

Association between HIV-1 Nef-mediated MHC-I downregulation and the maintenance of the replication-competent latent viral reservoir in individuals with virally suppressed HIV-1 in Uganda: an exploratory cohort study

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Summary

Background The persistence of a replication-competent latent viral reservoir (RC-LVR) during antiretroviral therapy (ART) is a barrier to the development of a cure for HIV-1, but the role of viral genes in influencing RC-LVR size is unclear. We aimed to assess whether the magnitude by which the HIV-1 accessory protein Nef evades the adaptive immune response by downregulating MHC-I or CD4, or both, from the surface of infected cells is associated with the rate at which the RC-LVR in people with HIV-1 changes during long-term ART (>1 year).

Methods We conducted an exploratory cohort study in which *nef* genes were sequenced from outgrowth viruses derived from the quantitative viral outgrowth assay (QVOA) for a group of people with ART-suppressed HIV-1 in Uganda between 2015 and 2020. Study participants were selected from the Rakai Health Sciences Program (RHSP) LVR cohort, a cohort of 90 adults (aged ≥ 18 years) who were HIV-1 positive, receiving ART, and had maintained viral suppression for at least 1 year at the time of study enrolment. For this study, participants were required to have available p24⁺ QVOA wells that contained a single viral outgrowth isolate, as assessed by next-generation sequencing. In cases where further sequencing identified wells containing multiple viral clones, all sequenced *nef* variants were included for functional analysis. The unique isolated *nef* variants were used to generate pseudoviruses, which were employed to measure cell surface CD4 and MHC-I downregulation in infected CD4⁺ Sup-T1 cells via flow cytometry. The size and rate of change of the RC-LVR in participants was estimated using previous QVOA results and a Bayesian model. We then assessed whether a correlation existed between the extent to which the Nef proteins downregulated cell surface MHC-I and CD4 and the calculated RC-LVR rate of change during the study period.

Findings 14 (15%) of 90 participants from the RHSP cohort met the inclusion criteria and were enrolled in this study. 49 *nef* sequences were isolated from these participants. We observed variability in participant-derived Nef-mediated cell surface MHC-I downregulation (median 114.88% [IQR 104.93–121.51] of the downregulation capacity of NL4-3 Nef) and CD4 downregulation (94.50% [84.05–100.16] of NL4-3 Nef). The estimated rate of change of the RC-LVR was positive for four participants. For one donor, the rate of change was significantly positive (7.4×10^{-4} logit infectious units per million [IUPM] per day [95% credibility interval 3.2×10^{-4} to 1.2×10^{-3}]) over the course of the study period (2015–20). The estimated rate of change of the RC-LVR for the remaining ten participants was negative, and significantly negative in four donors (-1.1×10^{-3} logit IUPM per day [95% credibility interval -1.8×10^{-3} to -3.7×10^{-4}]; -1.4×10^{-3} [-2.0×10^{-3} to -8.5×10^{-4}]; -7.0×10^{-4} [-1.3×10^{-3} to -1.6×10^{-4}]; and -2.0×10^{-3} [-2.9×10^{-3} to -1.1×10^{-3}]). A significant relationship between Nef-mediated MHC-I downregulation and the RC-LVR rate of change during the 5-year study period ($r=0.6088$ [95% CI 0.2366 to 0.9810]; $p=0.023$) was found, in which less efficient MHC-I downregulation correlated with faster RC-LVR decay during long-term ART. By contrast, Nef-mediated CD4 downregulation was not associated with RC-LVR rate of change during the 5-year study period (-0.1604 [-0.7311 to 0.4102]; $p=0.58$).

Interpretation Nef-mediated MHC-I downregulation might contribute to HIV-1 persistence during long-term ART. Strategies to inhibit Nef-mediated MHC-I downregulation could represent a viable therapeutic avenue to reduce the size of the latent reservoir in vivo, improving treatment outcomes in people with HIV-1.

Funding Canadian Institutes of Health Research, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the REACH Martin Delaney Collaboratory.

Lancet Microbe 2025

Published Online
<https://doi.org/10.1016/j.lanmic.2024.101018>

For the French translation of the abstract see Online for appendix 1

For the Luganda translation of the abstract see Online for appendix 2

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Introduction

Antiretroviral therapy (ART) suppresses HIV-1 replication and disease progression allowing people with HIV-1 to live near-normal lives.¹ However, ART regimens remain non-curative, as evidenced by rapid viral rebound following ART cessation.² HIV-1 persistence in vivo is due to the existence of stable, heterogeneous cellular reservoirs harbouring integrated latent HIV-1 proviruses.³ This latent viral reservoir (LVR) is formed shortly following HIV-1 infection and persists despite ART through replication and clonal expansion of latently infected cells.^{4,5} The inducible, replication-competent LVR (RC-LVR) decays very slowly, necessitating lifelong ART to control infection.^{5–7} Significant efforts are underway to identify factors that accelerate the decay of the RC-LVR. Host factors associated cross-sectionally with a smaller LVR include early ART initiation (<6 months after infection), younger age, female sex, and lack of co-infection with cytomegalovirus or Epstein-Barr Virus.^{8–11} Additionally, the magnitude of HIV-specific granzyme B responses, primarily from HLA-I-restricted

CD8⁺ cytotoxic T lymphocytes (CTLs), negatively correlates with the size of the RC-LVR.¹² Viral-specific factors could also influence reservoir size. Indeed, infection with non-B HIV-1 subtypes is associated with smaller reservoirs than infection with subtype B HIV-1 strains.^{13–15}

The HIV-1 accessory protein Nef facilitates evasion of host adaptive immune responses primarily by downregulating MHC-I and CD4 from the cell surface.^{16–19} Nef-mediated downregulation of cell surface CD4 renders infected cells more resistant to clearance via the antibody-dependent cellular cytotoxicity (ADCC) response.¹⁸ Additionally, Nef-mediated MHC-I downregulation reduces viral antigen presentation to MHC-I-restricted CTLs.^{16,20} In the context of the LVR, the proportion of HIV-1 proviruses containing intact *nef* open-reading frames (ORFs) increases in ex-vivo cultured infected CD4⁺ T cells exposed to HIV-1 specific CTLs, suggesting Nef could contribute to the persistence of latently infected cells by facilitating evasion of CTL recognition and killing.²¹

Research in context

Evidence before this study

We searched Web of Science on Oct 23, 2019, with no restrictions on language or calendar year using the following search terms: ("latent reservoir" OR "reservoir size") AND "HIV" AND "Nef" AND "subtype" AND ("ART" OR "cART"), which resulted in one manuscript published in *Journal of Virology* in April, 2019, the time at which this study commenced. A more recent search done on Jan 17, 2025 confirms that all relevant literature is still captured in this *Journal of Virology* study. This manuscript quantified the total latent reservoir by measuring proviral DNA loads and the size of the replication-competent latent viral reservoir (RC-LVR) using quantitative viral outgrowth assay (QVOA). This cross-sectional study involved 30 people with HIV-1 who were placed on antiretroviral therapy (ART) in the acute or early stages of infection (<6 months) and used QVOA to calculate the size of RC-LVR after 48 weeks following ART initiation. This study found that the extent to which the HIV-1 accessory protein Nef downregulated cell surface MHC-I, but not CD4, was positively correlated with the size of the RC-LVR after 48 weeks of ART, suggesting HIV-1 Nef as a viral factor contributing to the size of the RC-LVR accordingly. However, this cross-sectional study could not address the rate at which the RC-LVR changes over time during long-term ART (>1 year). Furthermore, it involved a cohort of participants with HIV-1 in North America, further highlighting a gap in knowledge regarding the RC-LVR for people with HIV-1 in areas where non-B HIV-1 subtypes predominate.

