Impact of natural HIV-1 Nef polymorphisms on SERINC5 antagonism

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Background

- Nef is a multifunctional accessory protein (~206 a.a) critical for viral pathogenesis. It was recently reported that Nef enhances HIV-1 infectivity by downregulating host restriction factor SERINC5 from the cell surface, preventing its incorporation into budding virions. Mutations at several highly-conserved Nef residues impair SERINC5 antagonism, but few studies have evaluated the impact of natural sequence variation on this function. Furthermore, no reports have examined this function for Nef alleles isolated from HIV-1 elite controllers (EC) who display low plasma viral load in the absence of therapy.

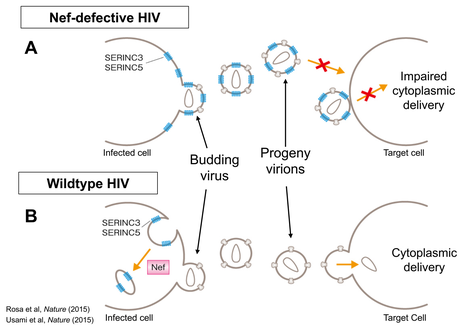

Figure 1. (A) The SERINC5 transmembrane protein restricts HIV infection when incorporated into progeny virions. **(B)** HIV-1 Nef protein counteracts SERINC5 restriction activity by downregulating it from the cell surface, preventing its incorporation into progeny virions

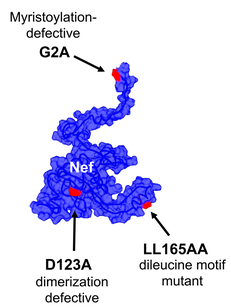

Figure 2. Canonical mutations in Nef (e.g. G2A, D123A, LLAA) can impair SERINC5 down-regulation function but these polymorphisms are rarely found in circulating sequences

Methods

Nef-mediated SERINC5 downregulation assay

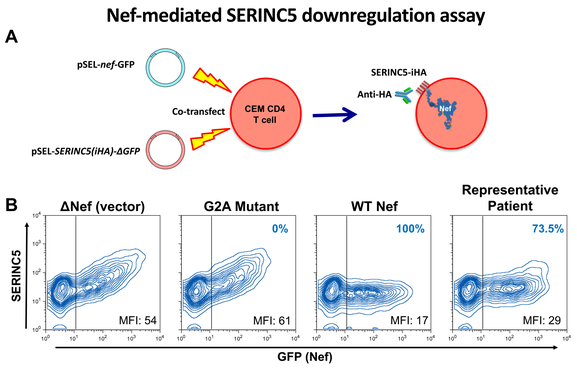

Figure 3. (A) To assess Nef-mediated SERINC5 downregulation, CEM CD4 T cells were cotransfected with a vector that expresses the Nef protein of interest alongside GFP which serves as a marker for transfection efficiency and a vector that expresses an internal HA tagged SERINC5 protein. Transfected cells were incubated overnight, stained with anti-HA-APC and analyzed by flow cytometry. **(B)** Representative flow plots of surface SERINC5 expression in CEM cells when transfected with ΔNef (empty vector), G2A mutant (defective Nef), WT Nef, and a patient Nef clone. Data was normalized by setting the function WT Nef to 100% and the G2A mutant to 0%

Acknowledgements

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Results

Result #1: Nef-mediated downregulation of SERINC5 is impaired in HIV Elite Controllers

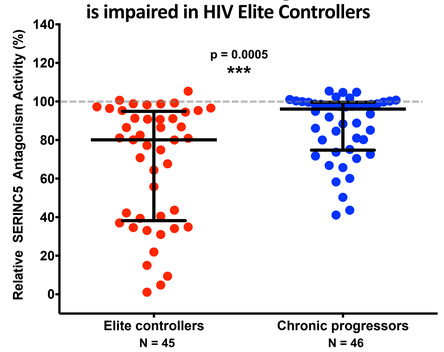

Figure 4. Primary Nef alleles were isolated from 45 HIV controllers and 46 progressors, cloned into pSEL-GFP vector and assessed for SERINC5 downregulation function. Nef alleles derived from controllers displayed significantly reduced ability to downregulate SERINC5 (median 80% [IQR 38-95%]) compared to Nef alleles derived from progressors (median 96% [IQR 75-100%]). Results shown were obtained from at least three independent experiments. Bars represent the median and interquartile ranges.

