Nef enhances HIV-1 replication and infectivity independently of SERINC3 and SERINC5 in CEM T cells

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Running Head: Attenuation of HIV-1Δnef without *serinc3* and *serinc5*

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Abstract

The lentiviral *nef* gene encodes several discrete activities aimed at co-opting or antagonizing cellular proteins and pathways to defeat host defenses and maintain persistent infection. Primary functions of Nef include downregulation of CD4 and MHC class-I from the cell surface, disruption or mimicry of T-cell receptor signaling, and enhancement of viral infectivity by counteraction of the host antiretroviral proteins SERINC3 and SERINC5. In the absence of Nef, SERINC3 and SERINC5 incorporate into virions and inhibit viral fusion with target cells, decreasing infectivity. However, whether Nef's counteraction of SERINC3 and SERINC5 is the cause of its positive influence on viral growth-rate in CD4-positive T cells is unclear. Here, we utilized CRISPR/Cas9 to knockout SERINC3 and SERINC5 in a leukemic CD4-positive T cell line (CEM) that displays robust *nef*-related infectivity and growth-rate phenotypes. As previously reported, viral replication was severely attenuated in CEM cells infected with HIV-1 lacking Nef (HIV-1ΔNef). This attenuated growth-rate phenotype was observed regardless of whether or not the coding regions of the *serinc3* and *serinc5* genes were intact. Moreover, knockout of *serinc3* and *serinc5* failed to restore the infectivity of HIV-1ΔNef virions produced from infected CEM cells in single-cycle replication experiments using CD4-positive HeLa cells as targets. Taken together, our results corroborate a recent study using another T-lymphoid cell line (MOLT-3) and suggest that Nef modulates a still unidentified host protein(s) to enhance viral growth rate and infectivity in CD4-positive T cells.

50 **Importance**

51

52 HIV-1 Nef is a major pathogenicity factor *in vivo*. A well-described activity of Nef is the
53 enhancement of virion-infectivity and viral propagation *in vitro.* The infectivity-effect has
54 been attributed to Nef's ability to prevent the cellular, antiretroviral proteins SERINC3 and
55 SERINC5 from incorporating into viral particles. While the activity of the SERINCs as
56 inhibitors of retroviral infectivity has been well-documented, the role these proteins play
57 in controlling HIV-1 replication is less clear. We report here that genetic disruption of
58 SERINC3 and SERINC5 rescues neither viral replication-rate nor the infectivity of cell-
59 free virions produced from CD4-positive T cells of the CEM lymphoblastoid line infected
60 with viruses lacking Nef. This indicates that failure to modulate SERINC3 and SERINC5
61 is not the cause of the virologic attenuation of *nef*-negative HIV-1 observed using this
62 system.

63

64

65 **Introduction**

66

67 Primate lentiviruses encode several accessory gene products that facilitate viral
68 reproduction and persistence by providing evasion of the host immune response. The
69 lentiviral Nef protein accelerates viral pathogenesis and progression to AIDS in SIV-
70 infected rhesus macaques (1) and enhances disease progression in humans infected with
71 HIV-1 (2, 3). Nef is a small, myristoylated, peripheral membrane protein with many well-
72 conserved activities, including the downregulation of cell surface proteins such as CD4
73 and MHC class-I (MHC-I), the modulation of T cell activation, and the enhancement of
74 viral infectivity and growth-rate (4).

75

76 To downregulate CD4, Nef binds the cytoplasmic domain of CD4 and links it to the clathrin
77 Adaptor Protein 2 (AP-2) complex, internalizing CD4 from the plasma membrane and
78 delivering it to lysosomes for degradation (5–8). The targeting of CD4 by Nef contributes
79 to viral replication in several ways. CD4 downregulation prevents superinfection of cells
80 and consequent premature cell-death, ensuring adequate time for viral replication (9). It
81 also contributes to inhibiting antibody dependent cellular cytotoxicity (ADCC) that
82 recognizes CD4-induced epitopes with Env (10). Potentially directly relevant to the work
83 presented here, CD4 incorporates into virions and inhibits virion-infectivity if not
84 downregulated by Nef (11, 12).

85

86 To downregulate MHC-I, Nef has been proposed to use two non-mutually exclusive
87 mechanisms: 1) Nef and the clathrin-adaptor AP-1 intercept *de novo* synthesized MHC-I

88 molecules within the *trans*-Golgi network (TGN), leading to lysosomal degradation (13–
89 16); and/or 2) Nef retains internalized MHC-I molecules in the TGN via induction of a Src-
90 family kinase/phosphoinositide-3-kinase signaling cascade (17–19). In either case,
91 reducing the expression of MHC-I molecules at the plasma membrane protects HIV-1
92 infected cells from lysis by cytotoxic T-lymphocytes, contributing to immune evasion (20).
93
94 Nef interacts directly with Src-family kinases including the lymphocyte specific kinase Lck,
95 which it downregulates from the cell surface (21). The many studies of Nef's influence on
96 T cell activation are difficult to reconcile, but transcriptional profiling suggests that the
97 expression of Nef mimics signaling through the T cell receptor (22). With the exception of
98 the model of MHC-I downregulation noted above, a clear mechanistic link between the
99 influence of HIV-1 Nef on T cell activation and its influence on the expression of cell
100 surface receptors is lacking.
101
102 Nef stimulates HIV-1 replication and enhances virion-infectivity in many cell culture
103 systems, including various T cell lines, primary CD4+ T cells, and human lymphoid tissue
104 (23–26). Nef alleles from various primate lentiviruses and isolated at different stages of
105 HIV-1 infection in humans maintain the ability to enhance HIV-1 replication and infectivity,
106 suggesting that these functions are important for establishing and maintaining persistent
107 infection (26–28). Expression of Nef within virion-producer cells and encoded either in *cis*
108 or in *trans* relative to the viral genome enhances HIV-1 replication and yields virions of
109 greater infectivity (29, 30). The ability of Nef to enhance infectivity requires cellular
110 components involved in vesicular trafficking (dynamin 2, AP-2, and clathrin) and is also