Added value of this study

To our knowledge, this is the first study to identify any factor associated with accelerated RC-LVR decay in people with HIV-1.

This study additionally provides valuable insights into the RC-LVR of people with HIV-1 in non-subtype B contexts, which have been studied less than subtype B contexts. Our study measured the RC-LVR via QVOA in a cohort of people with HIV-1 in Uganda over a 5-year sampling period. This allowed the RC-LVR rate of change to be calculated for each participant using a previously described Bayesian model. *Nef* sequences from 14 study participants were derived from QVOA outgrowth wells, and the ability of the different Nef proteins to downregulate cell surface MHC-I and CD4 within infected cells was calculated. We found that the extent to which Nef downregulates cell surface MHC-I, but not CD4, was inversely correlated with the rate of RC-LVR decay over time during ART. Namely, efficient Nef-mediated MHC-I downregulation was associated with a slower rate of RC-LVR decay over time.

Implications of all the available evidence

Our results identify Nef-mediated downregulation of cell surface MHC-I as an important viral correlate to longitudinal changes in the size of the RC-LVR during long-term ART. Because curative efforts are focused on eradicating the existing RC-LVR in people with HIV-1, our results and others suggest that pharmacologically targeting this Nef function could represent a viable therapeutic avenue to reduce the size of the RC-LVR in future curative approaches in both subtype B and non-subtype B contexts. Such strategies could represent an important step towards a functional cure for HIV/AIDS.

In a cross-sectional study published in 2019 of a cohort of people with HIV-1 who initiated ART during acute or early infection (<6 months after infection), the ability of Nef to downregulate MHC-I, but not CD4, correlated positively with both overall proviral burden and RC-LVR size after 48 weeks of virally suppressive ART.¹³ Because the RC-LVR decays slowly during virally suppressive ART,⁷ it remains unknown whether the ability of Nef to evade these adaptive immune responses influences the rate of RC-LVR decay during long-term virally suppressive ART. Most studies examining the RC-LVR have been done in high-income countries, which are typically dominated by subtype B HIV-1 strains.²² Thus, little is known regarding the effect of non-subtype B Nef proteins on the size of the RC-LVR. Here, we characterised whether the ability of non-subtype B Nef proteins to evade adaptive immune responses—by downregulating cell surface MHC-I and CD4—is associated with the maintenance of the RC-LVR during virally suppressive ART by comparing these functional outputs with the calculated RC-LVR change over a 5-year study period.

Methods

Study design and participants

We conducted an exploratory cohort study to examine longitudinal trends in the size of the HIV reservoir in Ugandan adults with non-subtype B HIV-1. This cohort (n=14) was nested within the Rakai Health Sciences Program (RHSP) LVR cohort (n=90), a longitudinal cohort of people with HIV-1 living in the Rakai district in Uganda.^{10,14,15} The overarching RHSP LVR cohort was recruited to examine longitudinal trends in the size of the HIV reservoir in Ugandan adults with non-subtype B HIV-1. Main inclusion criteria for the overarching cohort (n=90) were adults (aged ≥18 years) who were HIV-1 positive, receiving ART, and had maintained viral suppression for at least 1 year at the time of study enrolment. Specifically, participants had two plasma HIV-1 RNA measurements of less than 40 copies per mL, 10–18 months apart with no intervening detectable result. 70 participants who met these criteria were enrolled in 2015. A second group of 20 participants were recruited in 2016 who also met the previous criteria and were additionally required to have an estimated date of seroconversion (date of HIV negative test result available). Study visits included a blood draw (180 mL) for standard quantitative viral outgrowth assay (QVOA) to estimate the frequency of resting CD4⁺ T cells containing inducible replication-competent proviruses.^{7,23} For each participant, the rate of change of the RC-LVR (measured by QVOA) was estimated using a Bayesian model between enrolment (2015 or 2016) and September, 2020.¹⁵

For this nested study and the analysis of Nef protein function, we initially included all participants from the RHSP LVR cohort for whom p24⁺ QVOA wells that contained a single viral outgrowth isolate (as assessed by next-generation sequencing) were available. In cases where further

sequencing identified wells containing multiple viral clones, all sequenced *nef* variants were included for functional analysis. During the study timeframe (2015–20), Uganda implemented a nationwide change in the ART regimen beginning in 2018 to include the integrase strand transfer inhibitor dolutegravir. The subset of participants included in this study were all administered nucleoside reverse transcriptase inhibitor (NRTI)-based and non-nucleoside reverse transcriptase inhibitor (NNRTI)-based ART regimens before switching to dolutegravir-containing regimens. All participants were required to be followed up annually during the study timeframe (2015–20) upon study enrolment. Donor 45 withdrew after the 2017 timepoint (appendix 3 pp 2–3).

All participants provided informed written consent to study participation and use of stored samples for future research. Ethical approval was obtained from the Institutional Review Boards (IRBs) at the US National Institutes of Allergy and Infectious Diseases (14-I-N123), Uganda Virus Research Institute (GC/127/461), Uganda National Council for Science and Technology (HS1651), and Johns Hopkins Medical Institutions (CR00044266/IRB00038011). Research ethics approval was obtained from Western University (124581) for the analysis of de-identified data and stored samples.

