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Dynamic range of Nef-mediated evasion of HLA class II-restricted immune responses in early HIV-1 infection

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ABSTRACT

HLA class II-restricted CD4$^+$ T lymphocytes play an important role in controlling HIV-1 replication, especially in the acute/early infection stage. But, HIV-1 Nef counteracts this immune response by down-regulating HLA-DR and up-regulating the invariant chain associated with immature HLA-II (Ii). Although functional heterogeneity of various Nef activities, including down-regulation of HLA class I (HLA-I), is well documented, our understanding of Nef-mediated evasion of HLA-II-restricted immune responses during acute/early infection remains limited. Here, we examined the ability of Nef clones from 47 subjects with acute/early progressive infection and 46 subjects with chronic progressive infection to up-regulate Ii and down-regulate HLA-DR and HLA-I from the surface of HIV-infected cells. HLA-I down-regulation function was preserved among acute/early Nef clones, whereas both HLA-DR down-regulation and Ii up-regulation functions displayed relatively broad dynamic ranges. Nef's ability to down-regulate HLA-DR and up-regulate Ii correlated positively at this stage, suggesting they are functionally linked *in vivo*. Acute/early Nef clones also exhibited higher HLA-DR down-regulation and lower Ii up-regulation functions compared to chronic Nef clones. Taken together, our results support enhanced Nef-mediated HLA class II immune evasion activities in acute/early compared to chronic infection, highlighting the potential importance of these functions following transmission.

1. Introduction

It is becoming evident that, in addition to HLA class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTL), HLA class II (HLA-II)-restricted CD4$^+$ T lymphocytes also play an important role in controlling HIV-1 replication *in vivo*, particularly at the acute/early infection stage [1,2]. CD4$^+$ T cells provide help to mount antiviral CTL responses and humoral responses [3]. A key role of HLA-II restricted CD4$^+$ T cells in HIV-1 control is supported by the reports that 1) CD4$^+$ T cells exhibit direct killing of HIV-infected cells by recognizing HIV-derived peptides presented by HLA-II molecules [4,5], 2) HIV-1 can acquire escape mutations from HLA-II-restricted CD4$^+$ T cell responses [6], and 3) associations between vigorous HIV-specific CD4$^+$ T cell responses in acute/early infection and subsequent viral control [7].

The HIV-1 accessory factor Nef is a highly variable ~27 kDa myristoylated protein that is required for pathogenesis *in vivo* [8,9]. Nef exhibits multiple immune evasion functions, including down-regulation of HLA-DR [10] and up-regulation of the invariant chain associated with immature HLA-II (Ii) from the surface of HIV-1-infected cells [11], as well as down-regulation of HLA class I (HLA-I) [12,13]. It is thought that down-regulation of HLA-DR and up-regulation of Ii can subvert HLA-II-restricted antigen presentation and thus antigen-specific CD4$^+$ T cell stimulation [14,15], while down-regulation of HLA-I can subvert HLA-I-restricted CTL responses [12]. The stable expression of Ii prevents peptide binding to mature HLA-II [16], and immature HLA-II associated with Ii on the cell surface is nonfunctional in stimulating CD4$^+$ T cells [17]. As such, Nef-mediated up-regulation of Ii is thought to dampen the HLA-II-restricted immune response.

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Variation in Nef-mediated HLA-I down-regulation at the population level has been extensively characterized using clinically isolated sequences from acute [18,19] and chronic infection stages [20], and from elite controllers [21]. In contrast, few studies have investigated the functional breadth of Nef-mediated evasion of HLA-II-restricted immune responses. For example, HLA-DR down-regulation function was evaluated in a small number of patient-derived Nef clones from chronic infection [11] and in the context of pediatric infection [22]. Functional differences in Nef-mediated Ii up-regulation has also been studied in chronic progressive infection [20]. However, the extent of heterogeneity of these two Nef functions during acute/early infection remains uncharacterized.