111 determined by the Envelope (Env) glycoprotein (31, 32). A survey of cell lines identified
112 murine leukemia virus glycosylated Gag (MLV glycoGag), a protein structurally unrelated
113 to Nef, as an infectivity factor that rescues Nef-deficient HIV-1 virions (33). These and
114 other features suggested that Nef counteracts a cellular factor that restricts viral infectivity
115 and possibly replication.
116
117 Two groups identified the host transmembrane protein SERINC5, and to a lesser degree
118 SERINC3, as an inhibitor of HIV-1 virion-infectivity that is counteracted by Nef (34, 35).
119 Nef downregulates SERINC5 from the plasma membrane via a clathrin/AP-2 and Rab5/7
120 endo-lysosomal pathway (34, 36), reducing the incorporation of SERINC5 in HIV-1
121 virions. This in turn correlates with more efficient fusion of virions with target cells and
122 greater infectivity (34, 35). Nef's ability to counteract SERINC5 is conserved across
123 primate lentiviruses and correlates with the prevalence of these viruses in the wild (34,
124 37). Modulation of SERINC5 extends to other retroviral proteins, including S2 from equine
125 infectious anemia virus (EIAV) as well as MLV glycoGag (34, 35, 38). HIV-1 Env
126 glycoproteins are differentially sensitive to SERINC-mediated restriction when produced
127 from CD4-negative cells in single-round replication assays; sensitivity correlates to some
128 extent with the degree of Env-trimer openness and instability (34, 35, 39, 40).
129
130 SERINCs comprise a family of five genes that are evolutionarily conserved from yeast to
131 mammals (41). They encode multi-pass transmembrane proteins that support serine-
132 specific phospholipid biosynthesis (hence their name: <u>ser</u>ine <u>inc</u>orporator) (42), yet this
133 function does not seem to account for their anti-retroviral activity (43). Rather, SERINC5

134 appears to disrupt the formation of fusion pores between HIV-1 virions and target cells
135 (44) in an Env-conformation and CD4-dependent manner (45).
136
137 Initial studies of the Nef-SERINC relationship focused on the Jurkat T cell line, due to the
138 large defect in the infectivity of Nef-negative virions produced from these cells and their
139 relatively high levels of SERINC5 mRNA. Studies using another CD4-positive T cell line,
140 MOLT-3, have recently cast doubt on whether SERINC family proteins are sufficient to
141 explain the virologic phenotypes of Nef (46). In support of a SERINC-dependent
142 mechanism, Nef does not enhance HIV-1 infectivity and replication-rate in Jurkat T cells
143 when SERINC3 and SERINC5 are knocked out (34, 35). Moreover, a minimal MLV
144 glycoGag (termed glycoMA) can functionally replace Nef with respect to viral replication-
145 rate and virion-infectivity when the virus is propagated using Jurkat cells (46). In contrast,
146 Nef, but not glycoMA, enhances HIV-1 replication in MOLT-3 cells. The Nef-effect in Molt-
147 3 cells persists when the cells are knocked out for SERINC3 and SERINC5, indicating
148 that these restriction factors are not necessary for the virologic effects of Nef in this setting
149 (46). Remarkably, glycoMA cannot substitute functionally for Nef with respect to
150 stimulating viral replication in primary CD4-positive T cells. This suggests that the growth-
151 rate enhancing effect of Nef in primary T cells is unrelated to SERINC-antagonism (46),
152 even though the virion-infectivity enhancing effect of Nef reportedly is (34).
153
154 Given these conflicting results, we aimed to further test the hypothesis that Nef enhances
155 HIV-1 replication in a SERINC-dependent manner. To do this, we returned to the CD4-
156 positive T cell line in which we originally observed a striking stimulation of growth-rate by

157 Nef, an effect that was associated with Nef-mediated enhancement of virion-infectivity
158 (CEM) (23). Here, we show that neither the attenuated replication-rate of *nef*-deficient
159 HIV-1 in CEM T cells nor the reduced virion-infectivity of *nef*-deficient HIV-1 produced by
160 these cells is rescued by CRISPR/cas9 editing of *serinc3* and *serinc5*. These results
161 support those recently documented using MOLT-3 and primary CD4+ T cells and suggest
162 that how Nef stimulates HIV-1 replication remains unclear (46).
163
164
165 **Materials and Methods**
166 *Cell Lines and Plasmids:* HEK293T (a generous gift from Dr. Ned Landau) and HeLa
167 TZM-bl cells (Dr. John Kappes and Xiaoyun Wu,: NIH AIDS Reagent Program, Division
168 of AIDS, NIAID, NIH) (47–51) were grown in DMEM media (Thermo Fisher Scientific)
169 supplemented with 10% FBS (Hyclone) and 1% Penicillin/Streptomycin (Thermo Fisher
170 Scientific). HeLa P4.R5 (obtained from Dr. Ned Landau) were cultured in 10% FBS, 1%
171 Penicillin/Streptomycin and 1 μg puromycin. Both HeLa cell line derivatives express CD4,
172 CXCR4 and CCR5 and contain either a Tat-inducible β-galactosidase (HeLa P4.R5) or
173 both the β-galactosidase and luciferase (HeLa TZM.bl) genes under the transcriptional
174 control of the HIV-1 LTR. CEM cells, a leukemic T cell clone (a generous gift from Dr.
175 Douglas Richman), were cultured in RPMI 1640 media plus 10% FBS and 1%
176 Penicillin/Streptomycin (Thermo Fisher Scientific). The proviral plasmids pNL4-3 and
177 pNL4-3ΔNef have been previously described (23). LentiCRISPRv2 (Addgene; Catalog #:
178 52961) contains a single guide RNA (sgRNA) targeting Exon 2 of SERINC3 (5'-
179 ATAAATGAGGCGAGTCACCG-3') and was a gift from Dr. Massimo Pizzato (34). The

