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# Nef enhances HIV-1 replication and infectivity independently of SERINC5 in CEM T cells

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## Abstract

A primary function of HIV-1 Nef is the enhancement of viral infectivity and replication. Whether counteraction of the antiretroviral proteins SERINC3 and SERINC5 is the cause of this positive influence on viral growth-rate and infectivity remains unclear. Here, we utilized CRISPR/Cas9 to knockout SERINC3 and SERINC5 in a leukemic CD4-positive T cell line (CEM) that displays *nef*-related infectivity and growth-rate phenotypes. Viral replication was attenuated in CEM cells infected with HIV-1 lacking Nef (HIV-1 Nef). This attenuated growth-rate phenotype was observed regardless of whether the coding regions of the *serinc3* or *serinc5* genes were intact. Moreover, knockout of *serinc5* alone or of both *serinc5* and *serinc3* together failed to restore the infectivity of HIV1 Nef virions produced from infected CEM cells. Our results corroborate a similar study using another T-lymphoid cell line (MOLT-3) and indicate that the antagonism of SERINC3 and SERINC5 does not fully explain the virology of HIV-1 lacking Nef.

## 1. Introduction

Primate lentiviruses encode several accessory gene products that facilitate viral reproduction and persistence, in some cases by providing evasion of the host immune response. The

lentiviral Nef protein accelerates viral pathogenesis and progression to AIDS in SIV infected rhesus macaques (Kestler et al., 1991) and enhances disease progression in humans infected with HIV-1 (Kirchhoff et al., 1995; Deacon et al., 1995). Nef is a small, myristoylated, peripheral membrane protein with many well conserved activities, including the downregulation of cell surface proteins such as CD4 and MHC class-I (MHC-I), the modulation of T cell activation, and the enhancement of viral infectivity and growth-rate (Kirchhoff, 2010).

To downregulate CD4, Nef binds the cytoplasmic domain of CD4 and links it to the clathrin Adaptor Protein 2 (AP-2) complex, internalizing CD4 from the plasma membrane and delivering it to lysosomes for degradation (Garcia et al., 1993; Aiken et al., 1994; Chaudhuri et al., 2007; Kwon et al., 2020). The targeting of CD4 by Nef contributes to viral replication in several ways. CD4 downregulation prevents superinfection of cells and consequent premature cell-death, ensuring adequate time for viral replication (Wildum et al., 2006). It also contributes to inhibiting antibody dependent cellular cytotoxicity (ADCC) that recognizes CD4-induced epitopes with Env (Alsahafi et al., 2016). Potentially directly relevant to the work presented here, CD4 incorporates into virions and inhibits virion-infectivity if not downregulated by Nef (Ross et al., 1999; Lama et al., 1999).

To downregulate MHC-I, Nef has been proposed to use two non-mutually exclusive mechanisms: 1) Nef and the clathrin-adaptor AP-1 intercept *de novo* synthesized MHC-I molecules within the *trans*-Golgi network (TGN), leading to lysosomal degradation (Kasper et al., 2005; Roeth et al., 2004; Noviello et al., 2008; Jia et al., 2012); and/or 2) Nef retains internalized MHC-I molecules in the TGN via induction of a Src family kinase/ phosphoinositide 3-kinase signaling cascade (Hung et al., 2007; Blagoveshchenskaya et al., 2002; Dikeakos et al., 2010). In either case, reducing the expression of MHC-I molecules at the plasma membrane protects HIV-1 infected cells from lysis by cytotoxic T-lymphocytes, contributing to immune evasion (Collins et al., 1998).

Nef interacts directly with Src-family kinases including the lymphocyte specific kinase Lck, which it downregulates from the cell surface (Haller et al., 2007). The many studies of Nef's influence on T cell activation are difficult to reconcile, but transcriptional profiling suggests that the expression of Nef mimics signaling through the T cell receptor (Simmons et al., 2001). Apart from the model of MHC-I downregulation noted above, a clear mechanistic link between the influence of HIV-1 Nef on T cell activation and its influence on the expression of cell surface receptors is lacking.

Nef stimulates HIV-1 replication and enhances virion-infectivity in many cell culture systems, including various T cell lines, primary CD4-positive T cells, and human lymphoid tissue (Chowers et al., 1994; Spina et al., 1994; Lundquist et al., 2002; Münch et al., 2007). Diverse *nef* alleles from humans and primates maintain the ability to enhance HIV-1 replication and infectivity, suggesting these functions are important for establishing and maintaining persistent infection (Münch et al., 2007; Carl et al., 2001; Crotti et al., 2006). Expression of Nef within virion-producer cells and encoded either in *cis* or in *trans* relative to the viral genome enhances HIV-1 replication and yields virions of greater infectivity (Aiken and Trono, 1995; Pandori et al., 1996). Nef's ability to enhance infectivity requires

cellular components involved in vesicular trafficking (dynamin 2, AP-2, and clathrin) and is also determined by the Envelope (Env) glycoprotein (Pizzato et al., 2007, 2008). A survey of cell lines identified murine leukemia virus glycosylated Gag (MLV glycoGag), a protein structurally unrelated to Nef, as an infectivity factor that rescues Nef-deficient HIV-1 virions (Pizzato, 2010). These and other features suggested that Nef counteracts a cellular factor or factors that restrict viral infectivity and possibly replication.

Two groups identified the host transmembrane protein SERINC5, and to a lesser degree SERINC3, as inhibitors of HIV-1 virion-infectivity that are counteracted by Nef (Rosa et al., 2015; Usami et al., 2015). Nef downregulates SERINC5 from the plasma membrane via a clathrin/AP-2 and Rab5/7 endo-lysosomal pathway (Rosa et al., 2015; Shi et al., 2018), reducing the incorporation of SERINC5 in HIV-1 virions. This in turn correlates with more efficient fusion of virions with target cells and greater infectivity (Rosa et al., 2015; Usami et al., 2015). Nef's ability to counteract SERINC5 is conserved across primate lentiviruses and correlates with the prevalence of these viruses in the wild (Rosa et al., 2015; Heigele et al., 2016). Modulation of SERINC5 extends to other retroviral proteins, including S2 from equine infectious anemia virus (EIAV) as well as MLV glycoGag (Rosa et al., 2015; Usami et al., 2015; Chande et al., 2016). HIV-1 Env glycoproteins are differentially sensitive to SERINC-mediated restriction when produced from CD4-negative cells in single-round replication assays; sensitivity correlates to some extent with the degree of Env-trimer openness and instability (Rosa et al., 2015; Usami et al., 2015; Beitari et al., 2017; Angerstein et al., 2020).