To investigate this question, we collected 47 *nef* alleles from individuals with acute/early HIV-1 subtype B infection and analyzed Nef's ability to down-regulate HLA-DR, up-regulate Ii, and down-regulate HLA-I. Whereas HLA-I down-regulation function was relatively well conserved among Nef clones, both HLA-DR down-regulation and Ii up-regulation functions displayed relatively broad dynamic ranges at this infection stage. Nef's ability to down-regulate HLA-DR and up-regulate Ii showed a statistically significant positive correlation, suggesting these activities are functionally linked *in vivo*. We thus report here substantial characteristics of Nef's immune evasion functions at the acute/early infection stage.

2. Materials and methods

2.1. Study subjects

A total of 47 acute/early progressors (AP) identified during acute/early HIV-1 infection as defined by the Acute Infection Early Disease Research Program (AIEDRP) criteria [23] from cohorts in Boston and New York, USA; Berlin, Germany; and Sydney, Australia as described previously [18]. For each AP, the earliest available plasma sample was studied; these were collected at a median of 59 [IQR 34-72] estimated days post-infection. The median CD4 count and pVL among AP were 491 [IQR 402-727] cells/mm^3 and 930,000 [IQR 12,600–631,000] RNA copies/ml. A total of 46 chronic progressors (CP) (median pVL 80500 [IQR 25121-221250] RNA copies/ml); median CD4 count 292.5 [IQR 72.5-440] cells/mm^3) were studied as described previously [20]. All AP and CP were untreated at the time of sample collection and infected with HIV-1 subtype B. This study was approved by the institutional review board of Massachusetts General Hospital, Boston USA; all participants provided written informed consent.

2.2. Preparation of recombinant viruses

Patient-derived *nef* genes were amplified from plasma HIV-1 RNA by nested RT-PCR as described earlier [20] and cloned into the pNL4.3 backbone plasmid. A median of 3 *nef* clones was sequenced per patient; and a single clone with an intact Nef reading frame closely resembling the original bulk sequence was selected for analysis. Genbank accession numbers for clonal *nef* sequences are LC043169-LC043215 (AP) and JX440926-JX440971 (CP). Recombinant NL4.3 viruses harboring *nef* from HIV strain SF2 (NL4.3-Nef$_{SF2}$), and lacking *nef* (NL4.3ΔNef) were used as positive and negative controls, respectively. Infectious viruses were generated as described [24]. Briefly, HEK-293T cells (1×10^6 cells) were transfected with each proviral clone (5 μg) and the DNA encoding vesicular stomatitis virus envelope glycoprotein (1 μg). Virus-containing supernatants were harvested 48hr following transfection.

2.3. Analysis of cell surface HLA molecules

A human lymphoblastoid cell line, 721.221 stably expressing HLA-A*24:02 (provided by M. Takiguchi, Kumamoto University, Japan) was exposed to the recombinant HIV-1 for 48hr, followed by staining with the following antibodies and reagents: anti-HLA-DR allophycocyanin-Cy7 antibody (clone: L243, BioLegend Co.), anti-Ii Alexor Fluor 647 antibody (clone: LN2, BioLegend Co.), and pan specific anti-HLA-I PE (clone: w6/32, BioLegend Co.), anti-HIV-1 p24 Gag FITC (clone: KC57, Beckman–Coulter), and 7-amino-actinomycin D (BioLegend Co). Mean fluorescence intensity (MFI) of each receptor in live p24^{Gag} positive and negative subsets was determined by flow cytometry (FACS Verse; BD Biosciences). Results were expressed as the mean of triplicate experiments, normalized to control strain NL4.3-Nef$_{SF2}$, such that values >100% and <100% indicated increased or decreased activity, respectively.