Procedures

Upon study enrolment, cohort participants were administered a questionnaire by study staff in the local language, in which gender was self-reported with the option of male or female. Detailed methodologies regarding the QVOA analysis and Bayesian estimates used to calculate the size and rate of change of the RC-LVR for study participants have been described previously, and are summarised in appendix 3 (p 8).^{15,24} QVOA wells with the presence of only one viral outgrowth population, based on NGS data for *gp41* or *pol*, or both, were selected, as previously described.²⁴ Viral RNA was extracted from positive viral outgrowth supernatants (QIAamp Viral RNA Kit for RNA Extraction, Qiagen, Germantown, MD, USA) and the *nef* genes were reverse-transcribed (Qiagen OneStep RT-PCR Kit, Qiagen). The reverse-transcribed primary *nef* genes were sequenced by Sanger sequencing (nucleotides 8787–9407 of the pNL4-3 proviral genome [Genbank accession number: AF324493]), and *nef*-specific primers were designed to amplify the *nef* genes selected from the positive viral outgrowth supernatants for cloning into the replication-deficient pNL4-3 Δ *gag/pol*-enhanced GFP-Nef proviral plasmid or synthesised using the GENEWIZ service (Azenta Life Sciences, Chelmsford, MA, USA), as described previously (appendix 3 p 8).¹⁷

The primary *nef* nucleotide sequences were aligned using the HMM-align alignment method of the HIValign tool hosted by the Los Alamos National Laboratory (LANL) HIV sequence database web server.²⁵ The aligned nucleotide sequences were inputted into the IQ-TREE ModelFinder

See Online for appendix 3

For the LANL HIV sequence database see <https://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html>

For IQ-TREE statistical software see <http://www.iqtree.org>

tool (version 1.6.12), where the TPM2u substitution rate model was selected for phylogenetic construction because this model had the lowest default Bayesian Information Criterion (BIC) score. The maximum likelihood phylogenetic tree relating the aligned primary nucleotide *nef* sequences was then constructed with IQ-TREE using the TPM2u nucleotide base substitution rate model, incorporated empirical state frequencies (+F), and rate variation represented by a discrete gamma model with four rate categories (+G4), and a proportion of invariable sites (+I). The maximum likelihood phylogeny was constructed with standard non-parametric bootstrap analysis with 10 000 replicate samples. The maximum likelihood tree was rooted on NL4-3 *nef*. The subtype of the participant-derived *nef* sequences was defined using the Recombinant Identification Program (RIP; version 3.0) hosted on the LANL web server using a window size of 500 and a confidence threshold of 99%.²⁶

HEK293T cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium containing 4 mM L-glutamine (Cytiva Life Sciences, Vancouver, BC, Canada) and 4.5 g/L glucose (Cytiva Life Sciences), and supplemented with 10% fetal bovine serum (FBS; Wisent, Saint-Jean-Baptiste, QC, Canada) and 1% penicillin and streptomycin (HyClone, Logan, UT, USA). Sup-T1 cells (ATCC) were maintained in Roswell Park Memorial Institute medium 1640 (Wisent) and supplemented with 1% penicillin–streptomycin (HyClone), 1% sodium pyruvate (HyClone), 1% non-essential amino acids (HyClone), 2 mM L-glutamine (HyClone), and 10% FBS (Wisent). All cells were grown at 37°C with 5% CO₂.

Methodologies related to the Nef-mediated cell surface MHC-I and CD4 downregulation assay are described in appendix 3 (pp 8–9). Briefly, vesicular stomatitis virus protein G (VSV-G)-pseudotyped viruses were generated upon transfection of HEK293T cells and used to infect Sup-T1 cells via spinoculation. Infection experiments were performed four times, and in each experiment the Nef-deficient (Δ Nef; proviral construct in which a premature stop codon is introduced in the N-terminal region of the protein, preventing the expression of full-length Nef) and NL4-3 Nef controls were performed in triplicate, whereas all other infections were performed in singlet. After 48 h, cells were stained with the Zombie near-infrared viability dye (BioLegend, San Diego, CA, USA), fixed with 1% paraformaldehyde (Thermo Fisher Scientific, Whitby, ON, Canada), and stained with fluorophore-conjugated antibodies (BioLegend) to detect cell-surface MHC-I and CD4. Cell-surface MHC-I and CD4 levels—quantified as geometric mean fluorescence intensity (gMFI)—were calculated via flow cytometry (appendix 3 p 9).

Outcomes

The primary objective of this nested study was to assess whether a correlation existed between the ability of Nef proteins derived from the study participants to downregulate cell surface MHC-I and CD4 and the calculated

rate of change of the RC-LVR during the defined study period (2015–20). Outcome measures of this study included quantifying the extent to which Nef proteins derived from single outgrowth viruses for the study participants downregulated cell surface MHC-I and CD4 within infected cells and quantifying the association between Nef-mediated MHC-I or CD4 downregulation, or both, and changes in RC-LVR size as a function of time during virally suppressive ART. These analyses enabled the characterisation of these Nef functions within individual participants (when more than one Nef protein was tested) and between participants.

Statistical analysis

Because this study was an exploratory analysis and the type of variability observed in the Nef responses was unknown at study initiation, a power calculation was not performed.

For Nef functional experiments, experimental replicates were omitted from analysis if cell viability was below 50%. Differences in Nef-mediated MHC-I and CD4 downregulation between Nef variants was first established using the Kruskal–Wallis equality-of-populations rank test, followed by post-hoc pairwise comparisons between each participant-derived Nef and the NL4-3 Nef positive control using a Dunn's test (appendix 3 p 9).

We next tested if participant-specific Nef outputs correlated with the change in RC-LVR size calculated using a Bayesian model (appendix 3 p 8).¹⁵ Because DTG initiation could be associated with a temporary increase in QVOA-based estimates in reservoir size,¹⁵ the analysis was limited to the Bayesian estimate for the change in RC-LVR size that included a correction for DTG initiation, as described (appendix 3 p 8).¹⁵ For participants with more than one Nef variant, the median Nef functional output across all variants from a participant was used to estimate their participant-specific Nef functional output (appendix 3 p 9). Outputs were then correlated with the participant-specific pre-DTG RC-LVR rates of change using Spearman's rank correlation. The 95% CIs on Spearman's correlation coefficients—denoted as *r*—were calculated by bootstrapping with 1000 repeats.

To test whether Nef functional outputs differed by subtype, variant-level and participant-level MHC-I and CD4 downregulation were compared. For variant-level comparisons, the median of four experimental replicates for each variant was used. For participant-level comparisons, the median function across all variants isolated from a participant was compared. Differences between subtypes were assessed using the Kruskal–Wallis equality-of-populations rank test. Post-hoc pairwise comparisons between subtypes were made using a Dunn's test. The median CD4 and MHC-I downregulation for each subtype, along with IQR, are presented.

For all statistical tests, *p* values less than or equal to 0.05 were considered statistically significant. Tests were performed using Stata (version 18). Figures were generated using GraphPad Prism (version 8). GraphPad Prism (version 8)

For RIP see <https://www.hiv.lanl.gov/content/sequence/RIP/RIP.html>

was used to graph the linear regression best-fit lines with 95% CIs.

Role of the funding source

The funders of the study had no role in study design, data analysis, data interpretation, or writing of the report.