SERINCs comprise a family of five genes that are evolutionarily conserved from yeast to mammals (Firrito et al., 2018). They encode multi-pass transmembrane proteins that support serine specific phospholipid biosynthesis (hence their name: serine incorporator) (Inuzuka et al., 2005), yet this function does not seem to account for their anti-retroviral activity (Trautz et al., 2017). Rather, SERINC5 appears to disrupt the formation of fusion pores between HIV-1 virions and target cells (Sood et al., 2017) in an Env-conformation and CD4-dependent manner (Zhang et al., 2019).

Initial studies of the Nef-SERINC relationship focused on the Jurkat T cell line, due to the large defect in the infectivity of Nef-negative virions produced from these cells and their relatively high levels of SERINC5 mRNA. Studies using another CD4-positive T cell line, MOLT-3, have recently cast doubt on whether SERINC family proteins are sufficient to fully explain the virologic phenotypes of Nef (Wu et al., 2019). In support of a SERINC-dependent mechanism, Nef does not enhance HIV-1 infectivity and replication-rate in Jurkat T cells when SERINC3 and SERINC5 are knocked out (Rosa et al., 2015; Usami et al., 2015). Moreover, a minimal MLV glycoGag (termed glycoMA) can functionally replace Nef with respect to viral replication rate and virion-infectivity when the virus is propagated using Jurkat cells (Wu et al., 2019). In contrast, Nef, but not glycoMA, enhances HIV-1 replication in MOLT-3 cells. The Nef-effect in MOLT-3 cells persists when the cells are knocked out for SERINC3 and SERINC5, indicating that these restriction factors are not necessary for the virologic effects of Nef in this setting (Wu et al., 2019). Remarkably, glycoMA cannot substitute functionally for Nef with respect to stimulating viral replication in primary CD4-positive T cells. This suggests that the growth rate enhancing effect of Nef

in primary T cells is unrelated to SERINC-antagonism (Wu et al., 2019), even though the virion-infectivity enhancing effect of Nef reportedly is (Rosa et al., 2015).

Given this background, we aimed to further test the hypothesis that Nef enhances HIV-1 replication in a SERINC-dependent manner. To do this, we returned to the CD4-positive T cell line in which we originally observed a stimulation of growth-rate by Nef, an effect that was associated with Nef-mediated enhancement of virion-infectivity (CEM) (Chowers et al., 1994). Here, we show that neither the attenuated replication-rate of *nef*-deficient HIV-1 in CEM T cells nor the reduced virion-infectivity of *nef*-deficient HIV-1 produced by these cells is rescued by CRISPR/cas9 editing of *serinc5,* either with or without additional editing of *serinc3*. These results support those documented using MOLT-3 and suggest that how Nef stimulates HIV-1 replication and virion infectivity remains unclear (Wu et al., 2019).

## 2.    Materials and methods

### Cell Lines and Plasmids:

HEK293T (a generous gift from Dr. Ned Landau) and HeLa TZM-bl cells (Dr. John Kappes and Xiaoyun Wu,: NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) (Platt et al., 1998; Derdeyn et al., 2000) were grown in DMEM media (Thermo Fisher Scientific) supplemented with 10% FBS (Hyclone) and 1% Penicillin/Streptomycin (Thermo Fisher Scientific). HeLa P4.R5 (obtained from Dr. Ned Landau) were cultured in 10% FBS, 1%

Penicillin/Streptomycin and 1 μg puromycin. Both HeLa cell line derivatives express CD4, CXCR4 and CCR5 and contain either a Tat-inducible β-galactosidase (HeLa P4.R5) or both the β-galactosidase and luciferase (HeLa TZM-bl) genes under the transcriptional control of the HIV-1 LTR. CCRF-CEM (a generous gift from Dr. Douglas Richman) and JTAg cells expressing (JTAg WT) or lacking SERINC3 and SERINC5 (JTAg SERINC3/ SERINC5 KO; kindly provided by Dr. Heinrich Gottlinger) are T cell leukemic clones that were cultured in RPMI 1640 media plus 10% FBS and 1% Penicillin/Streptomycin (Thermo Fisher Scientific). The proviral plasmids pNL4–3 and pNL4–3 Nef have been previously described (Chowers et al., 1994). LentiCRISPRv2 (Addgene; Catalog #: 52961) contains a single guide RNA (sgRNA) targeting Exon 2 of SERINC3 (5′-ATAAATGAGGCGAGTCACCG-3′) and was a gift from Dr. Massimo Pizzato (Rosa et al., 2015). The lentiviral packaging (pRSV-Rev, pMDLg/pRRE) and envelope (pMD2.G) plasmids were kindly provided by Dr. Dan Gibbs. LentiCRISPR-GFP encodes GFP in place of puromycin (Wallace et al., 2017). Five sgRNAs targeting either Exon 1 or Exon 2 of SERINC5 were designed using an online CRISPR tool (benchling.com) and cloned into LentiCRISPR-GFP using previously described methods (Sanjana et al., 2014; Shalem et al., 2014). The sgRNA sequences were as follows: sgRNA-SERINC5(1), 5′-ACAGCACTGAGCTGACATCG-3'; sgRNA-SERINC5(2), 5′GCACTGAGCTGACATCGC GG-3'; sgRNA-SERINC5(3), 5'-

