Impaired Nef function is associated with early control of HIV-1 viremia

Running title: Early Nef function in HIV controllers

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Abstract

Host and viral factors influence the HIV-1 infection course. Reduced Nef function has been observed in HIV-1 controllers during the chronic phase, but the kinetics and mechanisms of Nef attenuation in such individuals remain unclear. We examined plasma RNA-derived Nef clones from 10 recently infected individuals who subsequently suppressed viremia to less than 2000 RNA copies/mL within one year post-infection (acute controllers) and 50 recently infected individuals who did not control viremia (acute progressors). Nef clones from acute controllers displayed lower ability to downregulate CD4 and HLA class I from the cell surface and reduced ability to enhance virion infectivity compared to those from acute progressors (all p<0.01). HLA class I downregulation activity correlated inversely with days post-infection (Spearman’s R=-0.85, p=0.004) and positively with baseline plasma viral load (Spearman’s R=0.81, p=0.007) in acute controllers, but not in acute progressors. Nef polymorphisms associated with functional changes over time were identified in follow-up samples from six controllers. For one such individual, mutational analyses indicated that four polymorphisms selected by HLA-A*31 and B*37 acted in combination to reduce Nef steady state protein levels and HLA class I downregulation activity. Our results demonstrate that relative control of initial HIV-1 viremia is associated with Nef clones that display reduced function, which in turn may influence the course of HIV-1 infection. Transmission of impaired Nef sequences likely contribute in part to this observation; however, accumulation of HLA-associated polymorphisms in Nef that impair function also suggests that CD8+ T-cell pressures play a role in this phenomenon.

Keywords: acute infection, Nef, protein function, CTL escape, HIV pathogenesis
Importance

Rare individuals can spontaneously control HIV-1 viremia in the absence of antiretroviral treatment. Understanding the host and viral factors that contribute to the controller phenotype may identify new strategies to design effective vaccines or therapeutics. The HIV-1 Nef protein enhances viral pathogenesis through multiple mechanisms. We examined the function of plasma HIV-1 RNA-derived Nef clones isolated from 10 recently infected individuals who subsequently controlled HIV viremia, compared to those from 50 individuals who failed to control viremia. Our results demonstrate that early Nef clones from HIV controllers displayed lower HLA class I and CD4 downregulation activity, as well as a reduced ability to enhance virion infectivity. The accumulation of HLA-associated polymorphisms in Nef during the first year post-infection was associated with impaired protein function in some controllers. This work highlights the potential for host immune responses to modulate HIV pathogenicity and disease outcome by targeting CTL epitopes in Nef.
Introduction

Rare HIV-1 infected individuals who suppress plasma viral loads (pVL) to less than 50 RNA copies/mL (“elite controllers”, EC) or to less than 2000 RNA copies/mL (“viremic controllers”) in the absence of antiretroviral therapy provide an opportunity to identify host and viral determinants of spontaneous HIV-1 control that could aid development of vaccines or novel therapeutics. However, the mechanisms underlying the HIV-1 controller phenotype, particularly those acting at the acute/early infection stage, remain incompletely defined.

Host genetic and immune factors influence HIV-1 control. Protective human leukocyte antigen (HLA) class I alleles, notably B*57 and B*27 have been associated with lower pVL and delayed rates of disease progression in natural history (1, 2) and genome wide association studies (3-5), and these alleles are enriched among HIV-1 controllers (6-8). Polyfunctional cytokine production and rapid perforin or granzyme expression are also frequently observed in CD8+ cytotoxic T lymphocytes (CTL) from controllers (9-11), suggesting that qualitative immune characteristics also influence viremia (12). While CTL responses targeting the HIV-1 Gag protein are likely to be central mediators of immune control (13), responses against Nef might also be beneficial (14, 15). Altogether, cellular immune responses recognizing mutationally constrained viral epitopes presented by certain HLA alleles are believed to be key to effective HIV-1 suppression (16).

Viral genetic factors also influence HIV-1 pathogenesis. *In vitro* viral replication capacity independently associates with early clinical markers of pathogenesis (17). Moreover, Gag, Pol, and Nef proteins from chronically-infected EC have consistently displayed relative functional attenuation (18-21), suggesting that impaired viral function is a hallmark of this phenotype (22). In many cases, host expression of protective HLA alleles and/or the presence of
specific HLA-associated escape mutations are associated with even lower viral protein function in these individuals, suggesting that adaptation to host HLA-restricted CTL can further attenuate HIV-1. Consistent with the observed “genetic fragility” (i.e. mutationally sensitive nature) of the HIV-1 p24 capsid protein as a result of its critical role in virion assembly (23), functional costs of CTL escape have been observed most readily in Gag (24-28), but immune-driven functional costs have also been demonstrated in Pol (18, 29) and Env (19, 30). The observation that compensatory mutations that offset the functional impact of escape arise more frequently in progressors compared to controllers (28, 31) (likely due in part to severely reduced viral replication in the latter individuals) further complicates the study of HIV-1 adaptation to its host and its pathogenic consequences.

A major gap in our knowledge of HIV-1 controllers is a poor understanding of early events following infection that contribute to clinical outcome in these individuals (22). HIV-1 controller cohorts are generally comprised of individuals identified during the chronic phase; as such, sequence/function relationships in early controller viruses and the role of early host immune responses in modulating viral pathogenesis remain incompletely defined. A recent study by our group demonstrated reduced in vitro replication capacities of recombinant HIV-1 strains encoding gag-protease from 18 recently infected individuals who subsequently controlled pVL to less than 2000 RNA copies/mL compared to those from 45 individuals who progressed with higher viremia, which was likely due to both transmission of less-fit strains and selection of CTL escape mutations by protective HLA alleles (32). It remains to be determined whether other viral proteins in controllers exhibit similar early evidence of relative attenuation. In particular, we hypothesized that HIV-1 Nef, a ~27kD accessory protein that enhances viral pathogenesis, may play an early role in determining HIV-1 control. Nef interacts with a number...
of host proteins and performs multiple functions (33), including downregulation of cell-surface
CD4 (34) and HLA class I (35), upregulation of HLA class II invariant chain (CD74) (36),
enhancement of virion infectivity (37), stimulation of viral replication in PBMC (38), and
alteration of T cell receptor signaling (39, 40). Nef’s relevance to pathogenesis is illustrated by
exceptionally slow disease progression exhibited by individuals infected with nef-deleted or nef-
defective HIV-1 (41-44).

