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Concise Communication

An HIV-1 Nef genotype that diminishes immune control mediated by protective HLA alleles

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Running Title: Nef subverts protective HLA effect

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Abstract

Objectives: Certain HLA-B alleles (protective alleles) associate with durable immune control of HIV-1, but with substantial heterogeneity in the level of control. It remains elusive whether viral factors including Nef-mediated immune evasion function diminish protective allele effect on viral control.

Design: The naturally occurring non-Ser variant at position 9 of HIV-1 subtype C Nef has recently exhibited an association with enhanced HLA-B downregulation function and decreased susceptibility to recognition by CD8 T cells. We therefore hypothesized this Nef genotype leads to diminished immune control mediated by protective HLA alleles.

Methods: Nef sequences were isolated from HIV-1 subtype C-infected patients harboring protective alleles and several Nef functions including downregulation of HLA-A, HLA-B, CD4, and SERINC5 were examined. Association between Nef non-Ser9 and plasma viral load was examined in two independent South African and Botswanan treatment-naïve cohorts.

Results: Nef clones isolated from protective allele⁺ individuals encoding Nef non-Ser9 variant exhibited greater ability to downregulate HLA-B when compared to the Ser9 variant, while other Nef functions including HLA-A, CD4 and SERINC5 downregulation activity were unaltered. By analyzing a cohort of South African subjects chronically

infected with subtype C HIV-1, Nef non-Ser9 associated with higher plasma viral load in patients harboring protective alleles. Corroboratively, the Nef non-Ser9 correlated with higher plasma viral load in an independent cohort in Botswana.

Conclusions: Taken together, our study identifies the Nef genotype, non-Ser9, that subverts host immune control in HIV-1 subtype C infection.

Keywords

HIV-1; subtype C; Nef; HLA; downregulation; protective allele;

Introduction

The natural course of HIV-1 infection varies greatly among HIV-1 infected individuals, with some progress rapidly to acquired immune deficiency syndrome within a year of seroconversion while others remain asymptomatic and maintain durable control of the virus for decades ^[1,2]. This could be a result of a complex interaction between host immune system and the virus. The most notable host factor associated with disease progression rate, as evidenced by genome-wide association studies, is the human leucocyte antigen (HLA) class I locus ^[3,4], in particular the HLA-B locus. HLA-B alleles such as HLA-B*57, HLA-B*58:01 and HLA-B*81:01 have been consistently shown to associate with lower viral loads in chronic HIV-1 subtype C infection in South Africa ^[5,6]; however extensive heterogeneity in levels of viral control exist among the infected individuals bearing such protective HLA alleles, suggesting additional host or viral factors modifying viral control.

The protective effect associated with certain HLA alleles is thought to be mediated by CD8+ cytotoxic T lymphocyte (CTL) responses. Individuals bearing protective alleles have profound ability to curtail HIV-1 replication through mounting strong CTL responses ^[7-9]. As such, viral mechanisms that may undermine such CTL responses could play a role in diminishing host immune control among the individuals

bearing the protective HLA alleles. It has been well established that HIV-1 employs Nef-mediated HLA-A and HLA-B downregulation to evade from CTL responses ^[10, 11]. This Nef function is impaired in elite controllers who show durable control of HIV-1 below detection limit ^[12, 13]. In addition, HLA-B alleles exhibited less susceptibility to Nef-mediated downregulation compared to HLA-A alleles ^[10, 14, 15], an observation that partly explains the dominant effect of HLA-B alleles in immune control of HIV-1 infection ^[5].

In our previous study, a naturally arising polymorphism non-Ser variant at Nef position 9 was identified from 168 Nef clones isolated from 168 HIV-1 subtype C chronically infected subjects, and recombinant viruses harboring this variant exhibited enhanced HLA-B downregulation activity as well as reduced susceptibility to HIV-specific CD8⁺ T cell recognition ^[15]. We postulated in the present study that this non-Ser9 genotype in Nef may act as a viral factor that subverts immune control mediated by protective HLA alleles.