180 lentiviral packaging (pxRSV-Rev, pMDLg/pRRE) and envelope (pMD2.G) plasmids were
181 kindly provided by Dr. Dan Gibbs. LentiCRISPR-GFP encodes GFP in place of
182 puromycin. Five sgRNAs targeting either Exon 1 or Exon 2 of SERINC5 were designed
183 using an online CRISPR tool (benchling.com) and cloned into LentiCRISPR-GFP using
184 previously described methods (52, 53). The sgRNA sequences were as follows: sgRNA-
185 SERINC5(1), 5'- ACA GCACTGAGCTGACATCG-3' ; sgRNA-SERINC5(2), 5'-
186 GCACTGAGCTGACATCGC GG-3' ; sgRNA-SERINC5(3), 5'-
187 CTTCGTTCAAGTGTGAGCTG'3' ; sgRNA-SERINC5(4), 5'-
188 CATCATGATGTCAACAACCG-3' ; sgRNA-SERINC5(5), 5'-
189 TGAGGGACTGCCGAATCCTG-3'. Briefly, sgRNA oligos were designed to produce the
190 same overhangs after *BsmBI* digestion (5'- *CACCG*(sgRNA Oligo #1)-3' ; 3'-*C*(sgRNA
191 Oligo #2)-*CAAA*-3'). The oligos were phosphorylated (T4 Polynucleotide Kinase ; NEB)
192 and annealed in a thermal cycler according to the following conditions: 37°C for 30
193 minutes; 95°C for 5 minutes with a ramp down to 25°C at 5°C/minute. Diluted oligos
194 (1:200) were ligated (T4 ligase; NEB) into dephosphorylated (Fast AP; Fermentas) and
195 *BsmBI* digested (Fast *BsmBI*; Fermentas) LentiCRISPR-GFP by overnight incubation at
196 16°C, followed by transformation into Stbl3 bacteria (Thermo Fisher Scientific). Plasmid
197 DNA was isolated from overnight bacterial cultures and verified via Sanger sequencing.
198 *Generation of stable cell lines using CRISPR-Cas9:* To produce 3rd generation lentiviral
199 stocks, HEK293T cells were transfected with a total of 22.5 μg total plasmid according to
200 the following equimolar ratios: 10 μg LentiCRISPR transfer plasmid (empty or containing
201 sgRNAs against either SERINC3 or SERINC5), 5.9 μg pMDLg/pRRE, 2.8 μg pxRSV-Rev
202 and 3.8 μg pMD2.G. Forty-eight hours later, concentrated lentivirus-containing

203 supernatant was harvested following low-speed centrifugation, filtration (0.45 μm), and
204 mixture with Lenti-X concentrator (Takara Bio) according to the manufacturer's
205 instructions. Briefly, 1 volume Lenti-X concentrator was mixed with 3 volumes clarified
206 supernatant. The mixture was incubated for 30 minutes at 4°C, centrifuged at 1,500 x g
207 for 45 minutes at 4°C, resuspended in 1 ml complete DMEM media and immediately
208 stored at -80°C in single-use aliquots (100 μL).
209 CEM cells knocked out for SERINC3 (S3-KO) were generated by spinoculation of 1 x 10^6
210 cells with 100 μL lentivirus (LentiCRISPRv2-SERINC3 ; (34)) at 1,200 x g for 2 hours at
211 25°C. Puromycin (1 μg/ml) was added to cell cultures 72 hours post transduction to select
212 for positive clones. Two weeks post-selection, genomic DNA was isolated from mock or
213 lentiCRISPRv2-SERINC3 transduced CEM cells using the DNeasy Blood and Tissue Kit
214 (Qiagen) according to the manufacturer's instructions. Genome editing was assessed by
215 Tracking of Indels by Decomposition (TIDE; tide.deskgen.com). PCR amplicons
216 encompassing exon 2 of SERINC3 were produced using Taq 2x Master Mix (NEB) and
217 the following primers: 5'-CAAATTACAACCAACTTGATTAACAACGACG-3' and 5'-
218 CTATAAAGCCTGATTTGCCTCGCTTTCTCTTC-3'. Twenty single cell clones were
219 isolated from bulk edited cultures using an array dilution method (IDT), followed by
220 genomic DNA isolation and PCR amplification as described above. PCR products
221 indicative of genome editing were sub-cloned into the TOPO TA vector (Thermo Fisher
222 Scientific) and plasmid DNA isolated from overnight bacterial cultures. Genome editing
223 was verified via Sanger sequencing of at least 10 independent bacterial clones.
224 To generate double-knockout cells, CEM S3 KO cells were spinoculated as described
225 above with either empty lentivirus (LentiCRISPR-GFP) or each of the five gRNAs

226 targeting SERINC5. Cells were sorted (BD FACS Aria) based on GFP expression
227 seventy-two hours post-transduction. Isolation of genomic DNA, PCR amplification and
228 TIDE analysis were carried out similarly to the generation of SERINC3 KO cells. We
229 clonally expanded, TOPO TA cloned and sequence verified sgRNA-SERINC5(4) since
230 this showed abundant genome editing and produced frameshift mutations in SERINC5
231 Exon 2. The following primers were used to generate PCR amplicons for TOPO TA
232 cloning and TIDE analysis: 5'-AGTGCCTGGCCATGTTTCTT-3' and 5'-
233 CATAGAGCAGGCTTCAGGAA-3'.
234 *HIV-1 production and titer:* To produce replication-competent viruses, HEK293T cells (4
235 x 10^6 /10 cm plate) were transfected with 24 μg of an infectious molecular clone of HIV-1
236 (NL4-3) or a mutant lacking the *nef* gene (NL4-3ΔNef) using Lipofectamine 2000 reagent
237 according to the manufacturer's instructions (Thermo Fisher Scientific). Virus-containing
238 supernatants were collected forty-eight hours post transfection, clarified by low-speed
239 centrifugation, passed through 0.45 μm filters and stored at -80°C. Viral titers were
240 measured by infecting HeLa P4.R5 cells with diluted viral stocks in duplicate in a 48-well
241 format for 48 hours. The cells were then fixed (1% formaldehyde; .2% gludaraldehyde)
242 for 5 minutes at room-temperature, followed by overnight staining at 37°C with a solution
243 composed of 4 mM potassium ferrocyanide, 4mM potassium ferricyanide, 2 mM $MgCl_2$
244 and 0.4 mg/ml X-gal (5-bromo-4chloro-3indolyl-β-D-galactopyranoside). Infected cells
245 (expressed as infectious centers (IC)) were quantified using a computer image-based
246 method (54) The p24 antigen concentrations of each stock were measured by ELISA
247 (ABL Bioscience).