To enhance our understanding of early Nef function in HIV-1 controllers, we examined
the sequence and in vitro function of plasma RNA-derived clonal nef sequences from 10 recently
infected individuals who subsequently suppressed pVL to less than 2000 RNA copies/mL in the
absence of treatment (acute controllers; “AC”), and 50 recently infected individuals who failed to
suppress viremia (acute progressors, “AP”). Nef clones were assessed for their ability to
downregulate CD4 and HLA class I, and to enhance virion infectivity. Longitudinal assessments
were also performed on Nef clones from six AC for whom follow-up samples were available,
including one individual for whom the impact of HLA-associated polymorphisms on Nef
function was explored in detail using site-directed mutagenesis. Overall, our results indicate that
HIV-1 viremia control is associated with the presence of early Nef clones that display reduced in
vitro function. This reduced function is likely a result of acquisition of partially attenuated viral
strains at transmission, combined with the subsequent selection of escape mutations by host CTL
responses that impair one or more Nef activities.
Methods

Study subjects. This study was approved by the Research Ethics Boards at Simon Fraser University (Burnaby, BC Canada) and the Massachusetts General Hospital (Boston, MA USA). As described previously (32), participants were identified at sites in the USA, Australia, and Germany during acute/early HIV-1 infection as defined by the Acute Infection Early Disease Research Program (AIEDRP) criteria (45). Study subjects included N=10 acute controllers (AC) who spontaneously suppressed HIV-1 plasma viremia to less than 2,000 RNA copies/mL during the first year of infection, and N=50 acute progressors (AP) who failed to suppress HIV-1 viremia to less than 2,000 RNA copies/mL during this time (Figure 1). The earliest available plasma samples were studied: for AC and AP these were collected an estimated median of 72 [Interquartile Range (IQR) 57-99] days and 56 [IQR 39-75] days post-infection respectively (Table 1). Additional follow-up analyses were conducted for six AC for whom samples were available (Table 1). All participants remained untreated for a minimum of 1 year post-infection.

Viral (HIV-1 Nef) and Host (HLA class I) genotyping. HIV-1 nef gene products were amplified from plasma RNA as described previously (32). Briefly, nested RT-PCR was performed using HIV-1 specific primers, where the second round forward and reverse primers included EcoRI and SacII restriction sites, respectively, used for cloning. Amplicons were ligated into pRES2-EGFP (Clontech), transformed into E. coli 10G cells (Lucigen), and selected on LB agar plates containing kanamycin. Colonies were screened by restriction enzyme digest to verify nef insertion. Nested RT-PCR amplicons and pRES2-nef-EGFP clones were sequenced bi-directionally on 3130xl or 3730xl automated DNA sequencer (Applied Biosystems, Inc.). Chromatograms were analyzed using Sequencher v5.0 (Genecodes) or RECall (46). In
bulk sequences, nucleotide mixtures were called if the subdominant peak height exceeded 25% (Sequencher) or subdominant peak area exceeded 20% (RECall) of the dominant peak. All sequences were confirmed to be HIV-1 subtype B using the Recombinant Identification Program (RIP; http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html). Nef sequences were aligned to HXB2 using an in-house tool based on the HyPhy platform (47) and phylogenetic analysis was conducted using PhyML (48, 49). Each clone was also compared to the original bulk sequence to enumerate the number of amino acid differences between them. These steps ensured that each Nef clone encoded an intact open reading frame, was free of gross genetic defects (e.g. large deletions), and that clones chosen for functional analysis were representative of each individual’s circulating viral quasi-species. HLA class I typing was performed using genomic DNA extracted from PBMC or plasma using sequence-based methods (50). All unique clonal nef sequences from AC have been deposited in Genbank (accession numbers KJ996014-KJ996066).

CD4 and HLA class I downregulation assays. Nef-mediated CD4 and HLA class I downregulation function was assessed by flow cytometry following transient transfection, as described previously (21). Briefly, 3 x 10^5 CEM-A*02+ cells in Opti-MEM medium (Life Technologies) were transfected with 5 µg of pIRES2-nef-EGFP by electroporation (square wave: 250 V, 2000 µF, infinite Ω, 25 ms; BioRad Gene Pulser MXcell), and recovered in R10+ medium (RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 µg of streptomycin/ml; Sigma). After 24 hours, unfixed transfected cells were stained with APC-labeled anti-CD4 and PE-labeled anti-HLA-A*02 antibodies (BD Biosciences), and surface expression of these molecules was detected using a Guava easyCyte 8HT flow cytometer (Millipore). For each Nef clone, the median fluorescence intensity (MFI) of
CD4 or HLA class I on GFP-positive (Nef-producing) cells was normalized to that of cells transfected with a positive control (pIRES2-EGFP containing SF2 strain Nef; NefSF2) and a negative control (empty pIRES2-EGFP) using the following formula: 

\[
\frac{\text{MFI}_{\text{patient Nef}} - \text{MFI}_{\text{negative control}}}{\text{MFI}_{\text{positive control}} - \text{MFI}_{\text{negative control}}}
\]

Normalized values less than 1.0 represent downregulation activities lower than the positive control NefSF2, while values greater than 1.0 represent downregulation activities higher than NefSF2. The calculated value of the negative control is zero. Each clone was tested in a minimum of 3 replicates, and results averaged.