Materials and methods

Study subjects

We characterized *HLA* class I genotype, plasma viral load and *nef* sequences from plasma viral RNA in antiretroviral naïve and chronically HIV-1 subtype C-infected

adults from two established cohorts in South Africa, Durban (N=530) ^[5] and Bloemfontein (N=138) ^[16], with a median log₁₀ viral load of 4.62 [IQR: 3.96-5.14] and 4.90 [IQR: 4.03-5.38] copies/ml, respectively. The levels of plasma viral load at both sites exhibited no significant difference (p=0.10), and we combined them as a South African cohort for further post hoc analyses in this study. In addition, as an independent cohort, we also characterized *HLA* class I genotype, plasma viral load and *nef* sequences from plasma viral RNA in antiretroviral naïve and chronically HIV-1 subtype C-infected adults from Botswana (N=193) ^[17], with a median log₁₀ viral load of 4.27 [IQR: 3.76-4.86] copies/ml. This study was approved by the institutional review board of relevant institutions; and all participants provided written informed consent.

Construction of patient-derived Nef clones

Patient derived *nef* sequences were obtained through a PCR amplification or custom synthesized (GeneWiz) based on the nucleotide sequence information generated in previous studies ^[5, 18]. The resultant *nef* sequences were cloned into both pcDNA-GFP ^[19] and pNL43-ΔNef ^[20], that enable expression of a fusion protein of Nef and green fluorescent protein as well as secretion of infectious viral particles, respectively.

Downregulation assay for Nef functions

Nef-mediated downregulation of cell surface molecules was assessed as

previously reported [12, 14, 15]. Jurkat cells and CEM cells that had been stably transfected with the gene encoding HLA-A*02:01 (A02) or A02 chimera where the cytoplasmic tail was replaced with that of a group of HLA-B molecules including HLA-B*57:01 (denoted as A02_{B57CT}) [15] were electroporated with either pcDNA-GFP alone or pcDNA-GFP harboring various patient-derived Nef sequences. After 24h, cells were stained with the HLA-A2 serotype-specific antibody and anti-human CD4 mAb followed by 7-amino-actinomycin D (7AAD) (all from BioLegend Co).

To assess Nef-mediated downregulation of SERINC5 from the cell surface, a Jurkat derivative, namely a JTA_g cell line in which SERINC3 and 5 were stably knocked out (JTA_g-S3/5^{-/-}; provided by M. Pizzato), was electroporated with pBJ5-SERINC5-internal HA tag (S5-iHA) (provided by H. Gottlinger) and pcDNA-GFP alone or harboring various patient-derived Nef sequences. After 24h, the cells were stained with anti-HA.11 followed by Zombie Aqua™ to remove dead cell fractions (all from BioLegend Co).

Note that in all systems above, live cells were gated, and the mean fluorescence intensity (MFI) of the target antigen in Nef-expressing cells (defined as the GFP⁺ subset, denoted MFI Nef⁺ in the below calculation) and non Nef-expressing cells (defined as the GFP⁻, denoted MFI Nef⁻) was analyzed by flow cytometry. The following formula was

used to calculate the downregulation activity of each Nef clone: $(\text{MFI Nef}^- - \text{MFI Nef}^+) / \text{MFI Nef}^- \times 100\%$, such that Nef function less than or greater than the control Nef_{SF2} is represented by values of <100% or >100%, respectively.

T cell recognition assay

T cell receptor (TCR)-based reporter cell co-culture assay was as previously described [14, 21]. Briefly, "effector" cells (5×10^4) were prepared by electroporation of Jurkat cells with plasmids encoding TCR- α and β chains specific for the HLA-A*02:01-restricted HIV-1 Gag FK10 epitope [Gag₄₃₃₋₄₄₂: FLGKIWPSYK], human CD8 α chain (Invivogen), and NFAT-driven luciferase reporter (Affymetrix). "Target" cells (5×10^4) were prepared by infecting Jurkat- A02_{B57CT} cells with HIV-1 encoding *nef* sequences of interest. Co-culture results in TCR-mediated calcium-flux and robust luciferase expression in the "effector" cells. After 6hr, NFAT-driven luciferase signal in effector cells was quantified.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 6.0. Non parametric tests were applied for all analyses. The Mann-Whitney U test and Wilcoxon test were used for un-paired and paired analyses, respectively. Statistical significance was defined as $p < 0.05$.