248 *HIV-1 infection and replication:* To conduct HIV-1 replication studies, 1 x 10^6 CEM cells
249 (wildtype (WT); SERINC3 knockout (S3 K.O.); SERINC3/SERINC5 knockout clone 6
250 (S3/S5 K.O. (6)); SERINC3/SERINC5 knockout clone 8 (S3/S5 K.O. (8))) were infected
251 with NL4-3 (hereafter termed Nef+) or NL4-3ΔNef (hereafter termed Nef-) at an multiplicity
252 of infection (MOI) = 0.001 for 4 hours at 37°C in a 24-well plate format. The cells were
253 then washed 3 times with 1 ml PBS (Corning), resuspended in 4 ml complete growth
254 media (RPMI 1640), transferred to T25 labeled flasks and incubated at 37°C in an
255 "upright" position for the duration of the experiment. Every three days, cultures were split
256 1:4 (1 ml cells ; 3 ml media) and an aliquot (1 ml viral supernatant) stored at -80°C for
257 quantification of HIV-1 replication (p24 antigen) by ELISA. To measure cell-associated
258 HIV-1 infection, mock infected or infected CEM cells were fixed and permeabilized
259 (Cytofix/Cytoperm; BD Biosciences) for 30 minutes at 4°C, washed (Perm Wash buffer;
260 BD Biosciences) and incubated with an HIV-1 core antigen(p24)-FITC antibody (clone
261 KC57; Beckman Coulter) for 30 minutes at 4°C. Cells were washed with Flow Buffer (1x
262 PBS + 3% FBS) and subjected to flow cytometry on a Novocyte 3000 (ACEA
263 Biosciences).
264 *Measurement of HIV-1 infectivity:* Viral infectivity was quantified from virions produced in
265 CEM cells infected with NL4-3 or NL4-3ΔNef at either day 9, 12 or 15 post-infection. A
266 20% sucrose cushion was used to concentrate virions via centrifugation according to the
267 following parameters: 23,500 x g ; 1 hour at 4°C. Viral pellets were resuspended in culture
268 medium and appropriate dilutions used to immediately infect 1.25 x 10^4 HeLa TZM.bl cells
269 in triplicate in a 96-well format. Forty-eight hours later, the culture medium was removed
270 and the cells were lysed using a luciferase reporter gene assay reagent (Britelite, Perkin

271 Elmer). Infectivity (luciferase activity) was measured using a luminometer with data
272 expressed as relative light units (RLU). These values were normalized to the p24
273 concentration within each sample and depicted as RLU/p24.
274 *Data analysis and presentation:* Datasets were analyzed and combined in Microsoft Excel
275 and GraphPad Prism 8.0 software. Where indicated, two-tailed unpaired t-tests were
276 performed. We utilized Adobe Photoshop and Illustrator CS6 for figure production.
277

278 **Results**

279 *Generation of CEM cells lacking SERINC3 and SERINC5*

280 We utilized CRISPR/Cas9 gene editing to determine whether the modulation of SERINC3
281 and SERINC5 is required for Nef to enhance HIV-1 replication in CEM cells. We chose
282 the CEM cell line due to its ability to support robust HIV-1 replication and virion-infectivity
283 in a Nef-dependent manner (23). We hypothesized that Nef would remain an important
284 factor in promoting viral spread whether or not CEM cells expressed SERINC3 and
285 SERINC5, a result in conflict with data obtained using Jurkat T cells but consistent with
286 data recently obtained using MOLT-3 T cells. To test this, we used lentiviral vectors
287 (LentiCRISPR) expressing both Cas9 and gene-specific gRNAs (52). CEM cells were
288 transduced with viruses generated from a LentiCRISPR vector targeting SERINC3 exon
289 2 (34). We enriched for transduced cells using puromycin, then isolated and expanded
290 clones after limiting dilution. Twenty CEM cell clones were assessed for genome editing
291 using Tracking of Indels by Decomposition (TIDE) (55), utilizing PCR products containing
292 the SERINC3 gRNA target site amplified from the genomic DNA of the individual cell
293 clones. We cloned some of these PCR products into a bacterial plasmid vector and

294 sequenced ten independent plasmid clones from each putative single-cell clone to verify
295 specific edits. We identified a CEM line bearing three out-of-frame indels and no wild-type
296 sequences; these three distinct edited sequences persisted after a second round of
297 single-cell cloning and sequencing (Figure 1A). We concluded that cells are likely triploid
298 for this chromosome, and we designated this line CEM SERINC3 knockout (CEM:S3KO).
299 To generate CEM cells lacking both SERINC3 and SERINC5, we designed five gRNAs
300 targeting either Exon 1 or Exon 2 of SERINC5. The gRNAs were subcloned into a lentiviral
301 vector that encodes GFP in place of puromycin (LentiCRISPR-GFP). We transduced
302 CEM:S3KO cells with distinct lentiviruses, each encoding a different gRNA targeting
303 SERINC5. Three days post-transduction, CEM cells were sorted based on GFP
304 expression and expanded in bulk. TIDE analysis revealed that gRNA4 targeting exon 2
305 yielded the most promising evidence of genome editing (data not shown). We single-cell
306 cloned and expanded these cells in a similar manner as described above for the editing
307 of SERINC3. We identified two clones containing indels within the SERINC5 target region.
308 PCR amplification from these putative single-cell clones and sequencing of individual
309 plasmid clones of the PCR products indicated that these consisted of either a mixture of
310 two triploid cell lines (“clone 6”) and a triploid edit (“clone 8”); these cell lines were termed
311 SERINC3/SERINC5 knockout clone 6 (CEM:S3/S5KO (6)) and CEM SERINC3/SERINC5
312 knockout clone 8 (CEM:S3/S5KO (8)) (Figure 1B). In both cases the cell lines encoded
313 out-of-frame indels in SERINC5 exon 2 and no wild-type sequences. The nine base-pair
314 insertion in clone 6 generated a stop codon. These three cells lines served as the basis
315 for our viral replication and infectivity studies.
316