2.4. Statistical analysis

Non-parametric statistics were employed throughout. The Mann–Whitney U test was used to test for differences between two groups; correlations were performed using Spearman's test. All tests of significance were two-tailed; a p-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Simultaneous detection of Nef activity in modulation of cell surface expression of HLA-DR, Ii, and HLA-I

A cell line 721.221 that had been engineered to stably express HLA-A*24 also expresses HLA-DR and Ii endogenously, thereby enabling us to simultaneously analyze Nef's ability to modulate cell surface expression of these molecules. To test this, recombinant NL4.3 viruses deficient in Nef (NL4.3ΔNef; negative control) or expressing a laboratory strain NL4.3-Nef$_{SF2}$ (positive control) were tested for their ability to modulate cell surface expression of these molecules. As expected, after infection of these cells with NL4.3ΔNef, no substantial difference in surface expression level of these receptors was observed (Fig. 1). In contrast, after infection with NL4.3-Nef$_{SF2}$, HLA-DR surface expression within the p24 Gag$^+$ (*i.e.* HIV-infected) subset was reduced to a mean ± SD of 74.8 ± 2.0% relative to that of the p24 Gag$^-$ (*i.e.* HIV-uninfected) subset. Furthermore, cell surface expression of Ii within infected cells increased to a mean ± SD of 390.8 ± 15.0% of that of uninfected cells (Fig. 1). Cell surface HLA-I expression in infected cells was reduced to a mean ± SD of 27.9 ± 3.0% compared to uninfected cells.

3.2. Differential activity of Nef clones at acute/early infection

Using this assay, five *nef* alleles derived from acute/early progressor (AP) subjects were initially tested for their ability to modulate cell surface expression of HLA-DR, Ii, and HLA-I. Nef function differed markedly based on the specific clone tested (Fig. 2A). For example, Nef of Subject #1 down-regulated HLA-DR to 75% of that of the uninfected (*i.e.* p24$^-$) subset – a level of function that was comparable to that of the NL4.3-Nef$_{SF2}$ control strain. Nef of Subject #5 down-regulated HLA-DR to 65% of that of the uninfected subset, a level of function that exceeded that of the NL4.3-Nef$_{SF2}$ control strain (Fig. 2A). Up-regulation of Ii and down-regulation of HLA-I of these Nef clones also varied to some extent (Fig. 2A).

To quantify the dynamic range of these Nef functions, we extended this analysis to 47 Nef clones isolated from 47 patients recruited during acute/early infection stage (one sample per