Result #2: Nef polymorphisms associated with differential SERINC5 antagonism activity

Table 1. Analysis of Nef residues associated with differential SERINC5 downregulation activity (p<0.05 and q<0.35)

Codon	Amino Acid	Number of subjects		Relative Nef activity (%)		Impact	p – value	q – value
		With AA	Without AA	With AA	Without AA			
51	T	95.3	80.0	43	45	↑	0.004	0.22
11	A	100.6	86.5	6	75	↑	0.005	0.22
51	N	80.0	96.0	42	46	↓	0.005	0.22
65	E	84.9	98.3	84	7	↓	0.010	0.25
19	K	100.7	85.6	9	82	↑	0.011	0.25
116	H	95.3	80.6	59	32	↑	0.012	0.25
94	E	50.3	88.6	5	86	↓	0.015	0.28
116	N	80.9	95.3	31	60	↓	0.020	0.30
182	Q	97.9	85.1	10	81	↑	0.021	0.30
43	V	39.4	88.4	5	85	↓	0.025	0.33
163	C	97.2	83.6	27	64	↑	0.028	0.33
55	C	90.8	73.7	82	9	↑	0.030	0.33
163	R	39.4	87.6	7	84	↓	0.033	0.33
114	V	89.8	74.9	70	21	↑	0.037	0.34
148	V	89.8	66.8	78	13	↑	0.041	0.34
28	D	85.1	93.4	47	44	↓	0.047	0.34
94	K	88.4	55.2	83	8	↑	0.047	0.34
170	L	81.0	91.1	55	36	↓	0.050	0.34

Result #3: Validation of selected Nef polymorphisms by site-directed mutagenesis in NL4.3

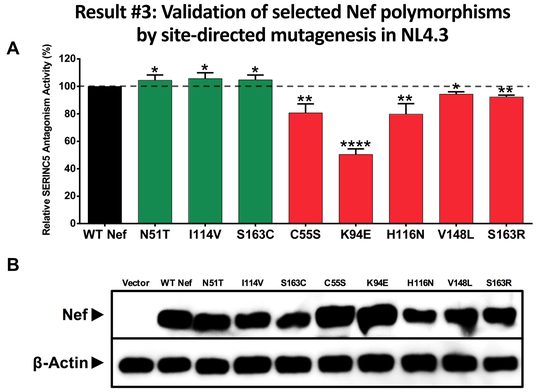

Figure 5. (A) Eight Nef polymorphisms were validated by site-directed mutagenesis in WT Nef (NL4-3 strain). Green bar represents point mutants that improved SERINC5 antagonism activity relative to WT Nef (100%) and red bars represents point mutants that impaired activity. The average function of ≥ 3 independent experiments is reported, and error bars indicate the standard deviation. All samples were statistically different from WT Nef (unpaired t-test: ; * = p ≤0.05, ** = p ≤ 0.01 and *** = p ≤ 0.001.) **(B)** Western blot analyses were used to assess steady-state protein expression of Nef mutants (upper blot). Blots were probed for the β-actin protein as a cellular loading control (lower blot).

Results

Result #4: HLA-associated escape mutations K94E and H116N impair Nef's ability to antagonize SERINC5

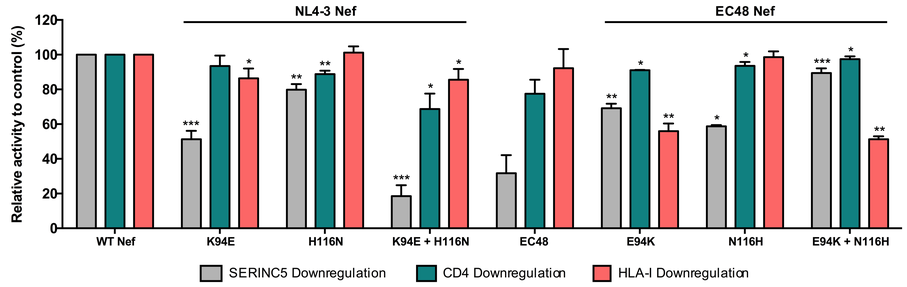

Figure 6. (Left) NL4-3 Nef mutants (K94E, H116N and K94E+H116N) was assessed for SERINC5, CD4 and HLA-I downregulation. **(Right)** EC48 patient Nef clone harboring E94 and N116 polymorphisms was defective for SERINC5 downregulation but retained activity for CD4 & HLA downregulation. Reversion mutations (E94K, N116H and E94K+N116H) were assessed for SERINC5, CD4 and HLA-I downregulation. Mean and SD from ≥ 3 independent experiments are shown. Unpaired t-test was performed to compare point mutants and WT Nef or EC48; * = p ≤0.05, ** = p ≤ 0.01 and *** = p ≤ 0.001.

Result #5: Inability of primary Nef clones to efficiently antagonize SERINC5 is correlated with impaired infectivity