Results

For this study, we selected 14 (15%) of 90 participants from the RHSP LVR cohort. Participant demographics are summarised in the table. The median age of participants at enrolment was 40·3 years (IQR 39·7–45·3). There were nine male participants and five female participants. The median duration of ART at study enrolment was 8·76 years (IQR 3·21–9·80). A switch from NRTI-based and NNRTI-based ART to dolutegravir-containing regimens was documented in 12 of 14 participants (appendix 3 pp 2–3).¹⁵

Overall, 49 *nef* sequences were isolated from the 14 participants. The *nef* sequences clustered with subtype D (24 [49%] of 49 *nef* sequences, six participants), subtype A1 (20 [41%] *nef* sequences, six participants), subtype C (four [8%] *nef* sequences, one participant), and one recombinant A1/D subtype (one [2%] *nef* sequence, one participant), which are the prevalent subtypes in Uganda (table, figure 1).^{22,27} One *nef* sequence from donor 17 (sequence identifier 17_4_1M13) encoded a premature stop codon located at position 91 of the amino acid sequence (relative to the NL4-3 Nef reference protein; figure 1; appendix 3 p 6) and is predicted to not express full-length Nef. This viral clone was observed in outgrowth assays as late as the 4-year timepoint. Of the remaining 48 *nef* sequences encoding full-length Nef proteins, the median length was 207 amino acids (IQR 207–207, range 203–218), with most of the variability occurring within the N-terminal region (appendix 3 p 6).

The gating schematic to identify single, live, and infected cells during the MHC-I and CD4 downregulation assay is detailed in figure 2A. The median functional outputs with IQR and associated p-values are described in the appendix 3 (pp 4–5). As expected, the Nef-deficient (Δ Nef) negative control did not downregulate cell-surface MHC-I (figures 2B, 2D, and 3A) and CD4 (figures 2C, 2E, and 3B). The least effective participant-derived *nef* was the variant encoding a premature stop codon (sequence identifier 17_4_1M13), the protein product of which minimally downregulated cell surface MHC-I (median 55·23% [IQR 54·41–57·69] of the downregulation capacity of NL4-3 Nef; figures 2B, 2D, and 3A) and CD4 (median 9·07% [7·72–12·01] of NL4-3 Nef; figures 2C, 2E, and 3B). Of the remaining 48 full-length Nef variants, we observed variability in functionality in the downregulation of both MHC-I (median 114·88% [104·93–121·51] of NL4-3 Nef; figure 3A) and CD4 (median 94·50% [84·05–100·16] of NL4-3 Nef; figure 3B). Participant-derived Nef variants generally downregulated cell-surface MHC-I more efficiently than NL4-3 Nef, with 23 (48%) of 48 full-length variants

Participants (n=14)	
Age at enrolment, years	40·3 (39·7–45·3)
Sex	
Male	9
Female	5
Pre-ART viral load, log ₁₀ copies per mL	4·47 (3·80–5·20)
Nadir CD4 ⁺ T cells, cells per μ L	203 (137–335)
ART duration at enrolment, years	8·76 (3·21–9·80)
HIV <i>nef</i> subtype*	
D	6
A1	6
C	1
Recombinant	1
HIV-1 Nef length, amino acids	207 (207–207)
Total primary <i>nef</i> sequences recovered	49
Unique <i>nef</i> sequences recovered per participant	
1	5
2	1
3	1
4	4
5	1
8	1
10	1

Data are n or median (IQR). ART=antiretroviral therapy. *Participant *nef* subtypes were identified using the Recombinant Identification Tool offered by the Los Alamos HIV Sequence database.²⁶

Table: Summary of clinical characteristics of study participants

downregulating MHC-I significantly more efficiently than NL4-3 Nef (figure 3A). By contrast, participant-derived Nef isolates were generally less efficient than NL4-3 Nef at downregulating cell-surface CD4, with 14 (29%) of 48 full-length variants downregulating CD4 significantly less than NL4-3 Nef (figure 3B). A summary of the median functional outputs across four experimental replicates with IQRs and associated p values is provided in the appendix 3 (pp 4–5).

Next, we examined whether Nef-mediated MHC-I or CD4 downregulation differed by HIV-1 subtype including all 49 tested Nef variants in this study. Because our cohort included only one individual with subtype C Nef (donor 19, four Nef variants), and one individual with subtype D/A1 recombinant Nef (donor 27, one Nef variant), we did not make statistical comparisons with these subtypes. We found no differences in Nef-mediated MHC-I downregulation between subtypes D and A1 (24 [49%] of 49 variants vs 20 [41%] variants; median 114·01% [102·91–121·75] of NL4-3 Nef vs 111·97 [104·25–118·98] of NL4-3 Nef, respectively; $p=0\cdot32$; appendix 3 p 7), but we did observe significantly more efficient CD4 downregulation by subtype D Nef variants than by subtype A1 Nef variants (median 97·75% [IQR 94·01–108·63] of NL4-3 Nef vs 84·24% [79·92–95·18] of NL4-3 Nef; $p=0\cdot0002$; appendix 3 p 7). Because Nef variants obtained from the same participant might not be independent, we also examined differences in the median MHC-I or CD4 downregulation for each