Results and Discussion

Non-Ser9 Nef genotype in HIV-infected patients harboring protective HLA alleles

A naturally occurring, but relatively minor, non-Ser variant at position 9 of subtype C HIV-1 Nef has been reported to be associated with enhanced HLA-B downregulation function and reduced antigen recognition by HIV-specific CD8⁺ T cells [15]. Because known protective HLA alleles are dominated by HLA-B, we hypothesized that the Nef non-Ser variant may be associated with impaired protective effect in HIV-infected patients. Indeed, in the South African cohort (N=668), the HIV-infected individuals harboring protective HLA alleles (defined as HLA-B*57, HLA-B*58:01, and HLA-B*81:01) [5] exhibited significantly lower plasma viral load than those harboring non-protective HLA alleles (p=0.0001) (Table 1). Prevalence of the non-Ser9 genotype was comparable (p=0.64) between those with and without protective HLA alleles (Table 1). Among the non-Ser9 variants, arginine was the most prevalent amino acid residue in both HIV-infected individuals with protective (n=4) and non-protective HLA alleles (n=15).

Functional effects of non-Ser9 nef genotype

We next examined the effects of the non-Ser9 Nef variant on several important

Nef functions. Seven *nef* clonal sequences encoding Ser (N=5) and Arg (N=2) at position 9 were isolated from seven protective HLA allele⁺ subjects. Mutations were introduced into these clones, from Ser to Arg or *vice versa* at position 9, to generate paired mutant Nef clones.

To test HLA-A and HLA-B downregulation activity, we used Jurkat cells that had been engineered to stably express the HLA-A*02:01 molecule (A02) and A02 chimeric molecule whose cytoplasmic tail had been replaced with that of HLA-B*57:01 (A02_{B57CT})^[15]. We transfected these cells with various Nef clones and quantified A02 or A02_{B57CT} downregulation activity. The ability to downregulate A02 was not significantly different between Nef clones encoding Ser9 and Arg9 (p=0.15); whereas the ability to downregulate A02_{B57CT} was significantly different between these genotypes (p=0.009) (Figure 1A). Notably, Nef clones encoding Ser9 downregulated A02 to greater extent than A02_{B57CT} (p=0.01), consistent with the previous reports^[10, 14, 15]; whereas Nef clones encoding Arg9 downregulated A02 and A02_{B57CT} comparably (p=0.64) (Figure 1A). Next, CEM cells were transfected with the same set of Nef clones and CD4 downregulation activity was quantified. No significant difference was observed between Nef clones encoding Ser9 and Arg9 (p=0.11) (Figure 1A). In addition, recent reports demonstrated that host proteins SERINC5, and to a lesser extent SERINC3, inhibit HIV-1 infectivity

and that this restriction activity is antagonized by Nef through downregulation of the SERINC proteins [22, 23]. A JTAG cell line with stable knockout of SERINC3 and 5 (JTAG-S3/5^{-/-}) was transfected with the same set of Nef clones together with a plasmid encoding SERINC5 harboring the internal HA tag (S5-iHA). Despite substantial functional heterogeneity across Nef clones, no significant difference was observed in Nef's ability to downregulate S5-iHA between the Nef sequences encoding Ser9 and Arg9 (p=0.93) (Figure 1A).

To test the effect of the enhanced HLA-B downregulation on T cell recognition of HIV-1-infected cells, we used previously established reporter T cell assay [14]. The Jurkat-A02_{B57CT} cells, as target cells, were infected with a set of paired recombinant viruses encoding Nef Ser9 or Arg9, and then co-culture with the effector cells. The cells infected with the virus encoding Arg9 were less susceptible to TCR recognition, compared to those infected with the virus encoding Ser9 (p=0.01) (Figure 1B).

Collectively, Nef clones encoding non-Ser9 isolated from protective allele⁺ patients associated with enhanced HLA-B downregulation ability and impaired T cell recognition of HIV-infected cells.