CTTCGTTCAAGTGTGAGCTG′3'; sgRNA-SERINC5(4), 5'-CATCATGATGTCAACAACCG-3'; sgRNA-SERINC5(5), 5'-

TGAGGGACTGCCGAATCCTG-3'. Briefly, sgRNA oligos were designed to produce the same overhangs after *BsmBI* digestion (5′-*CACCG*(sgRNA Oligo #1)-3'; 3′-*C*(sgRNA Oligo #2)-*CAAA*-3′). The oligos were phosphorylated (T4 Polynucleotide Kinase; NEB) and annealed in a thermal cycler according to the following conditions: 37 °C for 30 min; 95 °C for 5 min with a ramp down to 25 °C at 5 °C/min. Diluted oligos (1:200) were ligated (T4 ligase; NEB) into dephosphorylated (Fast AP; Fermentas) and *BsmBI* digested (Fast *BsmBI*; Fermentas) LentiCRISPR-GFP by overnight incubation at 16 °C, followed by transformation into Stbl3 bacteria (Thermo Fisher Scientific). Plasmid DNA was isolated from overnight bacterial cultures and verified via Sanger sequencing. *Generation of stable cell lines using CRISPR-Cas9:* To produce 3$^{rd}$ generation lentiviral stocks, HEK293T cells were transfected with a total of 22.5 μg total plasmid according to the following equimolar ratios: 10 μg LentiCRISPR transfer plasmid (empty or containing sgRNAs against either SERINC3 or SERINC5), 5.9 μg pMDLg/pRRE, 2.8 μg pRSV-Rev and 3.8 μg pMD2.G. Forty-eight hours later, concentrated lentivirus-containing supernatant was harvested following low-speed centrifugation, filtration (0.45 μm), and mixture with Lenti-X concentrator (Takara Bio) according to the manufacturer's instructions. Briefly, 1 vol Lenti-X concentrator was mixed with 3 vol clarified supernatant. The mixture was incubated for 30 min at 4 °C, centrifuged at 1500×$g$ for 45 min at 4 °C, resuspended in 1 ml complete DMEM media and immediately stored at −80 °C in single-use aliquots (100 μL).

We used previously validated sgRNAs to edit SERINC3 (Rosa et al., 2015), whereas each of the five sgRNAs targeting SERINC5 were screened and the sgRNA which caused the most efficient editing in bulk transduced cells was chosen (data not shown). To create CEM cells knocked out for SERINC3 (S3-KO), we spinoculated $1 \times 10^6$ cells with 100 μL lentivirus (LentiCRISPRv2-SERINC3; (Rosa et al., 2015)) at 1200×$g$ for 2 h at 25 °C. Puromycin (1 μg/ml) was added to cell cultures 72 h post transduction to select for positive clones. Two weeks post-selection, genomic DNA was isolated from mock or lentiCRISPRv2-SERINC3 transduced CEM cells using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Genome editing was assessed by Tracking of Indels by Decomposition (TIDE; (Brinkman et al., 2014)). PCR amplicons encompassing exon 2 of SERINC3 were produced using Taq 2x Master Mix (NEB) and the following primers: 5′-CAAATTACAACCAACTTGATTAACAACGACG-3′ and 5′-CTATAAAGCCTGATTTGCCTCGCTTTCTCTTC-3'. Clonal cell lines were isolated from bulk edited cultures using single-cell dilutions in a 96-well plate, followed by genomic DNA isolation and PCR amplification. Genome editing was initially verified via TIDE analysis. PCR amplicons were then cloned into the pCR2.1-TOPO-TA vector following the manufacturer's instructions (Invitrogen). Ten colonies were selected, miniprepped, and sent for Sanger Sequencing with a universal T7 primer probe to validate editing.

To generate either single knockout or double-knockout cells lacking SERINC5, CEM WT or CEM S3-KO cells were spinoculated as described above with either empty lentivirus (LentiCRISPR-GFP) or virus containing sgRNA-SERINC5(4). Isolation of genomic DNA, PCR amplification and TIDE analysis were carried out 72 h post transduction in a similar manner to the generation of S3-KO cells. We then expanded cells, which yielded two single SERINC5 knockout (S5-KO) clonal lines, and one SERINC3/SERINC5 knockout clonal cell line. We confirmed editing of each line via clonal sequencing as described

above. We named these lines: SERINC5 knockout clone 8 (S5-KO (8)), SERINC5 knockout clone 11 (S5-KO (11)) and SERINC3/SERINC5 knockout clone 9 (S3/S5-KO (9)). The following primers were used to generate PCR amplicons for TIDE analysis: 5′-AGTGCCTGGCCATGTTTCTT-3′ and 5′-CATAGAGCAGGCTTCAGGAA-3'.

### HIV-1 production and titer:

To produce replication-competent viruses, HEK293T cells ($4 \times 10^6$/10 cm plate) were transfected with 24 μg of an infectious molecular clone of HIV-1 (NL4–3) or a mutant lacking the *nef* gene (NL4–3 Nef) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Thermo Fisher Scientific). Virus-containing supernatants were collected 48 h post transfection, clarified by low-speed centrifugation, and stored at −80 °C. Viral titers were measured by infecting HeLa P4.R5 cells with diluted viral stocks in duplicate in a 48-well format for 48 h. The cells were then fixed (1% formaldehyde; 0.2% glutaraldehyde) for 5 min at room-temperature, followed by overnight staining at 37 °C with a solution composed of 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$ and 0.4 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Infected cells (expressed as infectious centers (IC)) were quantified using a computer image-based method (Day et al., 2006).

### HIV-1 infection and replication:

To conduct HIV-1 replication studies, $1 \times 10^6$ CEM cells (wildtype (WT); SERINC5 knockout clone 8 (S5KO (8))-; SERINC5 knockout clone 11 (S5KO (11); SERINC3/SERINC5 knockout clone 9 (S3/S5 KO(9) were infected with NL4–3 (hereafter termed Nef+) or NL4–3 Nef (hereafter termed Nef-) at a multiplicity of infection (MOI) of 0.01 overnight at 37 °C in 1.0 ml volume in wells of a 24-well plate. The cells were then washed 3 times with 1 ml PBS (Corning), resuspended in 4 ml complete growth media (RPMI 1640), transferred to T25 labeled flasks and incubated at 37 °C in an "upright" position for the duration of the experiment. Every three days, cultures were split 1:4 (1 ml cells; 3 ml media) and an aliquot (1 ml viral supernatant) stored at −80 °C for quantification of HIV-1 replication (p24 antigen) by ELISA.