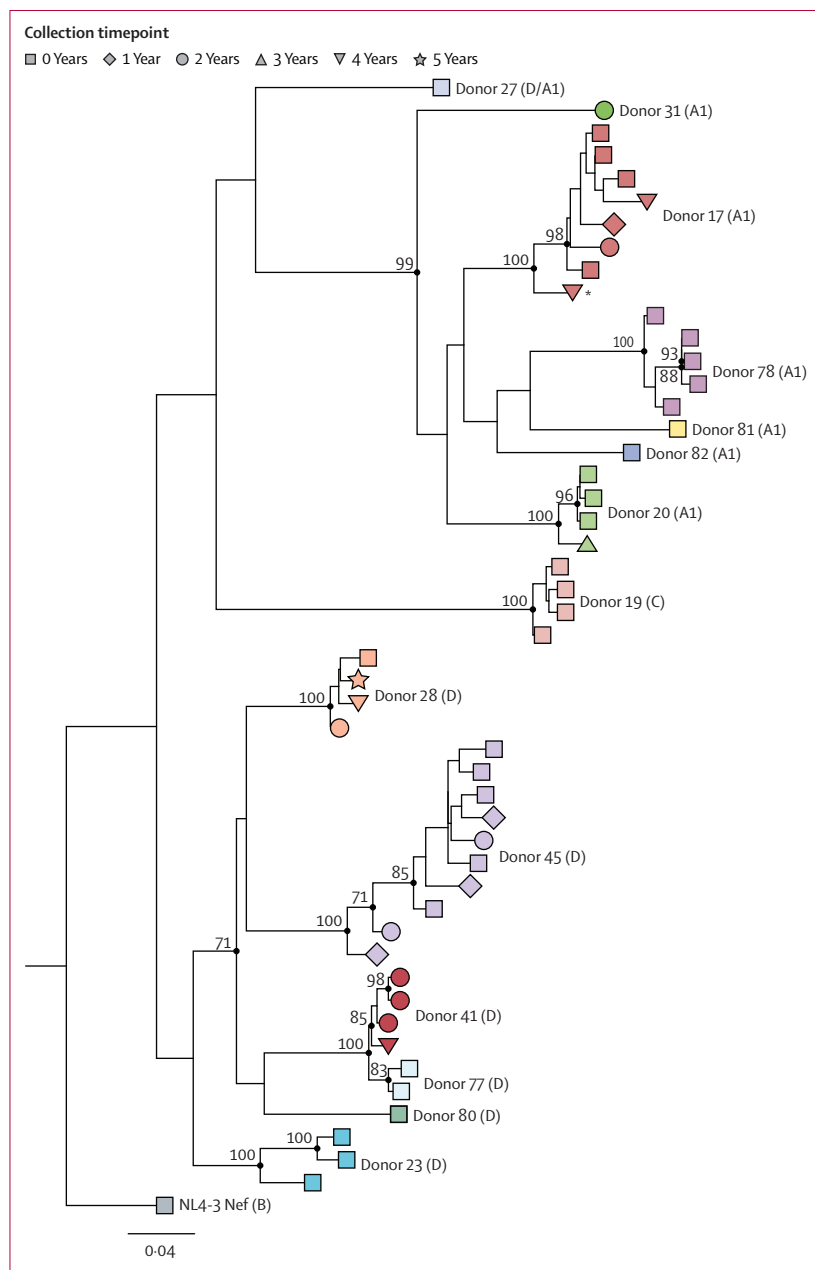


Figure 1: Maximum likelihood phylogeny relating primary HIV-1 *nef* nucleotide sequences used in the present study
 The maximum likelihood phylogenetic tree relating the aligned primary *nef* nucleotide sequences was constructed with IQ-TREE using a TPM2u base substitution rate model with standard non-parametric bootstrap analysis with 10 000 replicate samples. The scale bar represents the estimated number of nucleotide substitutions per site. Internal nodes of the Newick output tree from IQ-TREE were annotated with bootstrap support values as percentages; nodes with $\geq 70\%$ support are shown accordingly. The individual primary *nef* isolates are coloured according to the participant from which the sequences were derived. HIV-1 subtypes were identified via the Recombinant Identification Program tool hosted by Los Alamos National Laboratory and are listed after the participant identifiers.²⁶ The shape of the individual tips corresponds to the timepoint from which the *nef* sequences were collected. The phylogenetic tree is additionally rooted on the HIV subtype B reference strain NL4-3 *nef*. **nef* variant (sequence identifier 17_4_1M13) encoding a premature stop codon and predicted to not express full-length Nef.

participant (median of Nef variants isolated from a participant, for participants with more than one variant) between Nef subtypes D (six of 14 participants) and A1 (six of 14

participants). Between individuals with subtype D and A1 Nef, we did not observe significant differences in participant-level Nef-mediated MHC-I downregulation (median 113.95% [IQR 108.02–118.49] of NL4-3 Nef vs 105.97 [101.22–110.77] of NL4-3 Nef, respectively; $p=0.12$; appendix 3 p 7) or CD4 downregulation (96.59% [95.03–103.58] of NL4-3 Nef vs 87.64% [83.93–93.22] of NL4-3 Nef, respectively; $p=0.17$; appendix 3 p 7) in this small cohort.

We next sought to assess whether Nef-mediated MHC-I or CD4 downregulation, or both, were associated with changes in RC-LVR size as a function of time during virally suppressive ART. A negative rate of change indicates the size of the RC-LVR shrinks over time, whereas a positive rate of change indicates growth. We found that Nef-mediated MHC-I downregulation was significantly correlated with the rate of change of the RC-LVR ($r=0.6088$ [95% CI 0.2366 to 0.9810]; $p=0.023$; figure 4A). Of note, the estimated rate of change of donor 17's RC-LVR was significantly positive (7.4×10^{-4} logit infectious units per million [IUPM] per day [95% credibility interval 3.2×10^{-4} to 1.2×10^{-3}]; appendix 3 pp 2–3), and this participant also had the highest observed median Nef-mediated MHC-I downregulation (122.7% of NL4-3 Nef; figures 3A, 4A). Of the remaining 13 study participants, the estimated rate of change of three participants' RC-LVR was positive, whereas the estimated rate of change of ten participants' RC-LVR was negative. Of these ten participants, four donors had significantly negative estimated rates of change of the RC-LVR (donor 20: -1.1×10^{-3} logit IUPM per day [95% credibility interval -1.8×10^{-3} to -3.7×10^{-4}]; donor 31: -1.4×10^{-3} [-2.0×10^{-3} to -8.5×10^{-4}]; donor 41: -7.0×10^{-4} [-1.3×10^{-3} to -1.6×10^{-4}]; and donor 78: -2.0×10^{-3} [-2.9×10^{-3} to -1.1×10^{-3}]; figure 4; appendix 3 pp 2–3). By contrast, we found no association between Nef-mediated cell-surface CD4 downregulation and the rate of change in RC-LVR size ($r=-0.1604$ [95% CI -0.7311 to 0.4102]; $p=0.58$; figure 4B).

Discussion

Our analysis suggests Nef as a viral factor associated with changes in the size of the inducible RC-LVR in a cohort of people with non-subtype B HIV-1 in Uganda. Nef-dependent MHC-I downregulation was significantly correlated with the rate of change in RC-LVR size over time. Thus, more efficient Nef-dependent MHC-I downregulation could slow the rate of RC-LVR decay during long-term ART. To our knowledge, this is the first report of a viral or host factor associated with changes in the size of the RC-LVR over time during long-term ART.

Most research characterising the LVR in people with HIV-1 on ART has focused on cross-sectional analyses of predominantly subtype B HIV-1 infections, despite more than 80% of people with HIV-1 being affected by non-subtype B HIV-1.²² We previously reported that the RC-LVR was significantly smaller in this cohort from Uganda than in a cohort from the USA with exclusively