**Virion infectivity assays.** Nef clones were transferred into a pNL4.3 backbone plasmid as described (51) and confirmed by sequencing. Recombinant viruses encoding nef from HIV-1 strain SF2 (NL4.3-nefSF2) and lacking nef (NL4.3Δnef) served as positive and negative controls, respectively. Infectious viruses were generated by transfection of HEK-293T cells with each proviral clone and virus-containing supernatant was harvested at 48 hours, as described previously (52). Viral stocks were quantified using p24Gag ELISA (ZeptoMetrix Corp.) and aliquots stored at −80°C until use. Recombinant virus infectivity was determined by exposing 10^4 TZM-bl cells (NIH AIDS Reagent Program, Cat. #8129) to 3 ng p24Gag virus stock followed by chemiluminescence detection 48 hours later, as described (53). Infectivity values represent the mean of triplicate experiments, normalized to NL4.3-nefSF2, such that values less than 1.0 or greater than 1.0 indicated lower or higher activity than the positive control strain, respectively.

**Western blot analysis.** Steady-state Nef protein levels were measured by Western blot for a subset of AC and AP clones. For this, 5 x 10^6 CEM-A*02+ cells were transfected with 10 µg of pIRES2-Nef using electroporation. After 24 hours, cells were pelleted, lysed and analyzed as...
described previously (52), with modifications as follows. Total cell lysates were subjected to SDS-PAGE using Mini-PROTEAN TGX 4-20% gels (Bio-Rad Laboratories) and proteins were electro-blotted onto PVDF membrane. Nef protein was detected using a polyclonal rabbit (1:4000; NIH AIDS Reagent Program, #2949) (54) or sheep (1:2000; NIBSC Center for AIDS Reagents, #ARP444) antibody followed by staining with secondary donkey anti-rabbit (1:30000; GE Healthcare) or anti-sheep (1:35000; Jackson ImmunoResearch) antibody. For all experiments, expression of beta-actin was assessed simultaneously using primary mouse anti-actin (1:20000, Sigma) and secondary goat anti-mouse (1:20000, Jackson ImmunoResearch) antibodies. Band intensities were quantified with ImageQuant LAS 400 (GE Healthcare) and compared to positive control SF2 Nef.

**Site-directed mutagenesis.** Nef variants were generated using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions or using a PCR-based overlap extension method (55). Mutations were validated by bidirectional sequencing of the full-length nef insert prior to functional analysis.

**Statistics.** Statistical analyses were conducted in Prism 5.0 (Graphpad Software, Inc). The Mann-Whitney U test was used to compare Nef functions between groups. In patients with longitudinal (baseline vs. follow-up) Nef functional assessments, the Wilcoxon paired test was employed. Spearman’s correlation was used to investigate relationships between different Nef functions or with clinical HIV-1 parameters (e.g. CD4 T-cell count and plasma viral load). All tests were two-tailed with p<0.05 considered statistically significant.
Results

Isolation of early Nef clones from HIV-1 controllers and progressors

Clinical data from HIV-1 infected individuals enrolled at sites in the USA, Germany, and Australia were retrospectively screened to identify persons with acute/early infection who subsequently maintained viremia control below 2,000 RNA copies/mL during the first year post-infection, and for whom a baseline sample within 150 days of their estimated date of infection was available for study (32) (Figure 1). A comparison group of 50 acute progressors (AP) enrolled at the same sites, who displayed pVL greater than 2,000 RNA copies/mL over a similar period of time, were also assessed. Consistent with a previous study undertaken in this cohort (32), no significant enrichment of protective HLA class I alleles were observed in AC compared to AP (e.g. HLA-B*57 prevalence was 20% in AC versus 10% in AP, Fisher’s exact test p=0.3) (data not shown).

The timing of baseline sample collection differed slightly between cohorts: for AC, samples were collected a median 72 [interquartile range (IQR) 57-99] days following the estimated infection date, while those for AP were collected a median 56 [IQR 39-75] days post-infection (Mann-Whitney, p=0.065) (Table 1). Baseline pVL was also significantly lower in AC, which is not surprising given that these patients were selected based on their ability to subsequently control viremia. Nevertheless, we were concerned that differences in baseline pVL could be due in part to the earlier enrollment times for AP (who thus had a higher likelihood of begin sampled closer to peak viremia) and that this could potentially bias downstream analyses. Exclusion of AP with the earliest enrollment times (prior to 40 days post-infection) allowed us to identify a subset of 37 AP with baseline sample dates (median 72 [IQR 53-88] days) that were comparable to those of AC (p=0.42) (Table 1). For the remainder of our study, all primary
analyses were performed on the entire AP cohort, with secondary analyses performed using the AP subset matched for enrollment date. Results were consistent in all cases, even when not explicitly indicated in the text.

Participant-derived nef alleles were amplified from baseline plasma by nested RT-PCR and sequenced. As expected from an acutely-infected population, the median number of amino acid mixtures observed in bulk Nef HIV sequences was low: a median 0 [IQR 0-1] for AC compared to 0 [IQR 0-2] for AP (p=0.3). Nef amplicons were cloned into a CMV expression plasmid, and individual clones were isolated and resequenced to confirm an intact Nef open reading frame. For each AC, a minimum of 5 baseline nef clones (median 7, range 5-13) were isolated and sequenced. For each AP, 1-13 baseline clones (median 2) were isolated and sequenced. All clones were HIV-1 subtype B. Each Nef clone was verified to cluster closely with the original bulk plasma HIV RNA sequence (Figure S1). Overall, the median number of amino acid differences between clonal and original bulk sequences was 0 [IQR 0-1] for both AC and AP. For each patient, the first isolated clone was arbitrarily designated a “representative clone” while the most frequently observed clone was designated the “predominant clone” (Figure S1). AC and AP Nef sequences did not display substantial intra-cohort clustering in a combined phylogeny (Figure 2A and Figure S1), consistent with previous analyses of gag and pol genes from these persons (32). Together with previous results, our data indicate that early HIV-1 control in AC is unlikely to be due to gross HIV sequence defects or recent descent from a common attenuated viral ancestor. We confirmed Nef expression by Western blot for the predominant clone isolated from all 10 AC, and compared detection levels to a random subset of 10 AP. No significant differences in band intensity were observed between cohorts.
(representative data shown in Figure 2B), suggesting that Nef proteins from AC exhibited comparable in vitro stability to those from AP.