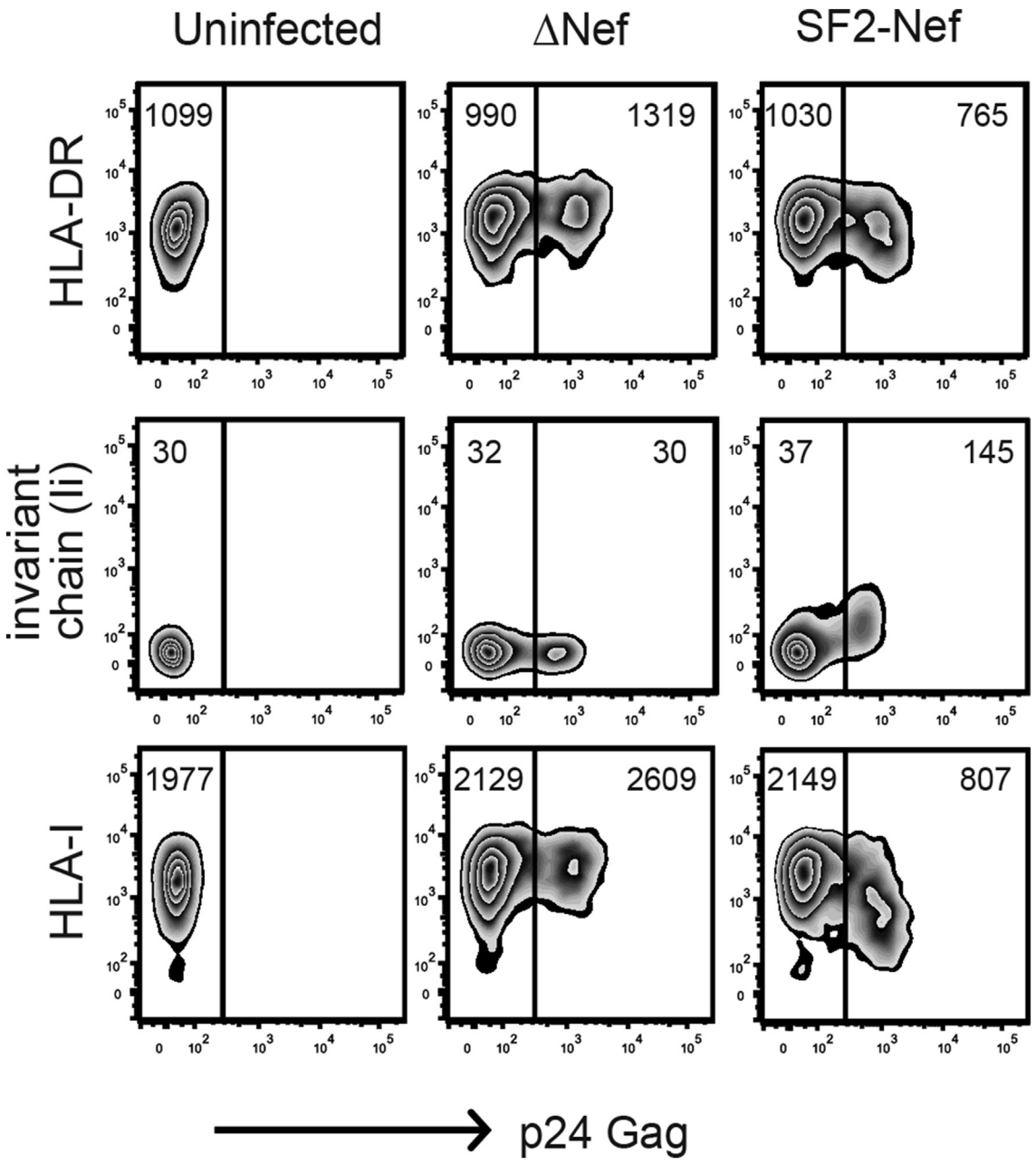

Fig. 1. Modulation of cell surface expression of HLA-DR, Ii, and HLA-I by recombinant HIV-1 NL4.3 control strains. Representative flow cytometry plots of uninfected 721.221 cells and those infected with recombinant NL4.3 viruses deficient in Nef (NL4.3ΔNef) or carrying Nef_{SF2} (NL4.3-Nef_{SF2}). Cells were stained with antibodies to HLA-DR, Ii, and HLA-I, followed by intracellular staining with antibody to p24 Gag. Numbers indicate the mean fluorescence intensities (MFI) of p24 Gag negative (uninfected) and positive (infected) cell subsets.

patient). In this analysis, the activity of each patient-derived Nef clone was normalized to that of the control strain NL4.3-Nef_{SF2}, which was set to 100%. Relative to control Nef_{SF2}, the 47 AP Nef clones exhibited a relatively broad range of HLA-DR down-regulation activities: median 136.7 [IQR 126.9-155.5]%. The range of Nef-mediated Ii up-regulation activities was similarly broad: median 81.4 [IQR 42.83-121.1]%. In contrast, HLA-I down-regulation activities of these 47 Nef clones were relatively conserved and comparable in magnitude to that of the control strain NL4.3-Nef_{SF2}: median 106.4 [IQR 101.48-114.05]%) (Fig. 2B). Importantly, the range of HLA-I down-regulation functions measured in the present study using a recombinant virus method was highly consistent with that previously observed in this same AP cohort using a Nef transfection assay that assessed down-regulation of HLA-A*02 [18]. Moreover, Nef-mediated HLA-DR down-regulation activity (but not other Nef functions) correlated inversely with plasma viral load (Spearman, $R = -0.42$, $p = 0.003$). However, we observed no other relationships between Nef activities and other clinical parameters such as CD4 count or estimated days post-infection.