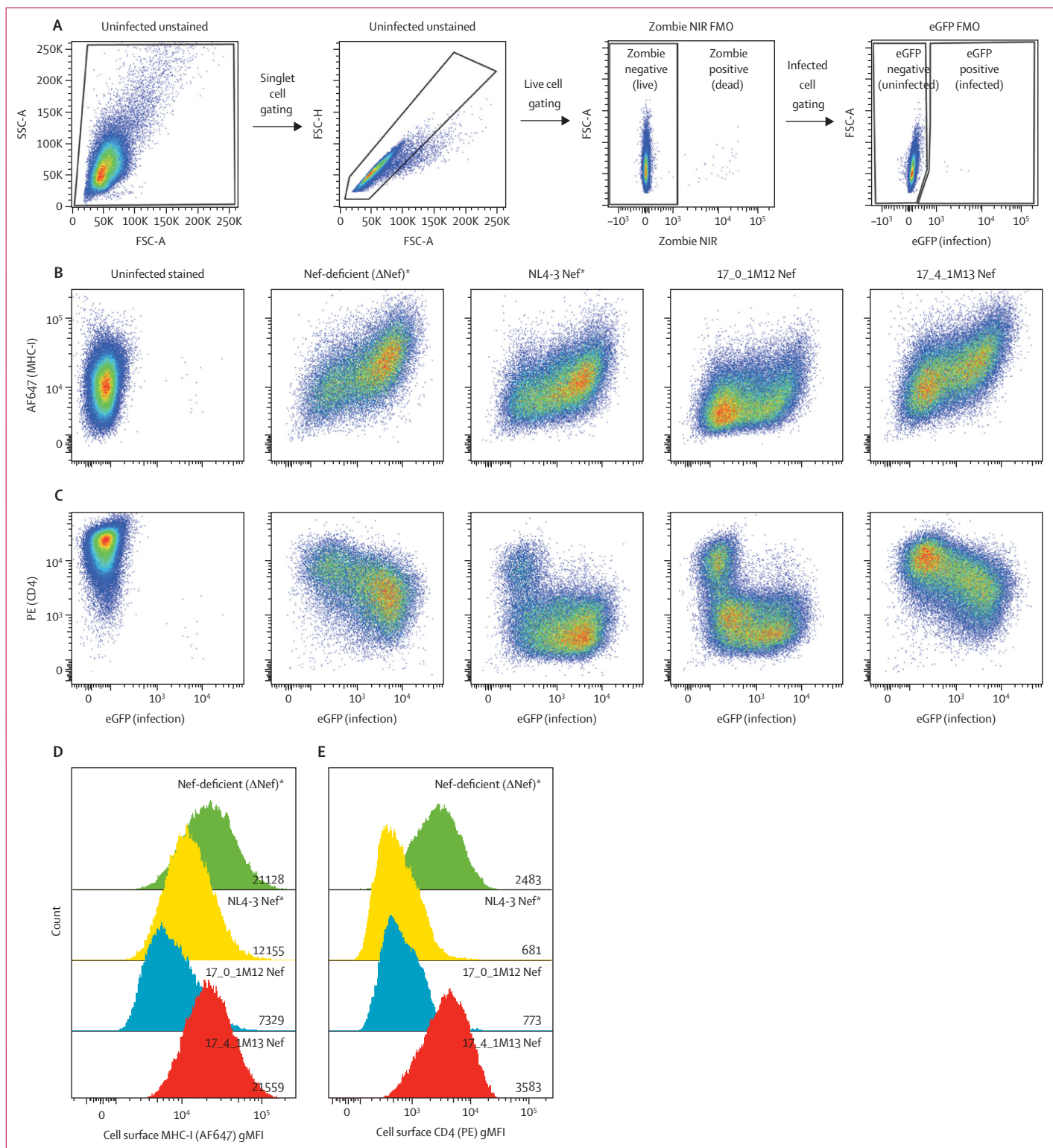
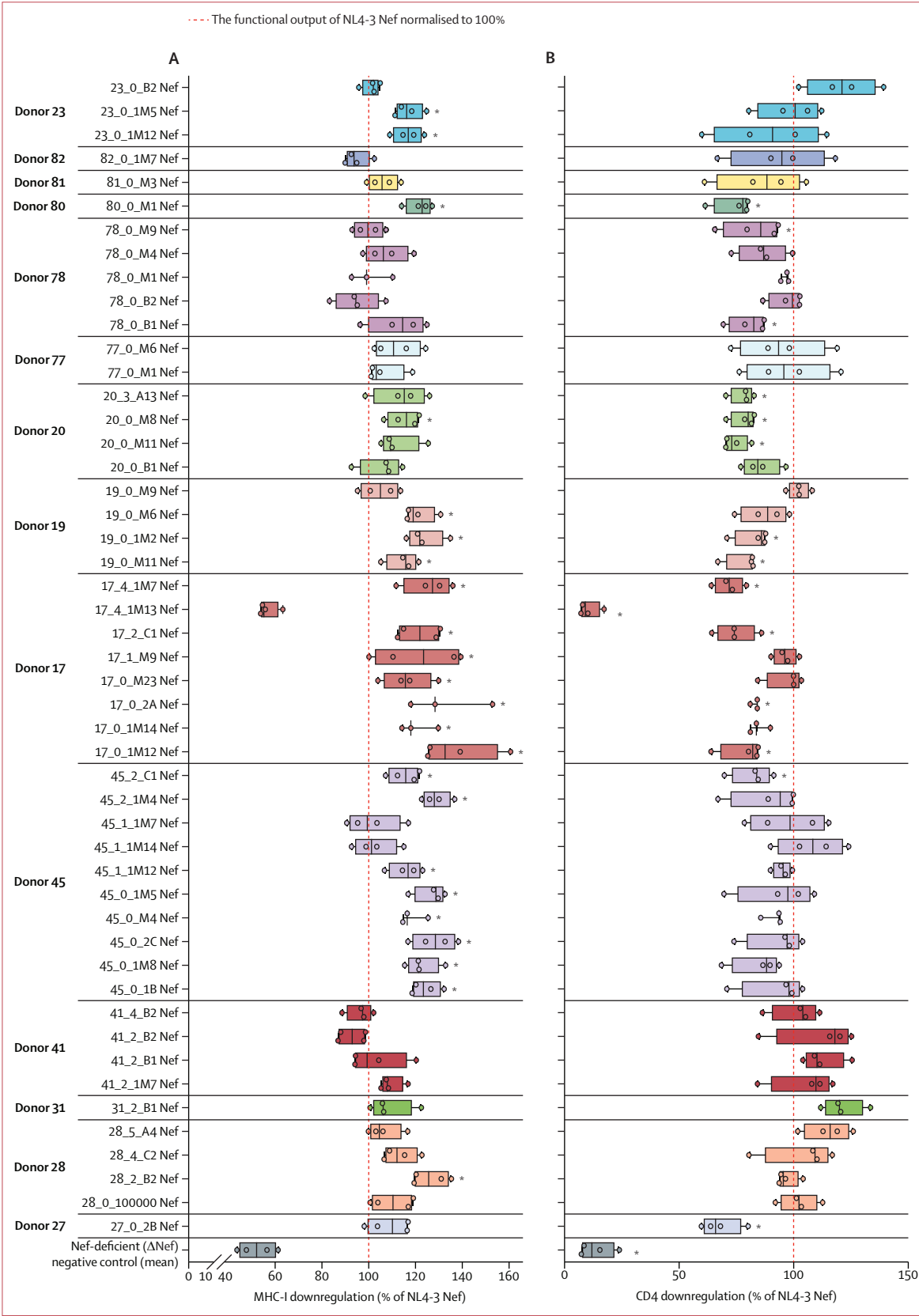


Figure 2: Gating schematic used to quantify cell surface MHC-I and CD4 levels within infected cells

Sup-T1 cells were infected with vesicular stomatitis virus protein G-pseudotyped viruses encoding primary Nef isolates. Cell-surface CD4 and MHC-I levels were quantified on infected cells via flow cytometry. (A) Gating schematic used for identifying infected Sup-T1 cells (based on FMO control): initial gating by FSC-A by SSC-A; FSC-A by FSC-H to exclude doublets; viability dye to exclude dead cells; and eGFP to identify infected cells. (B–E) Representative plots for cells infected with pseudoviruses that were Nef-deficient (Δ Nef negative control), pseudoviruses that expressed NL4-3 Nef (positive control), and pseudoviruses that encoded two participant-derived Nef samples: 17_0_1M12 and 17_4_1M13 (representing efficient and inefficient downregulation, respectively). Specifically: pseudocolour plots for MHC-I (B) and CD4 (C) and histograms (with gMFI shown) for MHC-I (D) and CD4 (E). AF647=Alexa Fluor 647, eGFP=enhanced green fluorescent protein, FMO=fluorescence minus one, FSC-A=forward scatter area, FSC-H=forward scatter height, gMFI=geometric mean fluorescence intensity, NIR=near-infrared, PE=phycoerythrin, SSC-A=side scatter area. *The plots for Nef-deficient (Δ Nef) and NL4-3 Nef pseudoviruses were derived from the first technical replicate of the given experiment.