Nef clones from AC display impaired CD4 and HLA class I downregulation function

The ability of Nef clones to downregulate cell-surface CD4 and HLA class I molecules was examined at 24 hours following transfection using flow cytometry (Figure 3). For each AC, the function of 2-5 unique clones was assessed. Results were normalized to those of a positive control (SF2Nef) that displays strong CD4 and HLA class I downregulation activities (21, 56), such that values less than 1.0 indicate lower Nef function and values greater than 1.0 indicate higher Nef function relative to SF2Nef. A negative control plasmid lacking nef did not downregulate either surface protein (Figures 3A and 3B); as such, its activity is 0 in this assay.

Based on an assessment of a representative clone isolated from each individual, the ability of Nef to downregulate CD4 in AC (median 0.94 [IQR 0.87-0.98]) was modestly lower than that observed in AP (0.99 [0.97-1.02]) (p=0.004) (Figure 3C). In addition, HLA class I downregulation activity was markedly lower in AC (0.83 [0.65-0.93]) compared to AP (1.00 [0.94-1.06]) (p<0.001) (Figure 3D). Secondary analyses using the “date of enrollment matched” AP subset were consistent with lower function of AC compared to AP Nefs (CD4: p=0.01, HLA: p=0.001; data not shown), indicating that our findings are not simply attributable to biases in sample collection date. Similarly, analyses comparing the median downregulation functions of all Nef clones isolated in AC vs AP also indicated significantly lower activities in the latter group (CD4: p=0.01, HLA: p<0.001, Figure S2 and data not shown), suggesting that our observations were not driven by biases related to analyses of only a single clone per patient.
Together, these results suggest that Nef-mediated HLA downregulation (and to a lesser extent CD4 downregulation) may contribute to early viremia control.

**Nef clones from AC show reduced ability to enhance virion infectivity**

We next constructed recombinant NL4.3 viruses encoding a representative early clonal nef sequence from 10 AC and 41 AP, and assessed virion infectivity. No difference in viral production was observed between stocks containing AC and AP nef sequences, as determined by p24\(^{\text{Gag}}\) ELISA, although there was a trend towards higher values among AC-derived viruses (Figure 4A). TZM-bl cells were inoculated with virus (3 ng p24\(^{\text{Gag}}\)) and infectivity was measured at 48 hours post-infection using chemiluminescence assays. Data were normalized to a positive control virus NL4.3-\(\text{nef}_{\text{SF2}}\), as described above. All recombinant viral stocks displayed infectivity values greater than NL4.3\(\Delta\text{nef}\) negative control, whose mean activity was 0.02 (standard deviation 0.009; N=8 replicates) in this assay. Overall, viruses encoding AC-derived nef alleles displayed significantly lower infectivity values (median 0.59 [IQR 0.44-0.71]) compared to those from AP (1.16 [0.71-1.51]) (p=0.003) (Figure 4B). Results were consistent in a secondary analysis using “date of enrollment matched” AP clones (p=0.01; data not shown).

**HLA class I downregulation activity correlates with baseline clinical parameters in AC**

We next wanted to characterize the relationship between these three Nef functions and markers of clinical status in the AC and AP cohorts (Table 2 and Table 3, respectively; and Figure S3). As expected for samples obtained during early infection following peak viremia, pVL correlated inversely with estimated days post-infection in both AC (Spearman; R= -0.77, p=0.01) and AP (R= -0.38, p=0.007). Consistent with previous observations in other cohorts (21,
Nef CD4 and HLA class I downregulation functions correlated positively in both AC (R=0.74, p=0.02) and AP (R=0.56, p<0.001). Given that these activities are largely genetically separable in mutational studies of HIV-1 reference strains (58, 59), this result supports the existence of secondary genetic determinants lying outside critical motifs in patient-derived Nef isolates that may modulate function and/or protein stability. Of note, the ability of Nef to enhance virion infectivity correlated significantly with baseline pVL in AP (R=0.54, p<0.001) but not AC. This may be due to the generally low activity seen among AC clones for this function, but it warrants further investigation. Surprisingly, Nef’s HLA class I downregulation activity correlated inversely with estimated days post-infection (R= -0.85, p=0.004) and positively with pVL (R=0.81, p=0.007) in AC. Similar associations were not seen for AP, despite enhanced statistical power to observed differences in this group. Together, these results suggest that longitudinal alterations in Nef function may contribute to spontaneous HIV-1 control in AC, but not AP.

Changes in Nef activity observed over time in some AC participants

To directly explore changes in Nef function over time in AC, we cloned plasma RNA Nef sequences at a single follow-up time point, collected a median of 283 [IQR 161-427] days after the date of baseline enrollment, from 6 AC individuals for whom a sample was available (Table 1). A median of 4 Nef clones (range 1-11) was isolated per patient at the follow-up time point. CD4 and HLA class I downregulation activities were measured for all unique clones, while a single nef allele was used to generate recombinant NL4.3 stocks to assess viral infectivity enhancement (Figure 5). In a paired analysis comparing the function of a representative Nef clone from baseline and follow-up samples, we observed no overall differences in the ability of
Nef to downregulate CD4 or HLA class I, or to enhance viral infectivity (Wilcoxon paired test; all p>0.5). This suggests that Nef functional alterations (whether they be increases or decreases over time) are not generalizable or consistent phenomena during the first year post-infection. However, we did observe substantial changes in these Nef functions in individual cases.