Our observation of relatively conserved HLA-I down-regulation activity supports this as an essential Nef function *in vivo* during acute/early HIV-1 infection. In contrast, the broader dynamic ranges of HLA-DR down-regulation and Ii up-regulation functions suggest inter-patient difference in Nef's ability to evade HLA-II-restricted immune responses. Alternatively, some functions may serve as surrogates of other Nef activities not assessed, such as enhancement

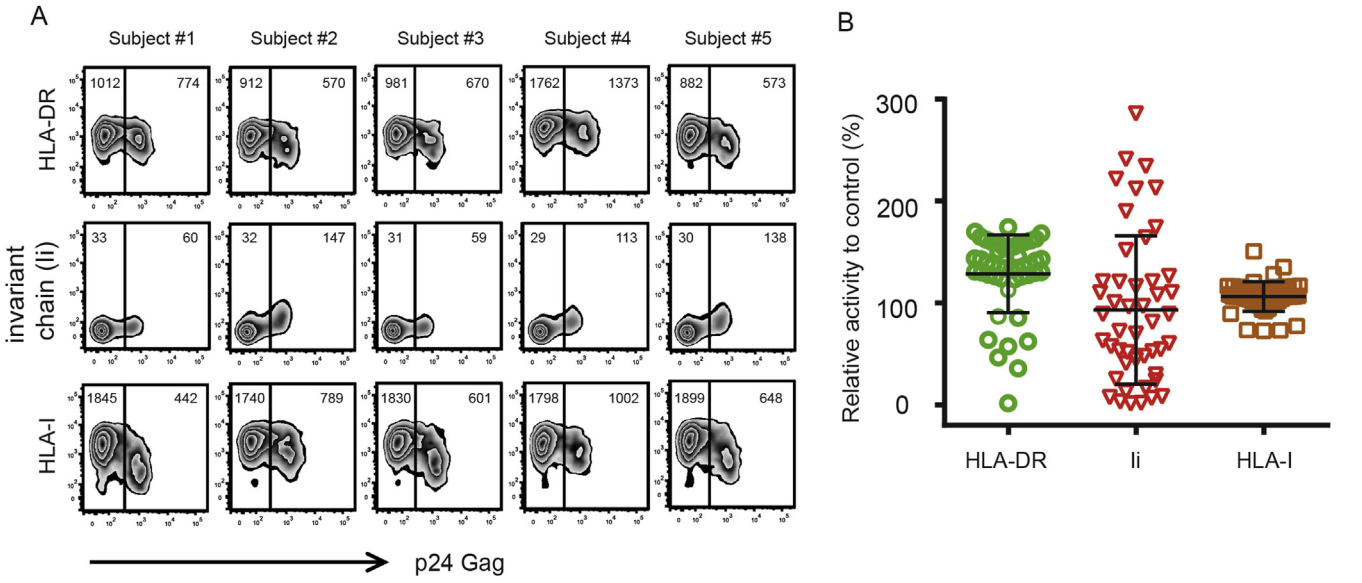

Fig. 2. Modulation of cell surface expression of HLA-DR, Ii, and HLA-I by acute/early Nef clones. 721.221 cells were infected with recombinant viruses expressing Nef clones derived from five subjects with acute/early infection to examine Nef-mediated modulation of cell surface expression of HLA-DR, Ii, and HLA-I (*panel A*). Cells were stained as in Fig. 1. Representative flow cytometry plots are shown; numbers indicate mean fluorescence intensities (MFI) of p24 Gag negative and positive subsets. The same functional activity of 47 Nef clones derived from acute/early infection, normalized to that of control strain NL4.3-$Nef_{SF2,}$ are shown (*panel B*). The values presented are means of 3 independent assays. Horizontal bars denote median and interquartile ranges.

of virion infectivity and CD4 down-regulation. Indeed, in a previous study of this AP cohort we demonstrated that enhancement of virion infectivity correlated positively with plasma viral load [18]. Also, a mechanistic link between Nef-mediated CD4 and Ii modulation is suggested by the observation that both functions involve interaction of Nef with clathrin adaptor protein 2 [25]. Nonetheless, our results extend our understanding of Nef functions that facilitate immune evasion in naturally occurring HIV-1 sequences soon after infection.