Nef-9 effects on plasma viral load

Finally, we analyzed the effect of Nef non-Ser9 on plasma viremia of protective allele⁺ subjects. Seven out of 85 protective allele⁺ subjects in South Africa were infected with viruses encoding Nef non-Ser9 (Table 1) and showed significantly higher plasma viral load compared to those encoding Nef Ser9 ($p=0.02$) (Figure 1C). We also performed the same analysis in an independent cohort from Botswana, where, as in South Africa, subtype C is the predominant HIV-1 subtype. However, it is reported that the protective effect of HLA-B*57, HLA-B*58:01 and HLA-B*81:01 has diminished in Botswana and instead HLA-B*42:01 is exhibited as a protective allele ^[17]. Indeed, in our Botswana cohort, subjects harboring HLA-B*42:01 showed lower plasma viral load compared to those harboring other alleles; whereas subjects harboring HLA-B*57, HLA-B*58:01 and HLA-B*81:01 did not show the protective effect (Table 1). Eight out of 67 protective (HLA-B*42:01) allele⁺ subjects were infected with viruses encoding Nef non-Ser9 (Table 1) and again showed significantly higher plasma viral load compared to those encoding Nef Ser9 ($p=0.01$) (Figure 1C). These data clearly indicate that Nef non-Ser9 genotype associates with higher plasma viral load in protective allele⁺ subjects infected with HIV-1 subtype C. However, it remains elusive why subtype C HIV-1 maintains Nef non-Ser9 in minority of infected hosts. This polymorphism may result in a detrimental effect on another Nef function (other than downregulation of CD4, HLA-A, and SERINC5 tested

here) at the expense of enhanced HLA-B downregulation function. Alternatively, Nef non-Ser9 may associate with one or more HLA alleles (as selective pressure from CTL responses), although non-Ser9 was not overrepresented in subjects harboring any HLA alleles including the protective ones in our dataset. Further analyses are needed to reveal what factor drives the amino acid variation at Nef position 9 in subtype C HIV-1 infection.

Conclusions

Our study shows that the naturally occurring non-Ser9 mutation selectively enhances HLA-B downregulation function while the other Nef functions including downregulation of HLA-A, CD4, and SERINC5 remain unaltered. This enhanced HLA-B downregulation conferred by Nef non-Ser9 is associated with higher plasma viral loads in HIV-1 subtype C-infected patients harboring protective HLA alleles in two independent cohorts. Thus, Nef non-Ser9 is a newly identified viral genotype that allows us to predict diminished immune control by protective HLA alleles.

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F.M. and T.U. designed the study; F.M., I.N., and M.T. performed the experiments; M.M., J.M., T.N., and P.G. provided access to patient samples and analyzed

clinical data; F.M., I.N., M.T., M.M., J.M, and T.U. analyzed data; and F.M., I.N., J.M, T.N., and T.U wrote the paper. All authors read and approved the final manuscript.