To address this further, we analyzed clonal nef sequences from baseline and follow-up time points in 4 AC who displayed the greatest changes in Nef function over time to identify polymorphisms that might contribute to these observations. Notably, in all 4 individuals, most observed sequence changes were linked to an HLA class I allele expressed by the host or to reversion of transmitted viral polymorphisms associated with HLA alleles presumably expressed in previous a host, as defined by reference lists of HLA-associated polymorphisms derived from published statistical association studies (60, 61). For example, selection of 4 polymorphisms associated with HLA-A*31 and B*37 in subject AC01 (discussed below) was accompanied by a 15% reduction in HLA class I downregulation activity, while in subject AC02, reductions in Nef-mediated infectivity enhancement corresponded with the appearance of a B*35-associated polymorphism, H40Q, and reversion to consensus at D63E (located in the acidic E62EEE65 cluster that binds PACS-2 (62)). In AC05, an 18% reduction in CD4 downregulation activity was observed in conjunction with D54E and the B*39-associated polymorphism N162S. Finally, recovery of wild type HLA class I downregulation function in follow-up clones from AC07 coincided with 10 polymorphisms in the C-terminal half of Nef, the most notable being reversion of a well-characterized A*23/A*24-associated escape mutation (F135Y) that is reported to alter this activity (63). These results indicate that certain host HLA-associated CTL pressures can select mutations that impact Nef function.
*HLA-driven polymorphisms act in combination to reduce Nef function in AC01*

AC01 was of particular interest because this subject displayed the greatest reduction in HLA class I downregulation activity and also controlled pVL to less than 50 RNA copies/mL within the first year. Of the 11 clones isolated from this patient at 383 days post-infection, 10 were identical and encoded 4 amino acid polymorphisms (R19K, H40R, M182L, and V194A) that together resulted in a 15% reduction in HLA class I downregulation compared to baseline clones (Figure 6A). The remaining follow-up clone contained a fifth polymorphism (E149K) and displayed 15% and 13% reductions in HLA class I and CD4 downregulation activities, respectively (data not shown). E149 is located at the base of a central loop in Nef (amino acids 149-179) that binds to AP-2 (64), suggesting that an E149K charge-reversal mutation may alter interactions with this clathrin adapter complex that are required for CD4 downregulation.

Published HLA-association studies (60, 61) indicate that mutations at Nef residues 19, 40, 182, and 194 are highly significantly associated with HLA-A*31 (codons 19 and 194) and B*37 (Nef codons 40 and 182), two alleles expressed by AC01. R19K is located in a putative A*31-restricted CTL epitope predicted based on this individual’s autologous baseline viral sequence (K\textsubscript{9}LAGWPTIR\textsubscript{19}, the site of mutation is underlined; prediction made using NetMHCpan2.8 (65, 66)); while V194A lies within a published A*31-restricted epitope (S\textsubscript{188}LAFRHI\textsubscript{VAR}\textsubscript{196}), although the baseline nef sequence in AC01 is slightly different (TLA\textsubscript{189}FHHVAR). Likewise, H40R is found within a published CTL epitope (R\textsubscript{35}DLEKHG\textsubscript{AI}\textsubscript{36}) detected in B*37-expressing vaccine recipients (68), while M182L resides in a predicted A*31-restricted epitope (V\textsubscript{180}LEWRFDSR\textsubscript{188}, based on consensus nef) as well as a putative B*37-restricted epitope that is predicted in AC01’s baseline viral sequence (K\textsubscript{178}EVLMKFDTRL\textsubscript{189}).
Viral adaptation to CTL in early infection commonly involves the appearance of transient polymorphisms in or near the epitope that is under immune pressure, from which the final escape form is ultimately selected (67, 69, 70). Baseline Nef clones from AC01 exhibited polymorphisms near codon 40 that are consistent with this phenomenon, notably D36N and E38K (Figure 6A). Furthermore, clones isolated at days 51, 65, and 93 encoded D36N, E38G, and K39T (adjacent to final escape site at codon 40) and H192Y (adjacent to the final escape site at codon 194) (data not shown).

These observations motivated us to undertake a more in-depth analysis of Nef-mediated HLA class I downregulation activity in AC01. Of these 4 polymorphisms, only R19K is located near a residue or motif that has been associated with this function (namely M20) (Figure 6B) (58, 63, 71-77). To directly examine their impact on Nef, forward mutations corresponding to each of the 4 polymorphisms were introduced individually into the baseline clone that most closely resembled consensus B/HXB2 nef (AC01b_F) and backward mutations were introduced into the predominant follow-up clone (AC01f_A) to revert each of these polymorphisms individually. Single amino acid changes in the baseline clone had only a modest impact on HLA class I downregulation function (~3% reduction in each case, Figure 6C). None of the forward mutations altered baseline Nef protein stability appreciably, but each backwards mutation restored expression of the follow-up clone to a level that was comparable to the baseline sequence (Figure 6D). Since none of these polymorphisms appeared to have a major affect on Nef function individually, and since Nef expression levels and in vitro function could be largely restored by reversion of any one of these mutations alone, our results suggest that all 4 HLA-associated polymorphisms acted in combination to reduce Nef stability, yielding reduced HLA class I downregulation activity in later clones from AC01. In summary, the emergence of HLA-
associated mutations that additively impaired Nef function over time in subject AC01 strongly suggests that Nef functional impairment in this individual is not solely attributable to transmission of an attenuated virus, but rather a direct consequence of HLA immunogenetics and CD8+ T cell selection \textit{in vivo}.
Discussion

In this study, we examined nef alleles from 10 recently infected individuals who controlled HIV-1 plasma viremia to less than 2,000 RNA copies/mL in the absence of antiretroviral therapy (acute controllers, AC) and 50 non-controllers (acute progressors, AP). While baseline Nef clones from both cohorts were generally functional (all patient-derived clones exhibited activities greater than the ΔNef control), those from AC displayed a median 5% reduced ability to downregulate surface CD4, 17% reduced ability to downregulate HLA class I molecules and 57% poorer enhancement of virion infectivity, compared to those from AP. Although we cannot draw definitive conclusions regarding the clinical significance of these observations, even small changes at the level of each virus-infected cell could be amplified through multiple cycles of infection, thereby influencing pathogenesis.