Figure 3: Participant-derived Nef-mediated downregulation of cell surface CD4 and MHC-I relative to NL4-3 Nef
Box plots show cell surface MHC-I (A) and CD4 downregulation (B)—compared with the mean Nef-deficient (Δ Nef) negative control—as a percentage of mean NL4-3 Nef for each primary Nef variant (median with IQR displayed, $n=4$). The red dashed line indicates the functional output of NL4-3 Nef normalised to 100%. *Significant differences in CD4 and MHC-I downregulation between each isolate and the NL4-3 Nef positive control are denoted by an asterisk ($p \leq 0.05$); exact p values and the functional output medians are provided in appendix 3 (pp 4-5).



subtype B HIV-1.¹⁴ Additionally, a study conducted in Canada showed that participants with non-subtype B HIV-1 had significantly smaller RC-LVR than did those with subtype B HIV-1.¹³ Although our cohort had no participants with subtype B HIV-1, subtype B Nef is generally more efficient at MHC-I downregulation than non-subtype B Nef.²⁸ Thus, variability in Nef functionality could contribute to differences in RC-LVR size when comparing HIV-1 subtypes. Although we did not observe significant differences in Nef function between subtypes A1 and D, the genetic variability between subtypes warrants a broader analysis.²⁸

The relationship between Nef-mediated cell surface MHC-I downregulation and the LVR observed in our study is consistent with a previous study, in which Nef-mediated MHC-I, but not CD4, downregulation positively correlated with RC-LVR size.¹³ This previous study predominantly included people with subtype B HIV-1 who started ART shortly after infection (<6 months) who had viral suppression for 48 weeks.¹³ The previous study and the current study suggest that Nef is a crucial viral determinant in both the establishment and maintenance of the RC-LVR during ART.¹³

Because Nef is expressed in latently infected rCD4⁺ T cells, our results suggest that efficient Nef-mediated MHC-I downregulation could shield latently infected cells from CTL recognition during long-term ART.²¹ This shielding could promote persistence and clonal expansion of these cells, thereby leading to very slow reservoir decay. Conversely, our analyses suggest that inefficient downregulation of MHC-I leads to accelerated RC-LVR decay, probably through increased CTL recognition of viral antigens, leading to enhanced elimination of latently infected cells. The genetic characterisation of proviruses derived from CD4⁺ T cells of people with HIV-1 on prolonged ART found that genetically intact proviruses were enriched in effector memory CD4⁺ T cells, suggesting effector memory CD4⁺ T cells comprise the majority of the RC-LVR.²¹ Latently infected effector memory CD4⁺ T cells had fewer known CTL escape mutations in *gag* and *pol* than central memory CD4⁺ T cells, suggesting these effector memory CD4⁺ T cells rely on Nef-mediated downregulation of MHC-I to be shielded from CTL elimination.²¹ Moreover, participant-derived CD4⁺ T cells infected with Nef-deficient HIV-1 were cleared more efficiently than cells infected with HIV-1 upon co-culture with autologous CTLs. This finding suggests that Nef-mediated MHC-I downregulation represents a primary mechanism protecting latently infected effector memory CD4⁺ T cells from CTL clearance.²¹

We found the size of the inducible RC-LVR in people with HIV-1 on long-term ART was estimated to possibly increase in four individuals. This was unexpected, given previous reports of RC-LVR decay on ART, but consistent with a study measuring the size of the RC-LVR in people with HIV-1 on very long-term ART (mean duration of 22 years).^{7,29} This study observed that the reservoir decays slowly during short-term ART, with an inflection point at approximately

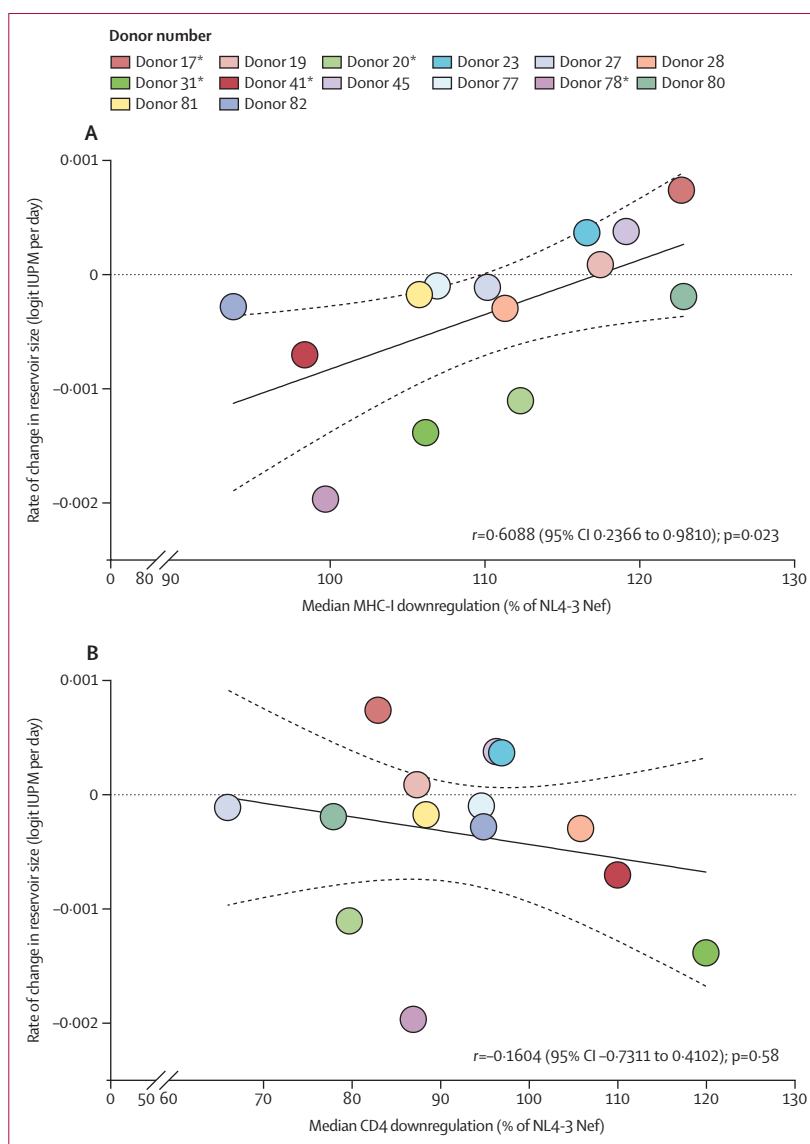


Figure 4: Correlation between the level of Nef-mediated MHC-I and CD4 downregulation and changes in RC-LVR size