### Measurement of HIV-1 infectivity:

Viral infectivity was quantified from virions produced in CEM cells infected with NL4–3 or NL4–3 Nef at day 12 post-infection. A 20% sucrose cushion was used to concentrate and partially purify the virions via centrifugation at $23,500 \times g$ for 1 h at 4 °C. Viral pellets were resuspended in culture medium and dilutions used to infect $1.25 \times 10^4$ HeLa TZM-bl cells in triplicate in a 96-well format. Forty-eight hours later, the culture medium was removed, and the cells were lysed using a luciferase reporter gene assay reagent (Britelite, Perkin Elmer). Infectivity (luciferase activity) was measured using a luminometer with data expressed as relative light units (RLU). These values were normalized to the p24 concentration of each sample and shown as RLU/p24.

**RT-qPCR and analysis:**

Total cellular RNA was extracted from $1 \times 10^6$ JTAg WT, JTAg S3/S5 KO, CEM WT, CEM S5KO (8), CEM S5KO (11), and CEM S3/S5 KO (9) cells using a Quick-RNA miniprep kit (Zymo Research), followed by treatment with RNase-free DNAse I (Zymo Research). Complementary DNA (cDNA) was generated from 250 ng of all extracted RNA samples using M-MLV RT (Thermo Fisher Scientific) and treated with RNaseOUT (Thermo Fisher Scientific). cDNA was mixed with the respective primer pairs and SyGreen Blue Mix (PCR Biosystems) following the manufacturer's protocol in biological triplicate and performed using a LightCycler 96 real-time PCR machine (Roche). Quantification cycle values were normalized to a reference gene (*GAPDH*) and relative *SERINC5* or *SERINC3* gene expression ratios were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The following primers were used for analysis: *SERINC5:* 5′-ATCGAGT TCTGACGCTCTGC-3′ and 5′-GCTCTTCAGTGTCCTCTCCAC-3′; *SERINC3* 5′-AATTCAGGAACACCAGCCTC-3′ and 5′- GGTTGGGATTGCAGGAAC GA-3'; *GAPDH* 5′-TGCACCACCAACTGCTTAGC-3′ and 5′-GGCATGGACT GTGGTCATGAG-3'.

**Data analysis and presentation:**

Datasets were analyzed and combined in Microsoft Excel and GraphPad Prism 8.0 software. Where indicated, two-tailed unpaired t-tests were performed. We utilized Adobe Photoshop, Illustrator CS6, and BioRender for figure production.

## 3. Results

### 3.1. Generation of CEM cells lacking SERINC3 and SERINC5

We utilized CRISPR/Cas9 gene editing to determine whether the modulation of SERINC3 and SERINC5 is required for Nef to enhance HIV-1 replication in CEM cells. We chose the CEM cell line due to its ability to support Nef-dependent HIV-1 replication and virion-infectivity (Chowers et al., 1994). We hypothesized that Nef would remain an important factor in promoting viral spread whether or not CEM cells expressed SERINC3 and SERINC5, a result in conflict with data reported using Jurkat T cells but consistent with data reported using MOLT-3 T cells. To test this, we created clonal cell lines lacking either SERINC5 or both SERINC3 and SERINC5 (described in Methods). We identified three clones containing indels within the SERINC5 target region: "clone 8" (S5-KO (8)), "clone 11", (S5-KO (11)) and "clone 9" (S3/S5KO (9)) (Fig. 1A Left panel). Clonal analysis revealed S5-KO (8) contained 4 and 13 bp deletions, S5-KO (9)) displayed 10 and 13 bp deletions, and S5-KO (11) contained 2, 11 and 13bp deletions (Fig. 1A Right Panel). Both S5-KO (8) and S5-KO (11) also contained sequences displaying large deletions upstream of the gRNA cut site, which may reflect why we observed mixed chromatogram signals well upstream of the cut site in our bulk sequences. Nonetheless, all sequences yielded out-of-frame mutations. SERINC3 knockout cells consisted of indels bearing a single base pair insertion or a single or double base pair deletion (S3/S5 KO (9); Fig. 1C).

We attempted to validate editing in these cell lines using a monoclonal antibody targeting the extracellular domain of SERINC5 (Molnar et al., 2020). However, this antibody did

not detect endogenous SERINC5 in either Jurkat or CEM cells (data not shown). Instead, we reasoned that CRISPR/Cas9 editing of *serinc3* and *serinc5* would lead to a quantifiable decrease in mRNA transcripts due to nonsense mediated decay (NMD; (Popp and Maquat, 2016)). To test this, we isolated RNA from CEM WT, CEM S5-KO (8), CEM S5-KO (11), CEM S3/S5-KO (9), and, as controls, JTAg WT and JTAg S3/S5-KO cells. In both JTAg and CEM, knockout clones expressed less *serinc5* (Fig. 1B) or *serinc3* (Fig. 1D) compared to wildtype clones. Given these results, these CEM cell lines served as the basis for our viral replication and infectivity studies.

## 3.2    Optimal HIV-1 spread and viral infectivity in CEM cells is dependent on Nef but independent of SERINC5.

To test whether Nef is required to counteract SERINC5 to enhance HIV-1 replication, we infected CEM wildtype (WT), S5-KO (8) and S5-KO (11) cells with either Nef expressing (hereafter termed Nef+: NL4–3) or Nef lacking (hereafter termed Nef-: NL4–3 Nef) HIV-1 viruses at a multiplicity of infection (MOI) equal to 0.01 infectious units per cell. The inocula for these growth-rate experiments were produced from HEK293T cells transfected with proviral plasmids, and the MOI was based on the infectivity of the virus stocks measured as infectious centers in cultures of CD4-positive HeLa-P4.R5 indicator cells (Day et al., 2006). To infect $1 \times 10^6$ CEM cells, we used amounts of Nef + or Nef-virus stocks that yielded 10,000 infectious centers in the HeLa indicator assay. Although the infectivity of the viruses to CEM cells might be different than to CD4-HeLa cells, we chose this approach rather than normalizing the inocula to the content of p24 capsid antigen to adjust for the reduced infectivity of Nef-virions produced by the HEK293T cells (data not shown). The CEM cell cultures were split every 3 days and viral replication quantified by the amount of viral capsid (p24) within the supernatant (Fig. 2A) measured by ELISA. The Nef + viruses propagated more rapidly than the Nef-viruses, accumulating more p24 antigen in the culture supernates at 12 days post-infection in CEM WT cells (Fig. 2B: Left panel). This corroborated the importance of Nef in enhancing viral replication in this *in vitro* system. If Nef-mediated modulation of SERINC5 were important for this phenotype, then the attenuated replication of Nef-virus should have been "rescued" in cells lacking SERINC5. Instead, Nef enhanced HIV-1 replication in the CEM S5KO (8) and CEM S5KO (11) cell lines (Fig. 2B: middle and right panels).