317 *Optimal HIV-1 spread in CEM cells is dependent on Nef but independent of SERINC3*
318 *and SERINC5.*
319 To test whether Nef is required to counteract SERINC3 and SERINC5 to enhance HIV-1
320 replication, we infected CEM wildtype (WT), S3KO, S3/S5 KO (6) and S3/S5 KO (8) cells
321 with either Nef expressing (hereafter termed Nef+: NL4-3) or Nef lacking (hereafter
322 termed Nef-: NL4-3ΔNef) HIV-1 viruses at a multiplicity of infection (MOI)=0.001 infectious
323 units per cell. The inocula for these growth-rate experiments were produced from
324 HEK293T cells, and the MOI was based on the infectivity of the virus stocks measured in
325 CD4-positive HeLa-P4.R5 indicator cells. Thus, to infect $1x10^6$ CEM cells, we used
326 amounts of Nef+ or Nef- virus stocks that yielded 1,000 infectious centers in the HeLa
327 indicator assay. Although the infectivity of the viruses to CEM cells might be different than
328 to CD4-HeLa cells, we chose this approach rather than normalizing the inocula to the
329 content of p24 capsid antigen to adjust for the reduced infectivity of Nef- virions produced
330 by the HEK293T cells (data not shown). The CEM cell cultures were split every 3-4 days
331 and viral replication quantified by the amount of viral capsid (p24) contained within the
332 supernatant (cell-free: p24 ELISA) or associated with cells (intracellular flow) (Figure 2A).
333 As expected, we observed low but equal p24 production among all four CEM genotypes
334 whether the cells were infected with either Nef + (red line) or Nef – (blue line) expressing
335 virus (Figure 2B) at three days post-infection. This indicated that the standardization of
336 the inocula was likely accurate and that the infections were initiated at the same nominal
337 MOI. However, after day three, the Nef+ viruses propagated much more rapidly than the
338 Nef-, accumulating anywhere from 10- to 50-fold more p24 antigen in the culture
339 supernates at 6, 9 or 13 days post-infection in CEM WT cells (Figure 2B: Top left panel).

340 This corroborated the importance of Nef in enhancing viral replication in this *in vitro*
341 system. If Nef-mediated modulation of SERINC3 and/or SERINC5 were important for this
342 phenotype, then the attenuated replication of Nef- virus should be "rescued" in cell lacking
343 SERINC3 and/or SERINC5. Instead, Nef enhanced HIV-1 replication in CEM cells
344 knocked out for SERINC3 (Figure 2B: top right panel) or knocked out for SERINC3 and
345 SERINC5 (Figure 2B: bottom two panels). We observed a similar trend when analyzing
346 cell-associated virus at day 9 post-infection (Figure 2C) or cell-free virus from an
347 independent viral spreading experiment that utilized independently produced and titered
348 viral stocks (Figure 2D). Taken together, these data indicated that modulation of
349 SERINC3 and SERINC5 is not the primary mechanism by which Nef increases viral
350 spread in CEM cells.
351
352 *Nef enhances HIV-1 virion-infectivity in CEM cells independently of SERINC3 and*
353 *SERINC5.*
354 Various systems have shown that Nef enhances virion-infectivity in a SERINC- and cell-
355 type dependent manner (34). Therefore, we next asked whether counteraction of
356 SERINCs is necessary for Nef to enhance the infectiousness of virions. To test this, we
357 collected virions from the infected CEM cells at day 9, 13 or 15 post-infection and
358 measured single-cycle infectivity using HeLa TZM.bl reporter cells, which express
359 luciferase under the transcriptional control of the HIV-1 LTR (Figure 3A). We used these
360 cells because the luciferase read-out is more sensitive that the infectious center read-out
361 of the HeLa-P4.R5 cells, and the concentration of Nef- virus produced by the CEM
362 cultures was relatively low. Virions produced by Nef+ virus (day 9 post-infection) were 10-

363 to 50-fold more infectious per amount of p24 capsid antigen than those produced by Nef-
364 virus (day 13 post-infection), and this trend was observed regardless of SERINC3 and/or
365 SERINC5 expression (Figure 3B). We confirmed this result in an independent experiment
366 utilizing different viral stocks and analyzing virions collected at later times (Nef+: Day 13
367 post-infection versus Nef-: Day 15 post-infection; Figure 2C). Altogether, the data indicate
368 that Nef increases virion infectivity as well as growth-rate in CEM T cells independently
369 of SERINC3 and SERINC5.
370
371 **Discussion**
372
373 In this study, we sought to determine whether Nef's ability to enhance viral replication
374 and/or infectivity in CEM cells is primarily linked to modulation of SERINC3 and/or
375 SERINC5. We present evidence that argues against this by showing that Nef increases
376 viral replication rate in CEM cells lacking SERINC3 and SERINC5 and that virions of Nef+
377 virus are much more infectious than those of Nef- virus regardless of whether SERINC3
378 and SERINC5 are expressed in the CEM cells that produced them.
379
380 Our results indicate that Nef enhances virion-infectivity independently of SERINCs in
381 CEM T cells. This contrasts with reports in Jurkat and primary CD4+ cells, where Nef-
382 mediated enhancement of infectivity appears to correlate with SERINC5 expression (34,
383 35). How might these disparate results be explained? One reason could be differences in
384 experimental design. Most HIV-1 infectivity assays utilize virions produced from transient

385 overexpression systems (34, 35). Here, we used virions produced from infected CEM
386 cells either expressing or lacking endogenous SERINC3 and SERINC5.
387
388 Perhaps more likely are cell-type-specific differences. Our observations herein using
389 CEM cells are very similar to recent results reported using MOLT-3 cells (46). In MOLT-3
390 cells, a chimeric HIV-1 virus bearing the SERINC5 antagonist (glycoMA) failed to
391 substitute for Nef in rescuing HIV-1 infectivity (a property glycoMA should have if the sole
392 function of Nef were to counteract SERINC5), and knockout of SERINC5 did not rescue
393 the reduced growth rate of Nef- virus (46). Together, these data suggest that the SERINC-
394 dependence of the Nef infectivity phenotype is cell-type dependent (34, 35, 46), with
395 Jurkat and presumable primary CD4+ T cells being SERINC-dependent and CEM and
396 MOLT-3 cells being SERINC-independent. Thus, comparing virions produced from
397 infected primary CD4+ T cells either lacking or expressing SERINC5 may be particularly
398 insightful to adjudicate the relevance of observations made using T cell lines.
399
400 How does Nef's ability to enhance viral replication correlate with modulation of the
401 SERINCs? Jurkat cells lacking SERINC3 and SERINC5 support similar viral replication
402 when infected with HIV-1 viruses either expressing or lacking Nef (35). Furthermore,
403 substituting glycoMA for Nef rescues viral replication in Jurkat cells (34, 46). This
404 indicates that Nef's ability to enhance viral replication in these cells rests primarily on
405 modulation of SERINCs. However, glycoMA failed to rescue the replication of HIV-1
406 lacking Nef in MOLT-3 and primary CD4+ T cells (46). Moreover, MOLT-3 cells lacking
407 SERINC3 and SERINC5 support Nef's ability to enhance viral replication (46). Our results