The output for both functions is represented as cell-surface MHC-I or CD4 downregulation compared with the mean Nef-deficient (Δ Nef) negative control followed by normalisation to mean NL4-3 Nef, as described in figure 3. For donors with more than one retrieved *nef* sequence, the median Nef functional output was selected for subsequent correlation analysis, and each dot represents an individual. The 17_4_1M13 sample with the premature stop codon located within the core region of the Nef protein was included in the median Nef functional output calculation. Correlation analysis comparing the slope of the change in reservoir size for each participant with HIV-1 involved in the study to median MHC-I cell-surface downregulation (A) and median CD4 cell-surface downregulation (B). To assess whether these correlations were significant, a Spearman correlation test was used to compare cell surface MHC-I or CD4 downregulation with the slope of the change in reservoir size, where a two-tailed p value of 0.05 or less was considered statistically significant. The Spearman correlation coefficients (r) are listed along with the 95% CIs. Linear regression best-fit lines and 95% CIs were plotted. Study participants with significant estimated RC-LVR rates of change are denoted in the colour key with an asterisk. An RC-LVR rate of change was deemed significantly positive or negative if the 95% credibility intervals were both positive or negative, respectively. ART=anti-retroviral therapy. IUPM=infectious units per million. RC-LVR=replication-competent latent viral reservoir.

7 years of ART in which the reservoir begins to slowly increase in size.²⁹ Because the median time on ART at the time of enrolment in our study was 8.76 years, many

participants would have been approaching or surpassing the inflection point, thereby representing a potential confounding factor. Additionally, the inducible RC-LVR becomes more clonal during long-term ART, suggesting selection occurs within the reservoir during virally suppressive ART.²⁹ This selection could be driven by Nef's ability to evade CTL recognition, because latently infected cells deficient in MHC-I downregulation would probably be readily cleared.^{13,21}

Our study also has several limitations. First, our study population was small; nevertheless, we selected multiple variants from several individuals, providing a more detailed examination of intra-individual variability. Moreover, the longitudinal estimations of change in reservoir size were derived from a Bayesian model informed by a much larger study, thereby increasing estimation accuracy.¹⁵ Second, we cannot discount long-term administration of ART longer than 7 years as a potential factor in our estimations of change in RC-LVR size because most participants were enrolled after this point. Third, given that the main study objective was to identify a potential relationship between Nef-mediated host adaptive immune evasion and rate of RC-LVR decay, we cannot assess whether Nef's ability to downregulate MHC-I influenced the persistence of specific clones over time. Fourth, although this study only explored the relationship between Nef-mediated MHC-I and CD4 downregulation and the rate of RC-LVR decay, we do acknowledge that other Nef functions not assessed in this study—such as SERINC3 and SERINC5 downregulation—could influence the rate of RC-LVR decay. Nonetheless, we speculate that the effects of such functions on the RC-LVR are probably minimal in a virally suppressive ART context.¹⁷ Finally, given the significant effect of switching to dolutegravir on RC-LVR size in this population, it would be interesting to examine whether differences in MHC-I or CD4 downregulation, or both, could be observed in primary Nef isolates derived before and after the dolutegravir switch.¹⁵

In conclusion, our results identify Nef-mediated downregulation of MHC-I as an important viral correlate to longitudinal changes in the size of the RC-LVR during prolonged ART. These findings suggest Nef could influence both the initial establishment of the RC-LVR before ART administration and the persistence of the RC-LVR during long-term (>1 year) ART.^{13,21} Considering pharmacological inhibition of Nef-mediated MHC-I downregulation sensitises latently infected cells to CTL clearance in vitro,³⁰ future studies are warranted to assess whether in-vivo administration of Nef inhibitors can therapeutically reduce an established LVR in subtype B HIV-1 and non-subtype B HIV-1 contexts. Such strategies could be an important step in developing a functional HIV/AIDS cure.³⁰

Contributors

JDD, MJM, JLP, and ADR conceptualised the study. MJM, JLP, ADR, JDD, XZ, and R-CF curated the data. MJM, JDD, JLP, ADR, XZ, and R-CF did the formal analysis. JDD, JLP, ADR, TCQ, AART, and SJR acquired funding. JDD, JLP, ADR, MJM, SS, JH, EEB, AAC, ORB, EK, JCM, CK, and BL did the

investigation and acquired the data. MJM, JDD, JLP, ADR, CRE, SMT, CF, and XZ developed the methodology. SJR, ST, SJ, AA, TK, and PB coordinated the participants. JDD, MJM, SJR, JLP, and ADR administered the project. JLP, ADR, JDD, TCQ, AART, and SJR obtained resources. JLP ran the statistical software. JDD, JLP, ADR, SJR, and AFYP supervised the study. JDD, MJM, JLP, and ADR validated and visualised the data and wrote the original draft. All authors contributed to the writing, editing, and reviewing of the manuscript. MJM, JDD, JLP, ADR, and AFYP directly accessed and verified the underlying data reported in this manuscript. All authors had full access to all the data in the study and accept responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

All data used to generate figures and tables are available upon reasonable request to the corresponding author and pending any International Review Board approvals. The accession numbers of the primary *nef* sequences deposited in GenBank are PP171563–PP171611.

Acknowledgments

The authors would like to thank the study participants and clinical study staff. This work was supported by the Canadian Institutes of Health Research (project grant number 186284 to JDD and project grant number 155990 to AFYP) and the Division of Intramural Research at the National Institute of Allergy and Infectious Diseases (NIAID), as part of the US National Institutes of Health (NIH). The REACH Martin Delaney Collaboratory (NIH grant 1-UM1AI164565) is supported by the following NIH co-funding institutes: the National Institute of Mental Health; the National Institute on Drug Abuse; the National Institute of Neurological Disorders and Stroke; the National Institute of Diabetes and Digestive and Kidney Diseases; the National Heart, Lung and Blood Institute; and the NIAID. JLP was supported by the Canada Research Chairs Program, the Canadian Foundation for Innovation John R Evans Leaders Fund, and the Ministry of Colleges and Universities Ontario Research Fund Small Infrastructure Fund. MJM was supported in part by an Ontario Graduate Scholarship provided by the Government of Ontario. R-CF was supported in part by a fellowship from the Ontario Genomics—Canadian Statistical Sciences Institute.

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