We next asked whether counteraction of SERINCs is necessary for Nef to enhance the infectiousness of virions produced by CEM cells, since Nef enhances virion-infectivity in a SERINC- and cell-type dependent manner (Rosa et al., 2015). To test this, we collected virions from infected CEM cells at day 12 post-infection and measured single-cycle infectivity using HeLa TZM-bl reporter cells, which express luciferase under the transcriptional control of the HIV-1 LTR (Fig. 2C). We used these cells because the luciferase read-out is more sensitive than the infectious center read-out of the HeLa-P4.R5 cells. Virions produced at day 12 post infection by Nef + virus were around 8-fold more infectious per amount of p24 capsid antigen than those produced by Nef-virus, and this trend was observed regardless of SERINC5 expression (Fig. 2D). Taken together, these data indicate that counteraction of SERINC5 is not the primary mechanism by which Nef increases viral spread or infectivity in CEM cells.

### 3.3   Nef enhances HIV-1 spread and virion-infectivity independently of SERINC3 in CEM cells.

Whereas CEM cells express slightly less *serinc5* RNA than Jurkats, they express markedly more *serinc3* RNA (Fig. 1, B and D). Thus, we sought to determine whether Nef-mediated modulation of SERINC3 influences viral growth rate and infectivity in these cells and might explain the *nef*-phenotype. We infected CEM WT and CEM cells lacking both SERINC3 and SERINC5 (S3/SKO (9)) with Nef+ and Nef – viruses and measured viral replication and infectivity in a similar manner as shown in Fig. 2A and C. The absence of SERINC3 in CEM cells did not "rescue" Nef-virus in terms of either viral replication rate (Fig. 3A, compare left and right panels) or virion infectivity (Fig. 3B). Altogether, this data indicates that Nef increases virion infectivity as well as growth-rate in CEM T cells independently of SERINC3 and SERINC5.

## 4.   Discussion

In this study, we sought to determine whether Nef's ability to enhance viral replication and/or infectivity in CEM T cells is primarily linked to modulation of SERINC5, either with or without SERINC3. We present evidence that argues against this by showing that Nef increases viral replication rate in CEM cells lacking SERINC5 and that virions of Nef + virus are more infectious than those of Nef-virus regardless of whether SERINC3 and SERINC5 are expressed in the CEM cells that produced them.

Our finding that Nef enhances virion-infectivity independently of SERINCs in CEM T cells contrasts with reports in Jurkat and primary CD4-positive cells, where Nef mediated enhancement of infectivity appears to correlate with SERINC5 expression (Rosa et al., 2015; Usami et al., 2015). On the other hand, our observations herein using CEM cells are similar to recent results reported using MOLT-3 cells (Wu et al., 2019). In MOLT-3 cells, a chimeric HIV-1 virus bearing the SERINC5 antagonist (glycoMA) failed to substitute for Nef in rescuing HIV-1 infectivity (a property glycoMA should have if the sole function of Nef were to counteract SERINC5), and knockout of SERINC5 did not rescue the reduced growth rate of Nef-virus (Wu et al., 2019). Together, these data suggest that the SERINC-dependence of the Nef infectivity phenotype is cell-type dependent (Rosa et al., 2015; Usami et al., 2015; Wu et al., 2019), with Jurkat being SERINC-dependent and CEM and MOLT-3 cells being SERINC-independent.

What mechanisms might explain Nef-mediated enhancement of infectivity and/or viral replication independently of SERINCs? One potential explanation could be Nef-mediated modulation of Src family kinases (SFKs). Nef binds several SFK members such as Lck, Hck, Lyn, and c-Src through a conserved proline-rich (PxxP) motif contained within Nef's Src homology region 3 (SH3) binding domain (Trible et al., 2006; Saksela et al., 1995), and primary Nef isolates from HIV-1 Group M display a conserved ability to activate SFK's (Narute and Smithgall, 2012). Nef mutants lacking the PxxP motif were reportedly unable to enhance HIV-1 replication in peripheral blood mononuclear cells (Saksela et al., 1995). However, other studies reported that mutation of Nef's PxxP motif yielded little to no difference in HIV-1 replication within MOLT-3 or primary CD4-positive T cells, and in CEM T cells the attenuated phenotype of mutants of the SH3 binding domain was modest

and seemed attributable to reduced expression of Nef (Lundquist et al., 2002; Wu et al., 2019; Craig et al., 1999). Analyzing HIV-1 replication in T cells lacking one or more SFKs may be necessary to adequately assess whether Nef's ability to enhance HIV-1 replication is mediated by these interactions.

One notable similarity between MOLT-3 and CEM lymphoblastoid cells that distinguishes them from Jurkat cells is that they do not express the T cell receptor (TCR) at their surfaces (Greenberg et al., 1988; Royer et al., 1985). Given that Nef reportedly mimics TCR-signaling (Simmons et al., 2001; Len et al., 2017), another possibility is that MOLT-3 and CEM cells reveal a SERINC-independent growth-rate Nef phenotype that is exaggerated by the absence of constitutive TCR signaling in these cells. This could be consistent with the initial observations that the activation-state of primary CD4-positive T cells affects the Nef growth-rate phenotype (Spina et al., 1994). Nonetheless, how TCR signaling would affect virion-infectivity is obscure.