We previously observed that multiple Nef functions were impaired in chronically HIV-1 infected EC (21), and results presented here demonstrate that similar impairments can be seen at early times post-infection. We therefore conclude that modest functional attenuation of Nef is a relatively common occurrence in individuals who spontaneously control HIV-1. Our results indicate that this may be attributed to both transmission of less-fit HIV-1 strains as well as the acquisition of HLA-associated mutations that compromise Nef function at early times post-infection – and that the precise mechanism may be unique to each individual. Notably, the in vitro replication capacity of gag-protease recombinant viruses is also reduced in this AC cohort through apparently similar means (32). Together, our data support a model where the early function of major HIV-1 structural and accessory proteins has a long-term impact on viremia control and disease progression, which is consistent with studies highlighting the impact of transmitted/founder viral sequence on clinical outcome (78-80).
In a cross-sectional analysis, baseline HLA class I downregulation function correlated inversely with baseline sampling date in Nef clones from AC (Table 2). This is consistent with early attenuation of Nef following infection as the transmitted/founder virus adapts to its new host through selection of CTL escape mutations. A similar correlation was not observed for AP (Table 3), suggesting that early changes in Nef sequence in these individuals were not associated with reduced function. Intriguingly, Nef-mediated enhancement of virion infectivity correlated directly with pVL in AP, but not AC; however, additional studies will be necessary to confirm a potential link between this Nef function in establishment of viral load set-point in HIV-infected persons who progress normally.

Longitudinal analyses of Nef clones from six AC revealed substantial changes in Nef function in individual patients over time, though neither the direction nor magnitude of these changes were consistent across patients (possibly due to the small number of individuals examined). However, among the four AC who exhibited substantial functional increases or decreases over time, these could be attributed to unique polymorphisms that arose in the first year of infection. Many were consistent with HLA-driven adaptations in the current host, or to reversion of HLA-associated polymorphisms present in the baseline sequence; however, only one (F135Y) had been previously described to affect Nef function (63).

Our detailed forward and reverse mutagenesis of clones from AC01 demonstrated that multiple substitutions acted synergistically to affect Nef function in this individual. In particular, four polymorphisms selected by HLA-A*31 and B*37 were required to reduce the ability of Nef to downregulate HLA class I. Together, these results indicate that host CTL pressure can select for escape mutations that directly reduce Nef function over the infection course, indicating that, in at least a subset of controllers, functional impairments are not solely attributable to acquisition
of attenuated strains, but rather a direct consequence of HLA-restricted CD8+ T cell selection in vivo. Reduced HLA class I downregulation function in follow-up clones from AC01 correlated with lower steady-state Nef protein levels in cells (Figure 5), and site-directed mutagenesis confirmed that all four HLA-associated polymorphisms were necessary to see both effects.

Intriguingly, we observed a 42% reduction in protein levels for the quadruple Nef mutant, but only a 15% reduction in its HLA class I downregulation function. This suggests that there may be a threshold effect, such that modest reductions in Nef expression are tolerated without a significant loss of function. It is well-established that a greater amount of Nef protein is required to promote efficient downregulation of HLA class I (as compared to CD4) (81); as such, HLA class I downregulation activity is likely to be more sensitive to alterations in protein expression or stability. Impaired HLA class I downregulation was thus likely due to reduced Nef expression in this case, rather than disruption of a critical functional motif. Further studies will be necessary to determine if this is a consistent observation for patient-derived Nef clones in other contexts.

Some limitations of this study merit discussion. First, the AC cohort represents a rare group of individuals enrolled during acute/early infection, therefore the number of individuals and specimens available were limited. When possible, we tested the downregulation activity of multiple Nef clones from each AC, allowing a better assessment of functional diversity within each individual. Similar results for CD4 or HLA class I downregulation were obtained when we analyzed a representative clone, a predominant Nef clone or the median function of all clones, indicating that our conclusions are not biased by the isolate used. Second, Nef performs multiple roles in virus-infected cells and we have examined only three in vitro functions in this study. Our results demonstrate significant impairments in CD4 and HLA class I downregulation function as well as infectivity enhancement for early Nef clones from AC. We have not assessed...
the ability of Nef to modulate other cellular proteins or functions (including TCR signaling) or potential differences in viral replication capacity using multi-cycle assays. Further investigation would therefore extend the observations of this study; however, we believe that such work is unlikely to alter our conclusions significantly. Similarly, it has been reported that the effect of Nef on host HLA expression may differ between HLA alleles (82). While we assessed only downregulation of HLA-A*02 here, a previous study by our group observed a strong correlation between the ability of patient-derived Nef clones to downregulate A*02 and B*07 in this same assay system (56). Changes in Nef function were observed between baseline and follow-up clones sampled up to 516 days later. Thus, it is not clear when these changes occurred. Even in the case of AC01, for whom some intermediate specimens were available, the 4 polymorphisms examined became fixed between days 93 and 383. Finally, even though acute controllers were recruited, on average, less than 2.5 months following their estimated date of infection, we cannot rule out the possibility of very early selection and fixation of immune-driven mutations prior to sampling. Despite these limitations, we believe that the data presented here provide a unique window into early events during natural HIV-1 infection that can help us to better understand pathways and mechanisms of viral attenuation that may contribute to the controller phenotype.