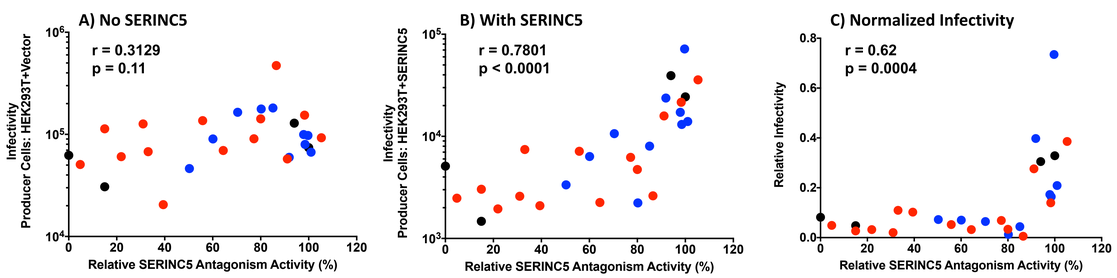

Figure 7. 24 primary Nef alleles (14 from controllers [red] and 10 from progressors [blue]) were sub-cloned into pNL4-3 and used to produce viral stocks in the absence or presence of SERINC5. Four control NL4-3 viruses (shown in black) were included: G2A Nef, ΔNef, NL4-3 Nef and SF2 Nef. The infectivity of each virus was assessed using TZM-bl cells where luminescence (absolute light units) was measured. Values on the y-axis represent the average luminescence from triplicate infection from a single experiment. Values on the x-axis represent the SERINC5 downregulation activity for each Nef allele, shown in Figure 4. The infectivity assay was performed twice, and results from a representative experiment was used for correlation analysis. **(A)** No correlation was observed between SERINC5 downregulation activity and infectivity when viruses were produced in the absence of SERINC5. **(B)** SERINC5 downregulation activity correlated with infectivity when viruses were produced in the presence of SERINC5. **(C)** Correlation between relative infectivity (SERINC+/SERINC-) and SERINC5 downregulation activity is shown.

Result #6: Viral Infectivity and replication capacity of HIV-1 NL4-3 viruses with K94E and/or H116N polymorphisms

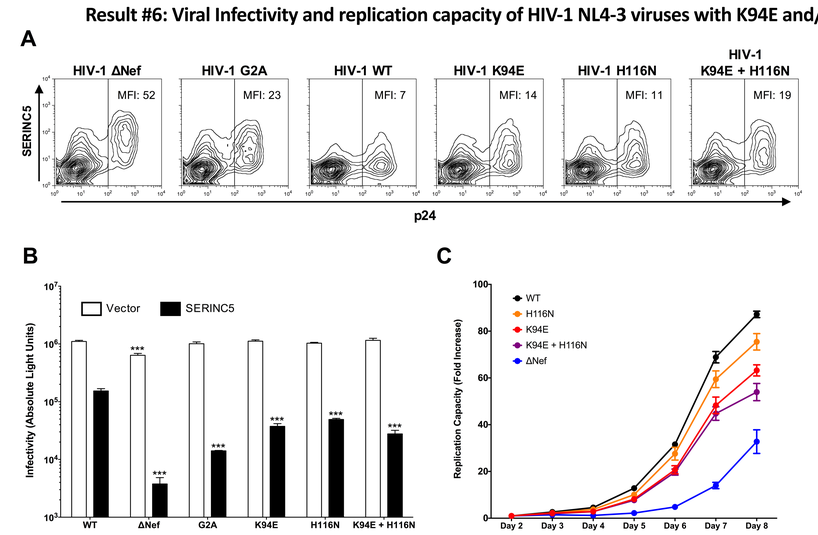

Figure 8. (A) Representative flow plot of HEK293T+SERINC5 virus producer cells when stained for cell surface SERINC5 (anti-HA-APC) and intracellular p24 (anti-p24-PE). MFI values of SERINC5 are shown in the p24+ gate. **(B)** Infectivity values (absolute light units) for HIV-1 NL4-3 viruses produced in HEK293T+vector (white bars) and HEK293T+SERINC5 (black bars). HIV-1 NL4-3 viruses encoding point mutations (G2A, K94E, H116N and K94E+H116N) in Nef displayed no apparent differences in infectivity from WT viruses when produced in HEK293T+vector cells, except for HIV-1 ΔNef, which was only 1.7-fold less infectious than WT (white bars). Whereas, when the same viruses were produced in HEK293T+SERINC5 cells, HIV-1 ΔNef viruses were ~41 fold less infectious than WT (comparable to the Nef+/Nef- infectivity ratio observed in Jurkat cells), while the G2A, K94E, H116N and K94E+H116N Nef viruses were ~11, ~4, ~3 and ~5.5 fold less infectious, respectively . An unpaired t-test was performed; *** indicates p ≤ 0.0001 **(C)** Replication capacity of WT and mutant viruses in Jurkat LTR-GFP R5 cells that express high levels of endogenous SERINC5. The mean fold increase from day 2 (MOI 0.0003) is reported from triplicate infections and error bars represent the standard deviation. HIV-1 ΔNef viruses replicated most poorly (33-fold increase on day 8) followed by the K94E+H116N double mutant (54-fold increase), K94E (63-fold increase), H116N (75-fold increase) and lastly, the WT virus (87-fold increase).

Conclusion

- **Results demonstrate that Nef's ability to counteract SERINC5 is modulated by natural sequence variation. Polymorphisms in two highly immunogenic CTL epitopes (restricted by B*08 and B*57) selectively impaired this Nef function, illustrating constraints on HIV-1 adaption and identifying potential targets for therapeutics to disrupt Nef-mediated SERINC5 antagonism.**