Lastly, the explanation might reside in Nef's ability to interact with components of clathrin-mediated trafficking pathways and to modulate many cellular membrane proteins, one of which might be currently unidentified but underlie the infectivity phenotype in MOLT-3 and CEM cells. Nef binds AP complexes via a conserved sorting signal near its C-terminus: 160ExxxLL164,165 (Sugden et al., 2016). This "di-leucine motif" is required for both Nef-mediated CD4 downregulation and optimal viral infectivity in CEM cells (Craig et al., 1998). The Nef LL164/165AA mutant, which is unable to bind AP-2 (Bresnahan et al., 1998), replicates poorly in MOLT-3 cells (Wu et al., 2019). Residues located within Nef's core domain and required for downregulating CD4 and interacting with Dynamin-2, a "pinchase" of clathrin coated pits, are also required for enhancement of viral replication in MOLT-3 cells (Wu et al., 2019; Cohen et al., 2000; Poe and Smithgall, 2009). Finally, Nef enhances HIV-1 replication in the absence of CD4 downregulation in MOLT-3 cells (Wu et al., 2019) and in CEM-derived cells (A2.01) engineered to express a CD4 lacking a cytoplasmic domain that is unresponsive to Nef (Chowers et al., 1995). These observations regarding CD4 are critically important, since CD4 is itself a potent inhibitor of infectivity that is counteracted by Nef (Ross et al., 1999; Lama et al., 1999). In the CEM experiments herein, CD4 downregulation by Nef could conceivably account for the observed infectivity and growth-rate phenotypes. Nonetheless, even if that were the case, the data would indicate that the contribution of CD4 to these phenotypes far outweighs the contribution of SERINC3 and SERINC5 in these cells and when using CXCR4-tropic viruses such as NL4–3, whose Env proteins are CD4-sensitive (Lundquist et al., 2004).

Our study has several caveats. As noted above, CEM cells, like MOLT3 cells, might not reflect the role of Nef in primary T cells or macrophages. Also, quantitative experimental variation is evident in the CEM system, with the *nef* virion-infectivity phenotype varying between 2- and 8-fold (compare Figs. 2 and 3). Nonetheless, a *nef*-phenotype was always apparent in these cells, and it was unaffected by knock-out of *serinc5* and *serinc3*.

Overall, whether Nef provides a direct, positive effect on viral replication or instead is counteracting a still unidentified restriction factor remains to be determined. Utilizing both

MOLT-3 and CEM cells might facilitate answering this question, providing a more complete understanding of the enigmatic yet important virologic effects of Nef.

## Acknowledgments

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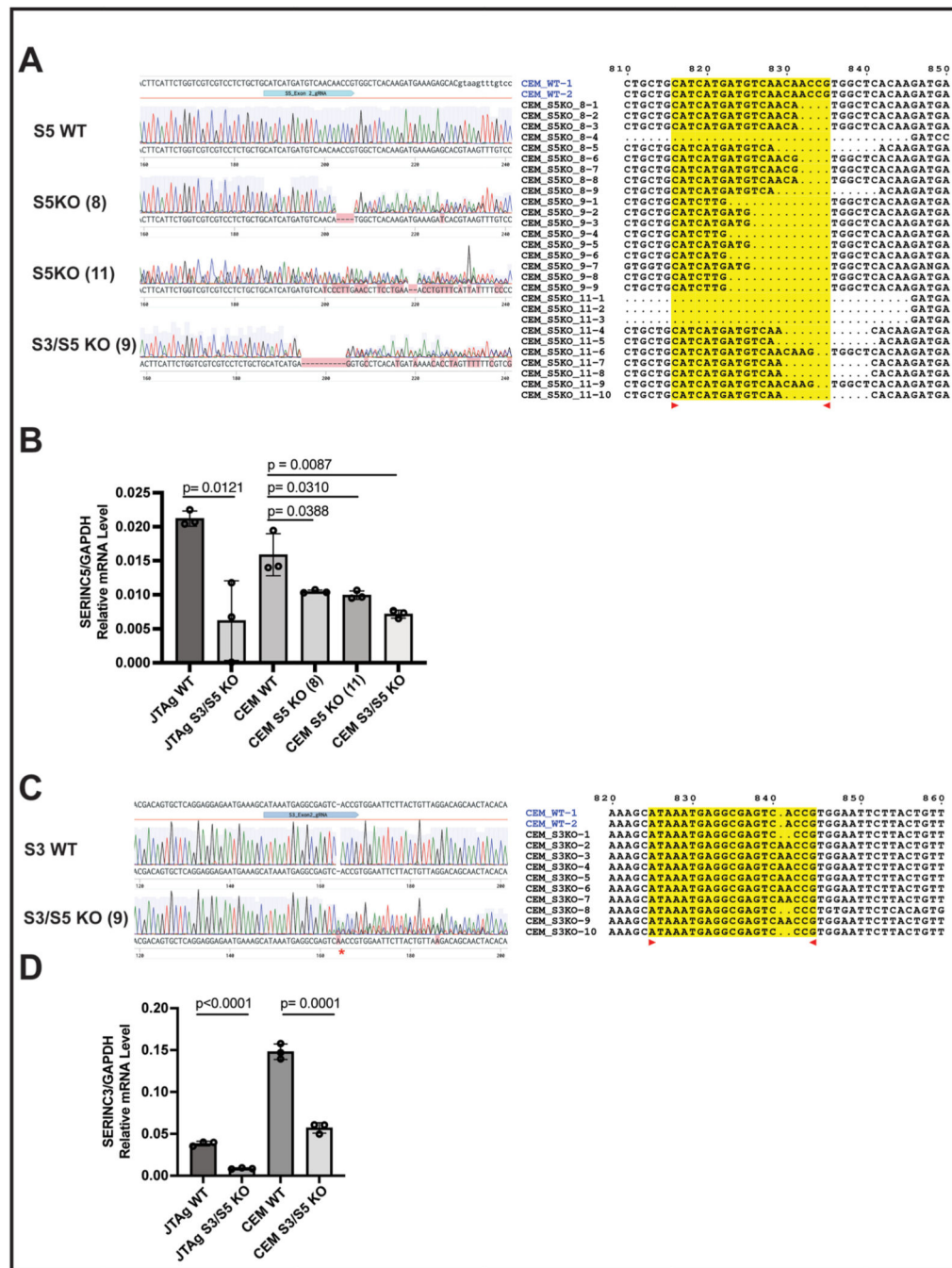
**Fig. 1. CRISPR/Cas9 editing of SERINC3 and SERINC5 in CEM cells.**
A.) *Left:* Chromatograms depicting a portion of exon 2 from SERINC5 wildtype (WT) or
three SERINC5 knockout clones: clone 8 (S5KO (8)), clone 11 (S5KO (11)) or clone 9
(S3/S5 KO (9)). The guide RNA (gRNA) target site is shown as a blue arrow/annotation.
Inserted nucleotides are highlighted red. Deletions are depicted as dashed line(s). *Right:*
Clonal analysis of SERINC5 knockout lines. The gRNA target site is highlighted in yellow.
S5KO (8) clonal sequences (CEM_S5KO_8–1 through 8–9) contained 4 and 13bp deletions,
S5KO (11) clonal sequences (CEM_S5KO_11–1 through 11–10) contained 2,11 and 13 bp

