limiting dilution were screened by flow cytometry after staining with anti-CD4 antibody (BioLegend; 300502) and PE-conjugated secondary antibody (Jackson ImmunoResearch; 115-116-146).

M3 triple-KO/CD4 and M3 triple-KO/CD4$_{\Delta CT}$ cells were obtained by retroviral transduction of M3 triple-KO cells with pCXbsrCD4 and pCXbsrCD4$_{\Delta CT}$, respectively, followed by selection with blasticidin

(5 μg/mL). The ectopic expression of CD4 or $CD4_{\Delta CT}$ was confirmed by flow cytometry. To examine the effects of Nef on CD4 surface levels, M3 triple-KO/CD4 and M3 triple-KO/$CD4_{\Delta CT}$ cells were transduced with empty MSCVpuro or $MSCVpuroNef_{LAI}$, followed by selection with puromycin (1 μg/mL). To facilitate the entry of R5-tropic viruses, M3 triple-KO/$CD4_{\Delta CT}$ cells were transduced with pCX4pur-synCCR5, followed by selection with puromycin (1 μg/mL).

M3 triple-KO cells expressing reduced amounts of the μ2 subunit of AP-2 (AP2M1) were obtained by delivering Cas9/sgRNA complexes targeting AP2M1 (target sequence, 5′-CGATGTCATCTCGGTAGACT-3′) into M3 triple-KO cells by nucleofection. Clones obtained by limiting dilution were screened by Western blotting with anti-AP50 (BD Biosciences; 611351) and anti-actin antibodies. To restore AP2M1 expression, clones expressing reduced amounts of AP2M1 were transduced with pCX4purAP2M1, followed by selection with puromycin (1 μg/mL).

Cell fusion. JTAg double-KO cells, which lack SERINC3 and SERINC5 and express only low levels of CD4 (12), were transduced with pCX4pur-Rluc8-GFP^{1-7}, and M3 triple-KO/$CD4_{\Delta CT}$ cells were transduced with pCX4pur-Rluc8-GFP^{8-11}, followed by selection with puromycin (1 μg/mL). To transiently express the NDV hemagglutinin-neuraminidase (HN) and fusion (F) proteins, JTAg double-KO cells expressing Rluc8-GFP^{1-7} were cotransfected with pCAGGS-HN and pCAGGS-F using Lipofectamine 2000 (Invitrogen). The transfected cells were then mixed with M3 triple-KO/$CD4_{\Delta CT}$ cells expressing Rluc8-GFP^{8-11}. After 2 days of incubation, GFP-positive cells were sorted with a Sony MA900 cell sorter.

Virus replication studies. Replication-competent HIV-1 was produced by transfecting 293T cells with plasmids containing full-length proviruses. Virus containing supernatants were clarified by low-speed centrifugation, passed through 0.45-μm filters, normalized for HIV-1 capsid (p24) antigen with an HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (XpressBio), and used to infect target cells.

MOLT-3 cells were infected in T25 flasks in 5 mL medium. FACS-sorted cells were infected in 96-well plates in 200 μL medium. PBMC were obtained from LRS chambers by Ficoll-Hypaque density gradient centrifugation and kept in RPMI 1640 medium supplemented with 15% human AB serum (Millipore Sigma). $CD4^+$ T cells were purified from fresh PBMC by negative selection with an EasySep human $CD4^+$ T cell enrichment kit (Stemcell Technologies). PBMC were seeded into 12-well plates in 2 mL medium and immediately stimulated with 2 μg/mL phytohemagglutinin (PHA) (Millipore Sigma). One day later, the PHA-containing medium was replaced by fresh medium, and the cells were infected in the presence of 20 U/mL interleukin 2 (Roche). $CD4^+$ T cells were seeded into 12-well plates and immediately infected at a p24 concentration of 1 ng/mL. On day 3 after infection, the cells were stimulated with 2 μg/mL PHA. The next day, the PHA-containing medium was replaced by fresh medium containing 20 U/mL interleukin 2.

Virus replication was monitored by comparing Gag protein expression levels in infected cells by Western blotting using anti-HIV-1 capsid (CA) antibody 183-H12-5C and anti-actin and/or by measuring the accumulation of p24 antigen in the culture supernatants over time with an HIV-1 p24 ELISA kit (XpressBio).

Analysis of virus infectivity. Vesicular stomatitis virus G (VSV-G) pseudotypes of NL4-3 or NL4-3/Nefstop were obtained by transfecting 293T cells, clarified by low-speed centrifugation, passed through 0.45-μm filters, normalized for p24 antigen, and used to infect M3 triple-KO/$CD4_{\Delta CT}$ cells. After overnight incubation, the infected M3 triple-KO/$CD4_{\Delta CT}$ cells were extensively washed to remove input virus, and resuspended in fresh medium. Supernatants harvested 24 h later were clarified by low-speed centrifugation, passed through 0.45-μm filters, normalized for p24 antigen, and used to infect MOLT-3/ZsGreen indicator cells (clone 45), which turn bright green upon infection (57). To limit virus replication to a single cycle, the entry inhibitor ADM3100 (5 μM) was added 16 h later. After another 2 days, the cells were fixed with 4% paraformaldehyde, and ZsGreen expression was analyzed by flow cytometry.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.05 MB.

FIG S2, PDF file, 0.04 MB.

FIG S3, PDF file, 0.04 MB.

FIG S4, PDF file, 0.2 MB.

FIG S5, PDF file, 0.3 MB.

FIG S6, PDF file, 0.1 MB.

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