3.3. *Functional co-dependencies of immune–evasion activities of Nef*

Mutational studies of laboratory Nef strains have revealed the genetic determinants of immune evasion functions. HLA-DR down-regulation is mediated by Nef motifs $EEEE_{62\text{-}65}$ and $PxxP_{72\text{-}78}$ [11], while Ii up-regulation is mediated by Nef motifs $EE_{154,155}$, $LL_{164,165}$, $DD_{174,175}$, and $ERE_{177\text{-}179}$ [11,26]. Nef motifs responsible for HLA-I down-regulation that extensively investigated are $RxR_{17\text{-}19}$ [26], M_{20} [27], $EEEE_{62\text{-}65}$ [28], and $PxxP_{72\text{-}78}$ [29]. However, the extent to which naturally-occurring genetic variation influences Nef's function remains incompletely known. For example, mutagenesis studies implicate Nef's $EEEE_{62\text{-}65}$ and $PxxP_{72\text{-}78}$ motifs in HLA-DR and HLA-I down-regulation function, but these motifs are highly conserved among the AP Nef clones examined here, suggesting secondary functional determinants. Similarly, the extent to which Nef's various activities are functionally independent remains incompletely characterized.

Pairwise correlations of Nef functions in our patient-derived sequences revealed a positive relationship between HLA-DR down-regulation and Ii up-regulation (Spearman's $R = 0.33$, $p = 0.002$) (Fig. 3A), suggesting shared molecular mechanisms and/or

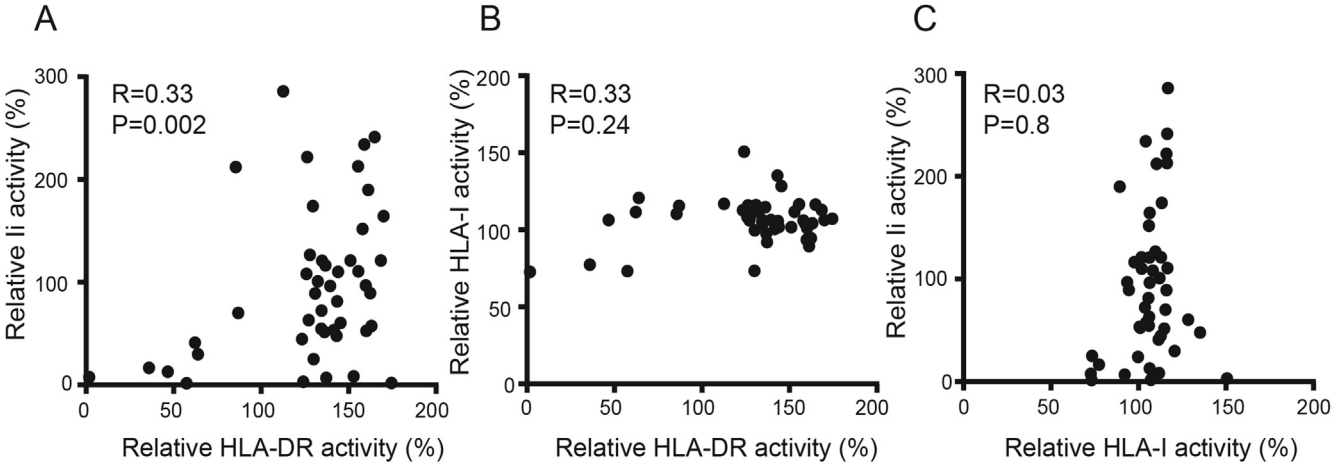

Fig. 3. Functional associations among Nef activities Graphs depicting pairwise correlations among the relative functional activities of acute/early Nef sequences: HLA-DR *vs.* Ii (*panel A*), HLA-DR *vs.* HLA-I (*panel B*), and HLA-I *vs.* Ii (*panel C*). Spearman's correlation test was used.

functional complementarity. Indeed, HLA-DR and Ii modulate antigen presentation [15]. Specifically, increased cell surface expression of Ii can be detrimental to this process as it increases internalization of HLA-DR molecules [30] and interferes with their cellular trafficking [31], thus impairing HLA-II-restricted antigen presentation [16]. Also, higher HLA-DR down-regulation and Ii up-regulation activities in some AP subjects suggests that there are substantial inter-patient differences in Nef's inherent ability to evade HLA class II-mediated immune responses in acute/early infection.