deletions and S3/S5 KO (9) clonal sequences (CEM_S5KO_9–1 through 9–9) contained 10 and 13bp deletions. B.) Quantitative PCR (qPCR) showing the ratio of SERINC5 to GAPDH mRNA in JTAg and CEM cells expressing or lacking SERINC5. Results depict the mean values and standard deviations and are representative of two independent experiments performed in triplicate. C.) *Left:* Chromatograms depict a portion of exon 2 from SERINC3 wildtype (WT) or SERINC3/SERINC5 knockout clone 9 (S3/S5 KO (9)). The guide RNA (gRNA) target site is shown as a blue arrow/annotation. Inserted nucleotides are highlighted red. *Right:* Clonal analysis of SERINC3 knockout line. The gRNA target site is highlighted in yellow. S3/S5KO (9) clonal sequences (CEM_S3KO-1 through 10) contained a 1bp insertion or 1 or 2 bp deletions. D.) Quantitative PCR (qPCR) showing the ratio of SERINC3 to GAPDH mRNA in JTAg and CEM cells expressing or lacking SERINC3. Results depict the mean values and standard deviations and are representative of two independent experiments performed in triplicate. Two-tailed unpaired t-tests were performed where indicated.
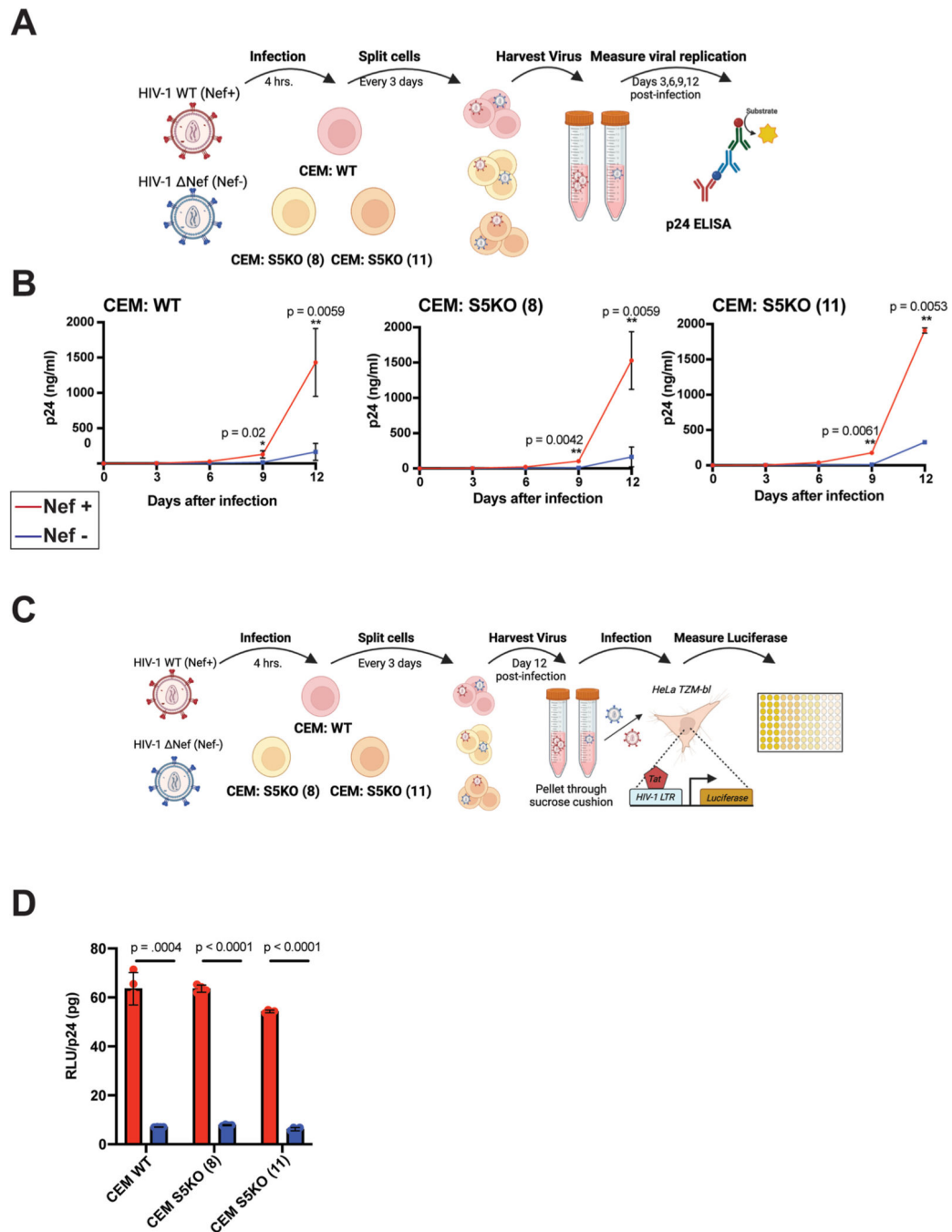
**Fig. 2.**