408 herein indicate that CEM cells lacking SERINC3 and SERINC5 similarly support Nef's
409 ability to enhance viral replication. Together with the MOLT-3 data, these results indicate
410 that Nef-mediated enhancement of viral propagation can occur in a SERINC-independent
411 manner. Whether primary CD4+ T cells lacking SERINC5 support the attenuated
412 phenotype of Nef-deficient viruses remains unknown and is an important question to
413 answer.
414
415 What mechanisms might explain Nef-mediated enhancement of infectivity and/or viral
416 replication independently of SERINCs? One potential explanation could be Nef-mediated
417 modulation of Src family kinases (SFKs). Nef binds several SFK members such as Lck,
418 Hck, Lyn and c-Src through a conserved proline-rich (PxxP) motif contained within Nef's
419 Src homology region 3 (SH3) binding domain (56, 57), and primary Nef isolates from
420 HIV-1 Group M display a conserved ability to activate SFK's (58). Nef mutants lacking the
421 PxxP motif were reportedly able to downregulate CD4 but unable to enhance HIV-1
422 replication in peripheral blood mononuclear cells (57). However, other studies reported
423 that mutation of Nef's PxxP motif yielded little to no difference in HIV-1 replication within
424 MOLT-3 or primary CD4+ T-cells, and in CEM T cells the attenuated phenotype of
425 mutants of the SH3 binding domain was modest and seemed attributable to reduced
426 expression of Nef (25, 46, 59). Analyzing HIV-1 replication in T cells lacking one or more
427 SFKs may be necessary to adequately assess whether Nef's ability to enhance HIV-1
428 replication is mediated by these interactions.
429

430 One notable similarity between MOLT-3 and CEM lymphoblastoid cells that distinguishes
431 them from Jurkat cells is that they do not express the T cell receptor (TCR) at their
432 surfaces (60, 61). Given that Nef reportedly mimics TCR-signaling (22, 62), another
433 possibility is that MOLT-3 and CEM cells reveal a SERINC-independent growth-rate Nef-
434 phenotype that is exaggerated by the absence of TCR signaling in these cells. This could
435 be consistent with the initial observations that the activation-state of primary CD4 T cells
436 affects the Nef growth-rate phenotype (24). Nonetheless, how TCR signaling would affect
437 virion-infectivity is obscure.
438
439 Lastly, the explanation might reside in Nef's ability to interact with components of clathrin
440 trafficking pathways and to modulate many cellular proteins, one of which might underlie
441 the infectivity phenotype in MOLT-3 and CEM cells. Nef binds AP complexes via a
442 conserved sorting signal near its C-terminus: 160ExxxLL164,165 (63). This "di-leucine
443 motif" is required for both Nef-mediated CD4 downregulation and optimal viral infectivity
444 in CEM cells (64). The Nef LL164/165AA mutant, which is unable to bind AP-2 (65),
445 replicates poorly in MOLT-3 cells (46). Residues located within Nef's core domain and
446 required for downregulating CD4 and interacting with Dynamin-2 are also required for
447 enhancement of viral replication in MOLT-3 cells (46, 66, 67). Finally, Nef enhances HIV-
448 1 replication in the absence of CD4 downregulation in MOLT-3 cells (46) and in CEM-
449 derived cells (A2.01) engineered to express a CD4 lacking a cytoplasmic domain that is
450 unresponsive to Nef (68). These observations regarding CD4 are critically important,
451 since CD4 is itself a potent inhibitor of infectivity that is counteracted by Nef (11, 12). In
452 the CEM experiments herein, CD4 downregulation by Nef could conceivably account for

453 the observed infectivity and growth-rate phenotypes. Nonetheless, even if that were the
454 case, the data would indicate that the contribution of CD4 to these phenotypes far
455 outweighs the contribution of the SERINC3 and SERINC5 in these cells.
456
457 Taken together, these results raise the intriguing possibility that Nef binds and internalizes
458 a novel factor within CEM as well as MOLT-3 cells that is necessary for enhancing viral
459 infectivity and replication. Utilizing both MOLT-3 and CEM cells might facilitate the search
460 for such a factor, providing a more complete understanding of the enigmatic yet important
461 virologic effects of Nef.
462
463
464
465
466

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680

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691 **Figure Legends**

692 *Figure 1: CRISPR/Cas9 editing of SERINC3 and SERINC5 in CEM cells.* A portion of the
693 wildtype (WT) sequence from exon 2 of either SERINC3 or SERINC5 is shown in each
694 panel, with the gRNA target site highlighted in bold. Inserted nucleotides are in green,
695 stop codons in red, and substituted nucleotides in blue. Deletions are depicted as a
696 dashed line. All mutant alleles sequenced produced frameshift mutations. A.) The
697 genotype in SERINC3 knockout (S3KO) cells consisted of alleles bearing a 1 bp insertion,
698 2 bp insertion, and 19 bp deletion. B.) We identified and utilized two different clones
699 knocked out for SERINC5: clone 8 (S3/S5KO (8)) and clone 6 (S3/S5KO (6)). S3/S5KO
700 (8) contained the following indels: 2 bp deletion, 4 bp deletion, and a 7 bp deletion (top)
701 while S3/S5KO (6) contained a 2 bp deletion, 3 bp deletion/1 bp insertion, 4 bp deletion,
702 9 bp insertion with the introduction of a stop codon, 10 bp deletion, and 31 bp deletion.

703

704 *Figure 2: Nef enhances HIV-1 replication independently of SERINC3 and SERINC5 in*
705 *CEM cells.* A.) Schematic of viral replication studies performed in CEM wildtype (WT),
706 SERINC3 knockout (S3KO), SERINC5 knockout clone 8 (S5KO (8)) or SERINC5
707 knockout clone (6 S5KO (6)) cells. Each cell line was infected with either NL4-3 (termed
708 Nef+) or HIV-1ΔNef (termed Nef-) at a low inoculum (MOI=0.001). The cultures were split
709 every 3-4 days and viral growth measured as indicated. B.) Viral replication quantified by
710 p24 Capsid ELISA in the supernatants of CEM WT, S3KO, S5KO (8) and S5KO cultures
711 (6). Results depict either three (WT, S3KO, S5KO (8)) or one (S5KO (6)) independent
712 infections measured in duplicate at each time point. C.) Flow cytometry of p24 positive
713 cells at day 9 post-infection. Data are compiled from three independent experiments. D.)