In contrast, HLA-I down-regulation showed no correlation with HLA-DR down-regulation or Ii up-regulation (Fig. 3B and C), suggesting that these functions are differentially regulated *in vivo*. This observation is consistent with a previous study from our group showing no correlation between HLA-I down-regulation activity and CD4 down-regulation, Ii up-regulation, enhancement of virion infectivity or stimulating of viral replication in PBMC by Nef clones derived from the same cohort of chronic progressors studied here [20]. Of note, no inverse relationships were observed between any of the three Nef immune-evasion activities tested in this study arguing against functional tradeoffs or the existence of particular substitutions or domains that enhance one function at the expense of another. Finally, we performed an exploratory sequence/function analysis in our dataset to identify Nef amino acids associated with particular functions; however no significant associations were identified after correction for multiple comparisons (not shown).

3.4. Differential Nef activities at different HIV-1 infection stages

Finally, we wanted to compare Nef's immune evasion activities between AP and CP, because Nef activities have shown to vary over the infection course [32,33]. Phylogenetic analyses of 47 AP and 46 CP clones showed no evidence of recent shared ancestry nor major clustering by infection stage (Fig. 4A); furthermore, no Nef amino acid residues were significantly enriched in either AP or CP cohorts (not shown).

Relative to control $Nef_{SF2,}$ the 46 CP Nef clones exhibited a median HLA-DR down-regulation value of 112.25 [IQR 81.98-124.7]%, a median Ii up-regulation value of 130.16 [IQR 88.85-166.42]% and a