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Figure legends

Fig. 1 Effects of Nef non-Ser9 on Nef functionality and plasma viral load

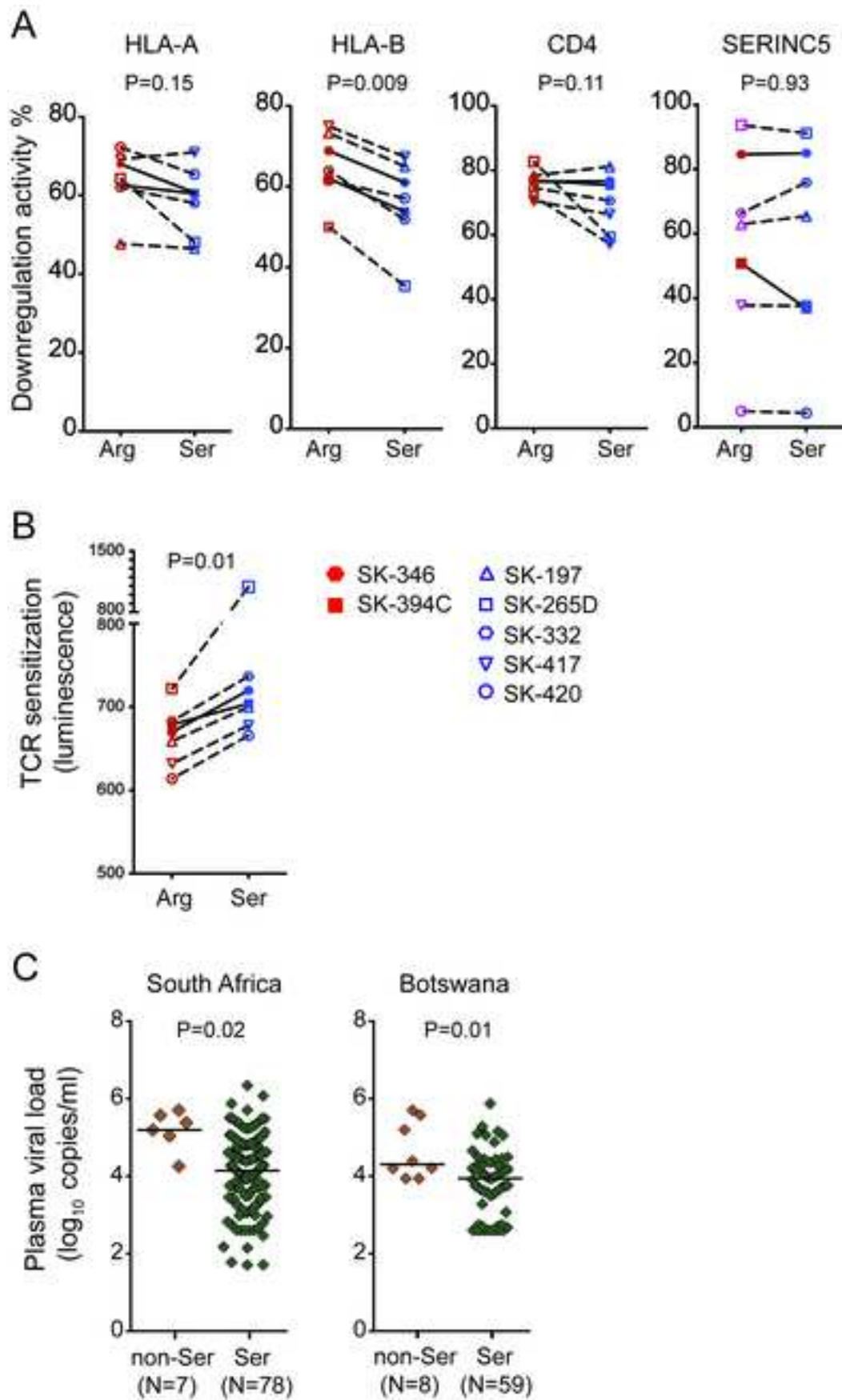
Effect of Nef non-Ser9 on several key Nef functions in vitro (*panel A*) and Effect of Nef's ability to downregulate HLA-B on TCR sensitization (*panel B*). Patient-derived Nef clones (N=7) from protective allele⁺ individuals harboring Arg (SK-346 and SK-394C) and Ser (SK-197, SK-265D, SK332, SK-417, and SK-420) at position 9 are cloned into pcDNA-GFP expression construct. After mutations are introduced from Ser to Arg or *vice*

versa to generate paired mutant Nef clones, Nef's ability to downregulate HLA-A (A02), HLA-B (A02_{B57CT}), CD4, and SERINC5 as well as Nef's effect on TCR sensitization were determined in Jurkat cell derivatives. Each plot represents mean of triplicate assays. Statistical analysis was done by Wilcoxon matched pairwise test. Effect of Nef non-Ser9 on plasma viral load in two independent cohorts (*panel C*). Plasma viral load values obtained in the protective HLA allele⁺ individuals infected with subtype C HIV-1 encoding Nef non-Ser9 and Ser9 in South Africa and Botswana. Protective HLA alleles are defined by B*57, HLA-B*58:01 and HLA-B*81:01 in South Africa, and B*4201 in Botswana. Horizontal lines represent median values and statistical analysis was performed using the Mann-Whitney U test.

Table 1 Summary of subjects infected with HIV-1 subtype C

Cohort	HLA alleles	Plasma viral load (log ₁₀ copy/ml) ^a			Nef non-Ser9 (prevalence) ^b		
		HLA(+)	HLA(-)	p value ^c	HLA(+)	HLA(-)	p value ^d
South Africa N=668	B*57, B*5801, B*8101	4.17 (3.60-4.90) N=85	4.65 (4.01-5.19) N=583	0.0001	7 (8.2%) N=85	38 (6.5%) N=583	0.64
Botswana N=193	B*4201	4.00 (3.50-4.39) N=67	4.38 (3.80-4.89) N=126	0.001	8 (11.9%) N=67	12 (9.5%) N=126	0.46
	B*57, B*5801, B*8101	4.16 (3.70-4.86) N=43	4.32 (3.77-4.85) N=150	0.39	3 (6.9%) N=43	13(8.6%) N=150	0.79

^a median and IQR in parenthesis; ^b the number of cases and % prevalence in the cohort; ^c Mann-Whitney U test; ^d Fisher's exact test



Revised Figure 1 Mwimanzi et al