714 HIV-1 replication measured by p24 ELISA using different viral stocks in a separate
715 independent experiment. Two-tailed unpaired t-tests were performed where indicated.
716
717 *Figure 3: HIV-1 requires Nef for optimal infectivity in CEM cells regardless of SERINC3*
718 *or SERINC5 expression.* A.) Schematic depicting measurement of single-cycle infectivity
719 with virions produced from infected CEM WT, S3KO, S5KO (8) or S5KO cultures (6).
720 HeLa Tzm.bl indicator cells contain a luciferase gene under the transcriptional control of
721 the HIV-1 LTR. B.) Infectivity data (relative luciferase units (RLU) normalized to p24
722 (RLU/p24)) from virions collected from cultures of either CEM WT cells or cells knocked
723 out for SERINC3 or SERINC3 and SERINC5 infected with either Nef+ (day 9 post-
724 infection) or Nef- (day 13 post-infection) viruses. Results are representative of one
725 experiment performed in triplicate. C.) Infectivity data (RLU/p24) for virions produced at
726 day 12 (Nef+) or day 15 (Nef-) post-infection of CEM WT or S3/S5 KO(8) cells. Results
727 represent one experiment that was fully independent from that shown in panel B and
728 performed in triplicate. D.) Combined RLU/24 data from Figure 3B and 3C in CEM WT
729 and S3/S5 KO(8) cells. Two-tailed unpaired t-tests were performed where indicated.
730
731
732
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734

Figure 1

A ***SERINC3 knockout: Exon 2***

Target Site

Gene: AATGAAAGC***ATAAATGAGGCGAGTCACCG***TGGAATTCT

S3:KO
AATGAAAGC***ATAAATGAGGCGAGTCAACCG***TGGAATTCT
AATGAAAGC***ATAAATGAG*** – – – – – – – – – – – – – – – – – – – G
AATGAAAGC***ATAAATGAGGCGAGTCATTCCG***TGGAATTCT

B ***SERINC5 knockout (clone 8): Exon 2***

Target Site

Gene: CCTCTGCTG***CATCATGATGTCAACAACCG***TGG

S5:KO (8)
CCTCTGCTG***CATCATGATGTCAACA*** – –***AG***TGG
CCTCTGCTG***CATCATGATGTCAA*** – – – –***CG***TGG
CCTCTGCTG***CATCATGATGT***– – – – – – –***CA***TGG

C ***SERINC5 knockout (clone 6): Exon 2***

Target Site

Gene: CCTCTGCTG***CATCATGATGTCAACAACCG***TGGCTCA

S5:KO (6)
CCTCTGCTG***CATCATGATGTCAACAA*** – –***G***TGGCTCA
CCTCTGCTG***CATCATGATGTCAAC*** – – – ***AAG***TGGCTCA
CCTCTGCTG***CATCATGATGTCAAC*** – – – – ***G***TGGCTCA
CCTCTGCTG***CATCATGATGTCATCTTGATTTGATCTTG***TGGCTCA
CCTCTGCTG***CATCATGAT*** – – – – – – – – – –***G***TGGCTCA
CC –TCA

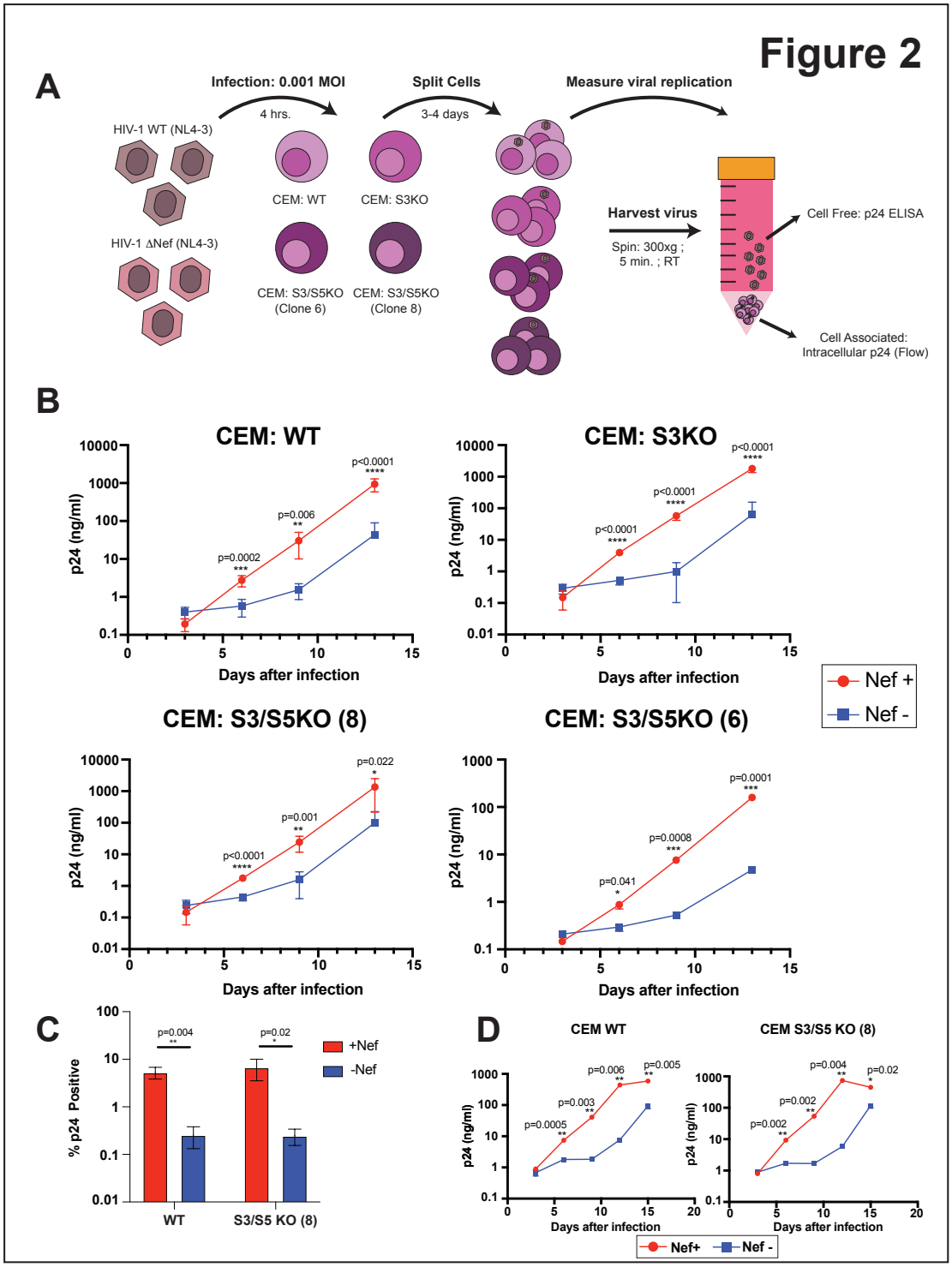
Figure 2
A
Infection: 0.001 MOI
4 hrs.
Split Cells
3-4 days
Measure viral replication
HIV-1 WT (NL4-3)
HIV-1 ΔNef (NL4-3)
CEM: WT
CEM: S3KO
CEM: S3/S5KO (Clone 6)
CEM: S3/S5KO (Clone 8)
Harvest virus
Spin: 300xg ; 5 min. ; RT
Cell Free: p24 ELISA
Cell Associated: Intracellular p24 (Flow)
B
CEM: WT
CEM: S3KO
CEM: S3/S5KO (8)
CEM: S3/S5KO (6)
p24 (ng/ml)
Days after infection
Nef +
Nef -
C
% p24 Positive
+Nef
-Nef
WT
S3/S5 KO (8)
D
CEM WT
CEM S3/S5 KO (8)
Nef+
Nef -