We conclude that impaired early Nef function is associated with spontaneous control of HIV-1 viremia. This observation is consistent with previous data showing reduced Nef activity in an independent cohort of chronically HIV-1 infected EC (21), and suggests that early functional deficits in Nef – via acquisition of modestly attenuated sequences at transmission or very rapid fixation of host-driven viral mutations - contribute to spontaneous viral control. Importantly, changes in Nef function during the first year post-infection frequently coincided with the appearance or reversion of HLA-associated polymorphisms, indicating that within-host
CTL pressures can drive the selection of mutations that modulate Nef activity in at least some individuals. These results highlight the potential for host immune responses to modulate HIV pathogenicity and disease outcome by targeting epitopes in Nef.
Acknowledgements

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We thank the clinicians, staff, and participants of the Acute Infection Early Disease Research Program (AIEDRP) for their critical contributions to this project. The following reagents were obtained from the NIH AIDS Research and Reference Reagents Program, Division of AIDS, NIAID: TZM-bl, Cat #8129, from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.; and HIV-1 Nef antiserum (rabbit), #2949, from Ronald Swanstrom. Sheep antiserum to HIV-1 Nef (Cat# ARP444) was obtained from the Centre for AIDS Reagents, NIBSC (UK) and was kindly donated by M. Harris.
References


Table 1. Description of Acute Controllers (N=10)

<table>
<thead>
<tr>
<th>Participant ID</th>
<th>HLA*</th>
<th>eDPI</th>
<th>pVL</th>
</tr>
</thead>
</table>
| AC01           | A*01:01/A*31:01  
                 |      | 33   | 25000 |
| Follow-up:     | 383  | 50   |      |
| AC02           | A*02:01/A*32:01  
                 | B*35/B*44       
                 | C*04:01/C*05    | 72   | 15200 |
| Follow-up:     | 469  | 774  |      |
| AC03           | A*02:01/A*24:01  
                 | B*15:01/B*41:01 
                 | C*03/C*17       | 86   | 1490  |
| AC04           | A*02:01/A*24:02  
                 | B*27:05/B*35:12 
                 | C*01:02/C*01:02 | 72   | 1140  |
| Follow-up:     | 251  | 50   |      |
| AC05           | A*24:02/A*31:01  
                 | B*39:01/B*52:01 
                 | C*07:02/C*12:02 | 71   | 7160  |
| Follow-up:     | 587  | 250  |      |
| AC06           | A*01:01/A*02:01  
                 | B*44:02/B*57:01 
                 | C*05/C*06:02    | 135  | 440   |
| AC07           | A*25:01/A*68:01  
                 | B*18:01/B*57:01 
                 | C*06:02/C*12:03 | 98   | 23900 |
| Follow-up:     | 204  | 1720 |      |
| AC08           | A*02:01/A*31:01  
                 | B*08:01/B*44:02 
                 | C*05/C*07       | 102  | 572   |
| AC09           | A*01:01/A*68:02  
                 | B*08:01/B*44:02 
                 | C*05/C*07       | 52   | 45700 |
| Follow-up:     | 268  | 110  |      |
| AC10           | A*01:01/A*01:01  
                 | B*15:17/B*49:01 
                 | C*07:01/C*12:03 | 59   | 22200 |

Median [IQR] for AC (N=10) at baseline

<p>| | | |</p>
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<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td></td>
<td>72 [62-95]</td>
<td>11,180 [998-24175]</td>
</tr>
</tbody>
</table>

Median [IQR] for all AP (N=50) at baseline

<p>| | | |</p>
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<tr>
<td></td>
<td>56 [39-75]</td>
<td>337,500 [35925-750100]</td>
</tr>
</tbody>
</table>

Median [IQR] for AP >40 eDPI (N=37) at baseline

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<tbody>
<tr>
<td></td>
<td>72 [53-88]</td>
<td>186,000 [22350-750005]</td>
</tr>
</tbody>
</table>

*Abbreviations: HLA: Human Leukocyte Antigen; eDPI: estimated days post-infection; pVL: plasma viral load
*Mann-Whitney p-values compared to AC cohort
### Table 2. Correlation Analyses for Acute Controllers (N=10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Statistic</th>
<th>Baseline pVL</th>
<th>Nef CD4 downregulation</th>
<th>Nef HLA downregulation</th>
<th>Nef Infectivity enhancement</th>
</tr>
</thead>
</table>
| eDPI          | R         | -0.77
               | 0.007       | -0.52                   | 0.1                      | 0.004                      | 0.6                       |
| Baseline      | p         | 0.48         | 0.81                    | 0.01                    | 1.0                        |
| pVL           | p         | 0.2          | 0.02                    | 0.9                     | 0.15                       |
| CD4 downregulation | R   | 0.74       | -0.05                  |                          |                            |
| HLA           | R         | 0.15         |                         |                          |                            |

Abbreviations: pVL: plasma viral load; HLA: Human Leukocyte Antigen; eDPI: estimated days post-infection

*Spearman R and p-values; significant associations are highlighted in bold type.*
Table 3. Correlation Analyses for Acute Progressors (N=50)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Statistic</th>
<th>Baseline</th>
<th>Nef CD4 downregulation</th>
<th>Nef HLA downregulation</th>
<th>Nef Infectivity enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline pVL</td>
<td>R</td>
<td>- 0.38b</td>
<td>0.05</td>
<td>0.05</td>
<td>- 0.11</td>
</tr>
<tr>
<td>eDPI p</td>
<td>p</td>
<td>0.007</td>
<td>0.7</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Baseline pVL</td>
<td>R</td>
<td>0.17</td>
<td>0.15</td>
<td>0.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pVL p</td>
<td>p</td>
<td>0.3</td>
<td>0.3</td>
<td>&lt;0.001</td>
<td>0.2</td>
</tr>
<tr>
<td>CD4 downregulation p</td>
<td>R</td>
<td>0.56</td>
<td>0.20</td>
<td>0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HLA downregulation p</td>
<td>R</td>
<td>- 0.10</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HLA: Human Leukocyte Antigen; eDPI: estimated days post-infection; pVL: plasma viral load

*Spearman R and p-values; significant associations are highlighted in bold type.*
**Figure Legends**

**Figure 1:** Plasma viral loads of AC and AP during the first year post infection. Lines illustrate the plasma viral load (HIV-RNA copies/mL) for each participant. (A) Data are plotted for 10 AC who achieved viremic control (<2,000 RNA copies/mL) within the first year of infection. (B) Data are plotted for 50 AP who did not control viremia during this time. Dashed lines (----) indicate the threshold value (2,000 RNA copies/mL) used to identify acute controllers.