Nef enhances HIV-1 replication independently of SERINC5 in *CEM cells.* A.) Schematic of viral replication studies performed in CEM wildtype (WT), SERINC5 knockout clone 8 (S5KO (8)) or SERINC5 knockout clone 11 (S5KO (11)) cells. Each cell line was infected with either NL4–3 (termed Nef+) or HIV-1 Nef (termed Nef-) at an MOI of 0.01. The cultures were split every 3–4 days and viral growth measured as indicated. Created with BioRender.com. B.) Viral replication quantified by p24 Capsid ELISA in the supernatants of CEM WT, S5KO (8) and S5KO (11) cultures. Results depict the averages

and standard deviations from either two (WT, S5KO (8)) or one (S5KO (11)) independent infection measured in duplicate at each time point. C.) Schematic depicting measurement of single-cycle infectivity with virions produced from infected CEM WT, S5KO (8) or S5KO (11) cultures. HeLa TZM-bl indicator cells contain a luciferase gene under the transcriptional control of the HIV-1 LTR. Created with BioRender.com. D.) Infectivity data (relative luciferase units (RLU) normalized to p24 (RLU/p24)) from virions collected at day 12 post-infection from cultures of either CEM WT or S5KO (8) and S5KO (11) cells. The virions were partially purified by centrifugation through a 20% sucrose cushion before measuring infectivity (RLU in the HeLa TZM-bl assay) and p24 concentration (ELISA). Results depict the mean values and standard deviations from one experiment performed in quadruplicate. Two-tailed paired t-tests were performed where indicated.
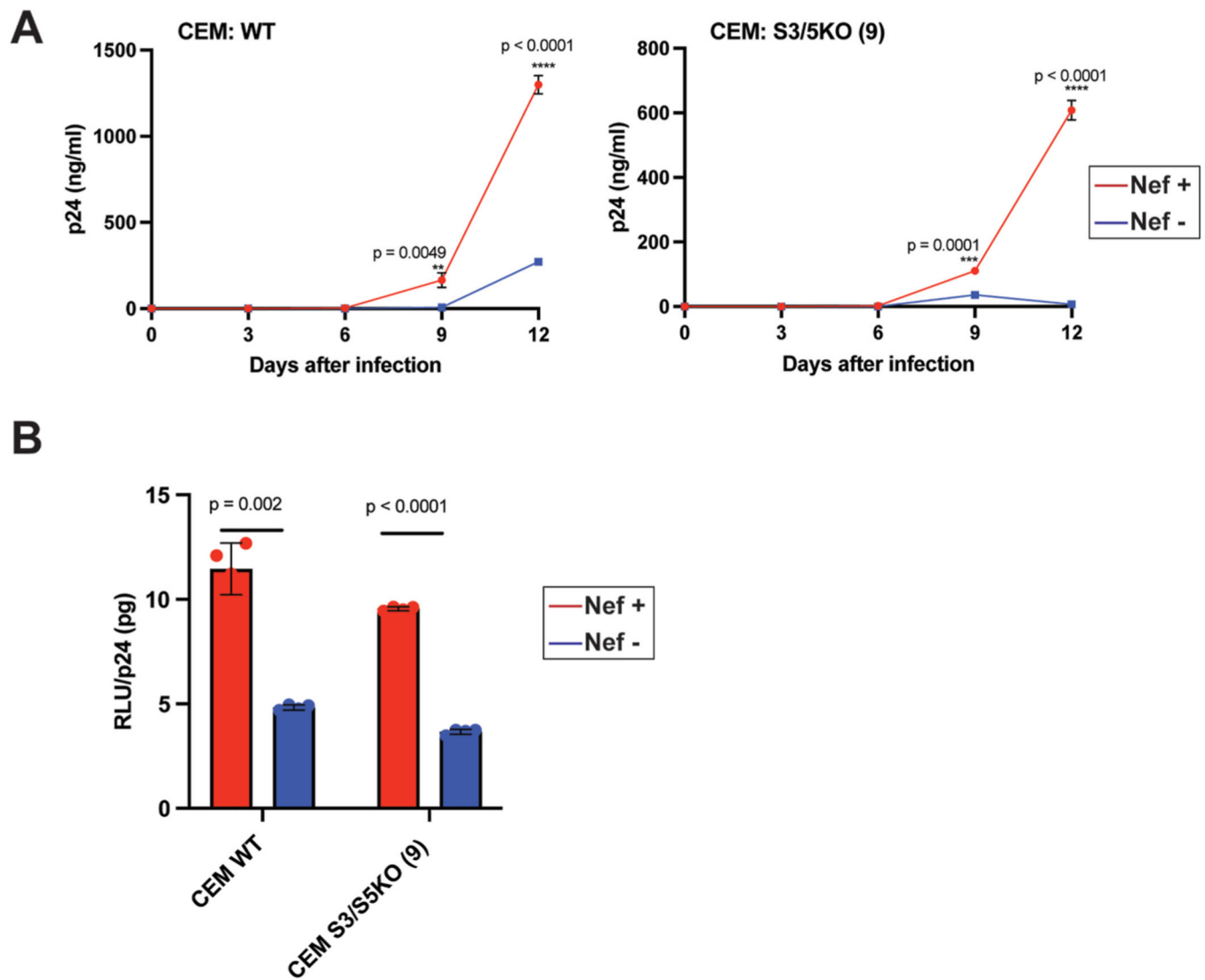
**Fig. 3.**
Nef enhances HIV-1 replication independently of SERINC3 in *CEM cells.* A.) Viral replication quantified by p24 Capsid ELISA in the supernatants of CEM WT or S3/S5 KO (9) cultures. Results depict the average and standard deviation of one independent infection measured in quadruplicate at each time point. B.) Infectivity data (relative luciferase units (RLU) normalized to p24 (RLU/p24)) from virions collected at Day 12 post-infection from cultures of either CEM WT or S3/S5KO (9) cells. Results depict the mean values and standard deviations from one experiment performed in quadruplicate. Two-tailed paired t-tests were performed where indicated.