737

738

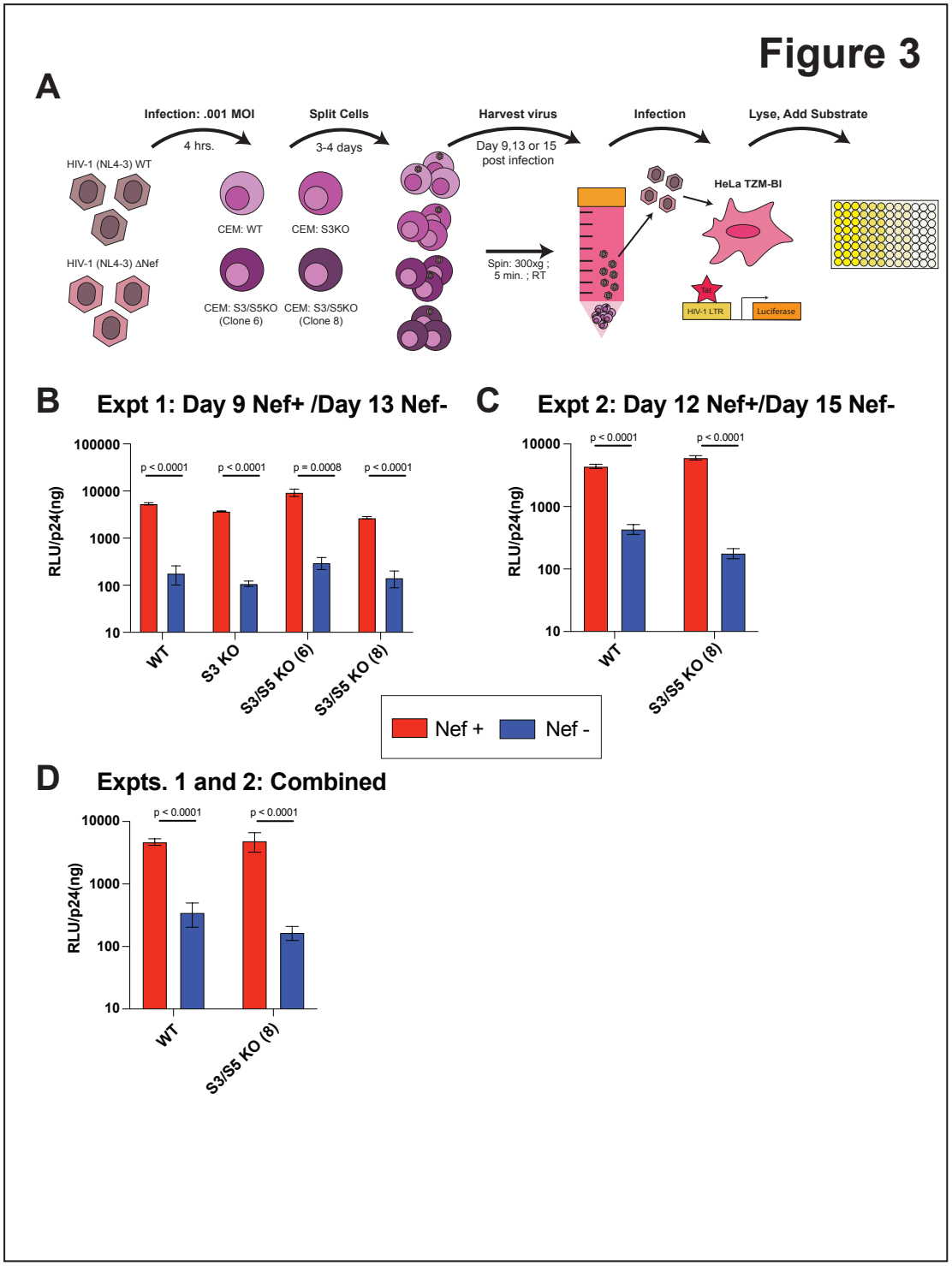
Figure 3
A
Infection: .001 MOI
4 hrs.
Split Cells
3-4 days
Harvest virus
Day 9,13 or 15
post infection
Infection
Lyse, Add Substrate
HIV-1 (NL4-3) WT
HIV-1 (NL4-3) ΔNef
CEM: WT
CEM: S3KO
CEM: S3/S5KO
(Clone 6)
CEM: S3/S5KO
(Clone 8)
Spin: 300xg ;
5 min. ; RT
HeLa TZM-Bl
HIV-1 LTR
Luciferase
B
Expt 1: Day 9 Nef+ /Day 13 Nef-
RLU/p24(ng)
100000
10000
1000
100
10
p < 0.0001
p < 0.0001
p = 0.0008
p < 0.0001
WT
S3 KO
S3/S5 KO (6)
S3/S5 KO (8)
C
Expt 2: Day 12 Nef+/Day 15 Nef-
RLU/p24(ng)
10000
1000
100
10
p < 0.0001
p < 0.0001
WT
S3/S5 KO (8)
Nef +
Nef -
D
Expts. 1 and 2: Combined
RLU/p24(ng)
10000
1000
100
10
p < 0.0001
p < 0.0001
WT
S3/S5 KO (8)

739