**Figure 2:** Nef sequences from AC and AP display no substantial phylogenetic clustering. (A) An unrooted maximum likelihood phylogenetic tree depicts the predominant Nef clone isolated from 10 AC (red) and 50 AP (blue), indicating that sequences did not exhibit substantial clustering by cohort. The HIV-1SF2 Nef sequence (black) is included for comparison. A phylogenetic tree that includes all clonal Nef sequences used for this study is shown in Figure S1. (B) Western blot was used to examine steady-state protein levels in AC and AP Nef clones, along with NefSF2 (positive control) and ΔNef (negative control). β-actin was used as a cellular protein control.

**Figure 3:** Reduced in vitro function is observed for AC Nef clones. Nef CD4 and HLA class I downregulation functions were assessed in ≥3 replicates, using flow cytometry. Representative flow cytometry plots display relative downregulation of CD4 (A) and HLA class I (B) for CEM-A*02 cells transfected with NefSF2 (positive control), ΔNef (negative control) or one participant-derived Nef clone. The x-axis (GFP+) indicates transfected cells and the y-axis indicates surface expression of CD4 or HLA class I molecules, respectively. Scatter plots depict normalized CD4 (C) or HLA class I (D) downregulation activities in representative Nef clones from AC (N=10,
red) compared to those from AP (N=50, blue). A dashed line (----) in each panel illustrates
normalized activity of 1.0, which represents the function of NefSF2. Values below 1.0 indicate
lower Nef function, while values above 1.0 indicate higher function, relative to NefSF2.

**Figure 4: Reduced ability to enhance virion infectivity in AC Nef clones.** Plots depict the
normalized ability of recombinant viruses encoding representative Nef clones from AC (N=10,
red) and AP (N=41, blue) to enhance viral production (p24Gag ng/mL) (A) and viral infectivity in
Tzm-bl cell assays (B). A dashed line (----) in each panel illustrates normalized activity of 1.0,
which represents the function of the positive control NL4.3-nefSF2. Values below 1.0 indicate
lower Nef function, while values above 1.0 indicate higher function, relative to NL4.3-nefSF2.
Each Nef-containing recombinant virus was tested in duplicate. Note that the negative control
NL4.3Δnef displayed a mean infectivity of 0.02 (SD 0.009, N=8 replicates) in this assay.

**Figure 5: No consistent changes in Nef activity are observed in AC patients over time.** The
ability of Nef to downregulate CD4 (A), HLA class I (B), or to enhance viral infectivity (C) is
shown for representative clones derived from 6 AC patients (AC01 – maroon, AC02 – green,
AC04 – orange, AC05 – red, AC07 – blue, and AC09 – purple) at baseline (closed circle) and at
follow-up (open circle) time points, described in Table 1. A dashed line (----) indicates
normalized activity of 1.0, which represents the function of the positive control NefSF2 in each
assay. The Wilcoxon p-value is shown; no significant differences in Nef function were observed
between baseline and follow-up clones in a paired test. Nef downregulation activities were tested
in ≥3 replicates, while viral infectivity was tested in duplicate.
Figure 6: Accumulation of HLA-associated polymorphisms is associated with reduced Nef function and expression. (A) An HXB2 alignment of unique Nef sequences derived from patient AC01 at baseline (N=5) and follow-up (N=2) time points is shown. Nef_{HXB2} is indicated for reference. Sequences are labeled by participant ID (AC01) followed by the time of sample collection (baseline, b; follow-up, f) and colony identifier (A, B, etc). A solid line distinguishes baseline clones (above the line) from follow-up clones (below the line). Four polymorphisms (R19K, H40R, M182L and V194A; highlighted in yellow) were observed in all follow-up clones and are associated with host HLA-A*31 or B*37, as discussed in the text. A model of the Nef protein structure (B) illustrates the location of residues/motifs previously associated with HLA class I downregulation function (green), the four polymorphisms observed in AC01 (red), and an area of potential overlap near R19 (magenta). A bar graph (C) depicts the normalized HLA class I downregulation activities of baseline clone AC01b_F and follow-up clone AC01f_A (red) and their corresponding single amino acid mutants (yellow) at the four polymorphic sites; all samples were tested in ≥4 replicates. A dashed line (----) indicates normalized activity of 1.0, which represents the function of the positive control Nef_{SF2}. Steady-state protein expression for Nef_{SF2} (positive control), ΔNef (negative control), AC01b_F, AC01f_A and their corresponding single amino acid mutants was examined by Western blot (D). β-actin was used as a cellular protein control. The relative band intensity (%) for each Nef clone was calculated by normalization to β-actin and then Nef_{SF2}. As such, a number >100% or <100% represents relative Nef expression greater or less than that of Nef_{SF2}. 
Figure 1

A.  
Plasma Viral Load (copies/mL)
est. Days Post-Infection

B.  
Plasma Viral Load (copies/mL)
est. Days Post-Infection
Figure 2

A.

B.

Sheep α-Nef

β-Actin
Figure 3

A. B. 

C. D. 

Normalized CD4 Downregulation

AC AP
0.4 0.6 0.8 1.0
p=0.004 (N=10) (N=50)

Normalized HLA-I Downregulation

Nef SF2 ∆Nef Patient -Nef

CD4-APC HLA-A*02-PE

Nef SF2 ∆Nef

GFP (Nef) GFP (Nef)
Figure 4

A. Normalized p24 Concentration

B. Normalized Viral Infectivity

AC (N=10)  AP (N=41)

p=0.1  p=0.003
Figure 5

A. Baseline Follow-up

B. Baseline Follow-up

C. Baseline Follow-up

Normalized Viral Infectivity

Normalized CD4 Downregulation

Normalized HLA-I Downregulation

AC01 AC02 AC04 AC05 AC07 AC09
Figure 6

A.

B.

C.

D.

Sheep α-Nef

β-Actin