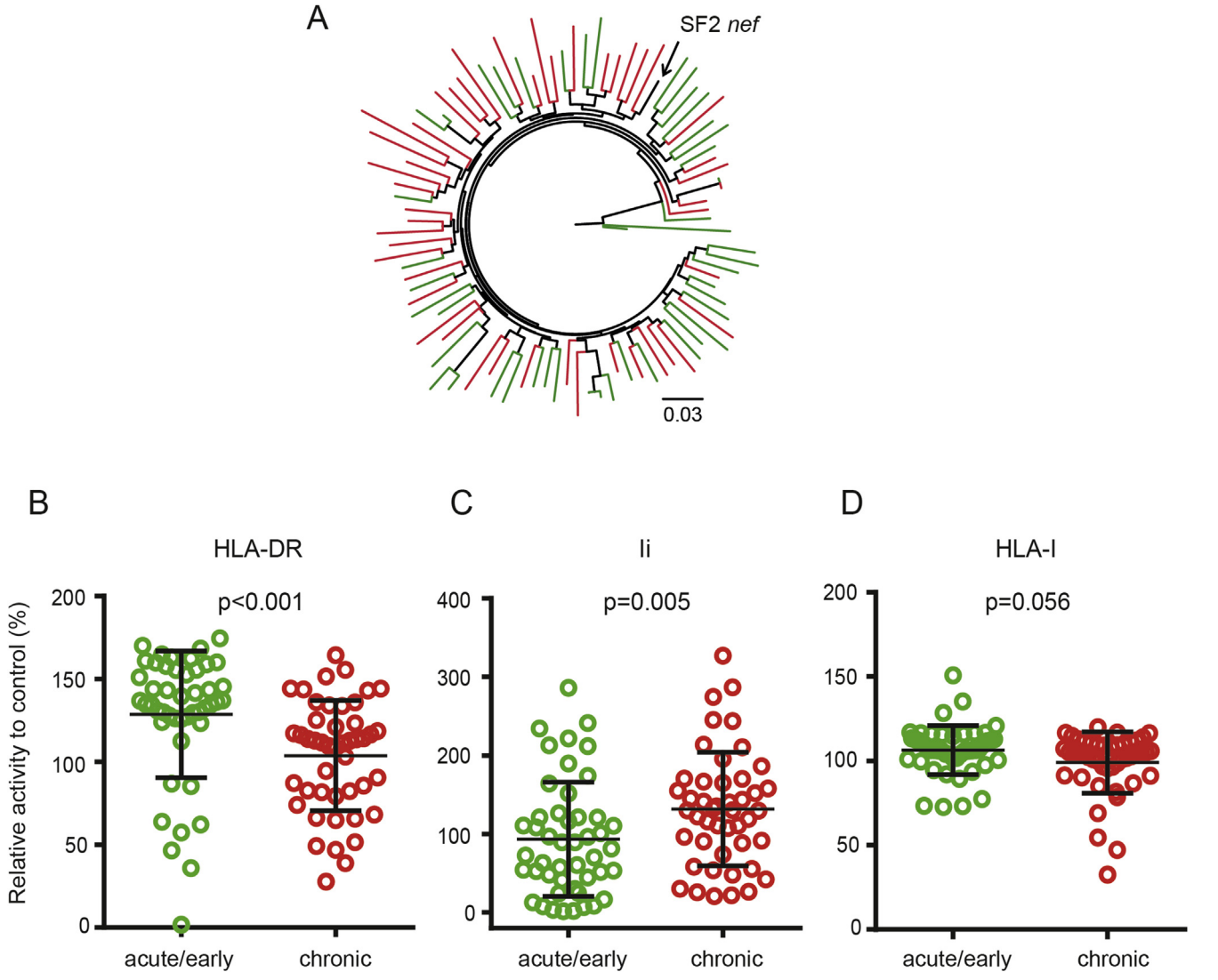

Fig. 4. Genetic and functional differences between infection stages. A maximum-likelihood phylogenetic tree constructed from *nef* sequences derived from N = 47 acute/early (*green*), N = 46 chronic progressors (*red*), and a control strain SF2 *nef* (*black*) (*panel A*). Functional comparison between acute/early and chronic Nef clones with respect to their HLA-DR down-regulation (*panel B*), Ii up-regulation (*panel C*), and HLA-I down-regulation (*panel D*) activities. The values presented are means of 3 independent assays and normalized to that of control strain Nef_{SF2} which was set to 100%. Horizontal bars denote median and interquartile ranges. Statistical significance was assessed using the Mann–Whitney U test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

median HLA-I down-regulation value of 104.62 [95.98-109.86]%) (Fig. 4). Importantly, the present data obtained for HLA-I down-regulation activity of CP Nef were highly consistent with those previously derived from testing the same set of Nef clones [20] (Spearman, $R = 0.82$, $p = 2.0 \times 10^{-7}$). Overall, Nef clones from AP exhibited higher HLA-DR down-regulation activities than those from CP ($p < 0.001$) (Fig. 4B), while Ii up-regulation activities of AP Nef clones were lower than those of CP ($p = 0.005$) (Fig. 4C). HLA-I down-regulation activities of AP Nef clones were marginally, though not significantly higher than that of CP Nef clones, ($p = 0.056$) (Fig. 4D).

Taken together, our results support the preservation of Nef's HLA class I down-regulation activity throughout infection. In contrast, HLA-DR down-regulation and Ii up-regulation functions of Nef varied between acute/early versus chronic stages, suggesting differential requirements for these two Nef functions during the infection course.

Viral genetic and functional studies of patient-derived Nef clones face numerous challenges and limitations. Although three Nef immune-evasion activities were simultaneously assessed in a human lymphoblastoid cell line, 721.221, Nef-mediated modulation of cell surface expression of HLA-DR, Ii, and HLA-I may vary in other cell types [11,15,34,35]. Furthermore, although HLA-DR down-regulation and Ii up-regulation functions are correlated, the relative contribution of these functions on impairment of HLA class II-mediated antigen presentation to $CD4^+$ T cells remains unclear. Similarly, the cross-sectional nature of the AP and CP cohorts precludes our ability to conclusively determine whether Nef-mediated HLA-DR down-regulation and Ii up-regulation function changes throughout infection within a given host. Nevertheless, our study characterizes the dynamic range of Nef immune evasion functions during acute/early infection, thus enhancing our understanding of the contribution of this key viral protein to HIV-1 pathogenesis shortly after transmission.

Conflict of interest

None.

Acknowledgments

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