HIV subtype and Nef-mediated immune evasion function correlate with viral reservoir size in early-treated individuals

by

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Abstract

The HIV accessory protein Nef is genetically diverse and modulates key immune evasion and pathogenic functions. Recent early HIV-specific adaptive immune responses were identified as correlates of HIV reservoir size. So, we hypothesized that viral factors facilitating evasion of such responses might also influence reservoir establishment and/or persistence. Plasma HIV RNA-derived *nef* clones were isolated from 30 acute/early-infected individuals and assessed for their CD4 and HLA-I downregulation function *in vitro*. We explored the relationships between baseline clinical, immunologic and virologic characteristics, and HIV reservoir size measured 48 weeks following initiation of suppressive cART. Nef-mediated HLA downregulation correlated positively with reservoir size. Furthermore, this function was retained in final multivariable models adjusting for established clinical and immunologic correlates of reservoir size. HIV subtype B infection also emerged as a significant correlate of reservoir size on cART. Results highlight potentially important role of viral factors in modulating viral reservoir establishment and persistence.

Keywords: HIV; Nef; viral reservoir; HLA-I downregulation; CD4 downregulation; viral pathogenesis

Dedication

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List of Acronyms

ADCC	Antibody dependent cellular cytotoxicity
AP2	Adaptor protein 2
cART	Combination antiretroviral therapy
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EF1	Human elongation factor-1
ELISA	Enzyme-linked immunosorbent assay
GFP	Green fluorescent protein
GTR	General time-reversible
HiFi	High fidelity
HIV-1	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T-cell leukemia-lymphoma virus
IQR	Interquartile range
IUPM	Infectious units per million
LANL	Los Alamos National Lab
LB	Luria-Bertani
LTNP	Long term non-progressor
LTR	Long terminal repeat
MAA	Multiassay algorithm
MAFFT	Multiple Alignment using Fast Fourier Transform
MFI	Median fluorescent intensity
Nef	Negative factor
OD	Optical density
PACS-1	Phosphofurin acidic cluster sorting protein 1
PACS-2	Phosphofurin acidic cluster sorting protein 2
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
pVL	Plasma viral load

RIP	Recombinant identification program
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
SBBC	Sidney blood bank cohort
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIV	Simian Immunodeficiency Virus
TGN	Trans-Golgi network

Executive Summary

While combination antiretroviral therapies (cART) have transformed HIV into a chronic manageable condition, they do not act upon the latent HIV reservoir and are therefore not curative. As HIV cure or remission should be more readily achievable in individuals with smaller HIV reservoirs, achieving a deeper understanding of clinical, immunologic and virologic determinants of reservoir size is critical to eradication efforts. We performed a post-hoc analysis of 30 participants of a clinical trial of early cART who had previously been assessed in detail for their clinical, immunologic and reservoir size characteristics. We observed that HIV subtype and autologous Nef-mediated HLA downregulation function correlated with viral reservoir size measured approximately one-year post-cART initiation. Our findings highlight virologic characteristics - both genetic and functional - as possible novel determinants of HIV reservoir establishment and persistence.

Chapter 1.

Functional analysis of primary HIV *nef* isolates

1.1. Introduction

1.1.1. The Nef protein

Human Immunodeficiency Virus (HIV) Negative Factor (Nef) protein is an accessory protein that is highly polymorphic in length (200-215 amino acids) and weight (27-35kDa) (1). It is located near the 3' end of the HIV genome, downstream the *envelope* gene, and partially overlaps with the U3 region of the 3' long-terminal repeat (LTR) (2). Nef lacks enzymatic activity and was initially thought to be dispensable for HIV pathogenicity (3). However, *in vivo* studies of both humans and primates have since redefined Nef as a pathogenic factor, in large part due to lack of disease progression resulting from infection with *nef*-deleted viruses (4–6). Among the extensively studied Nef functions include its immune evasion functions (primarily downregulation of CD4 (7, 8) and HLA-I (9, 10) molecules from the surface of infected cells), as well as its role in enhancing viral infectivity and replication (11, 12).

One important characteristic of HIV is its high evolutionary rate (13, 14) which enables it to adapt rapidly to its host immune environment (15). In addition, the virus undergoes recombination events (16, 17) which taken together gives rise to HIV genetic diversity. Indeed, HIV group M strains, which are responsible for the global pandemic, currently comprise 9 subtypes and 89 circulating recombinant forms that differ from one another at up to 30% of amino acid positions in some of HIV's more variable proteins (18). As a result, it is conceivable that this high *nef* genotypic diversity would in turn lead to diversity in Nef's major immune evasion functions, and thereby potentially influence pathogenesis. Whereas laboratory-adapted *nef* alleles have been extensively used to investigate the functions of the Nef protein *in vitro*, it is also vital to study the impact of naturally-occurring *nef* sequence variation on protein function - and in particular to quantify the dynamic range of function of naturally circulating *nef* sequences. Therefore, in this review we discuss studies that have analyzed primary *nef* isolates to investigate the relevance of genetic diversity on Nef's well-characterized immune evasion functions,

namely CD4 and Human Leukocyte Antigen class I (HLA-I) downregulation activities. We additionally discuss the evidence supporting *nef* genetic variation in influencing HIV pathogenesis, and more recently, HIV latency.

1.1.2. Global HIV epidemic and disease pathogenesis

Human Immunodeficiency Virus infection continues to affect populations across the world, though sub-Saharan Africa bears a disproportionate infection burden (19, 20). As of 2017, 36.9 million people were living with HIV (21). Of these, only 59% were accessing treatment, and only 47% were virally suppressed (21). Sadly, 1.8 million new infections were recorded in the same year with 0.9 million HIV-related deaths occurring in 2017 (21). Overall, people living with HIV infection continue to experience reduced quality of life compared to the general population (22). Advances in antiretroviral therapy since the mid-1990s (23) have transformed HIV infection from a fatal disease to a chronic manageable condition (24–29), but there remains no cure or vaccine. One or both will likely be required to ultimately contain the epidemic.

The HIV primarily infects CD4+ T lymphocytes, and CD4+ T cell depletion is a hallmark of HIV progression if the infection is left untreated (30, 31). CD8+ cytotoxic T lymphocyte (CTL) responses eliminate HIV-infected CD4+ T lymphocytes through recognition of specific viral peptides presented by human leukocyte antigen class I (HLA-I) molecules on the surface of the infected cells; for this reason, CTL responses are said to be HLA-restricted. HLA-restricted CTL responses emerge in acute HIV infection and play a major role in controlling viremia to setpoint levels, which are predictive of subsequent disease progression (32–35). The majority of HIV-specific $CD8^+$ T cell responses are directed at Gag, Pol and Nef proteins of HIV (36, 37). However, HIV, as a result of its high mutation rate (14), is able to develop mutations to evade CTL recognition, which are rapidly selected *in vivo* (38–42). These mutations are commonly referred to as "CTL escape mutations "or "immune escape mutations". Adaptation of HIV to HLA-restricted immune pressures is a major contributor to both within-host and population-level HIV evolution (43–46).

Given the HIV's extensive mutational capacity and global sequence diversity, elucidation of the impact of this variation on HIV pathogenesis is of critical importance. Whereas clinical, immunological and host genetic determinants of disease progression

have been characterized (47–52), there is comparably less knowledge on the impact of viral factors on HIV pathogenesis. Particularly, immune evasion mechanisms such as those mediated by Nef have been mostly described using *in vitro* systems that employ the use of transformed cell lines as well as laboratory adapted strains of HIV and/or HIV proteins. Therefore, studies on naturally circulating *nef* alleles are warranted, as such studies can shed light on pathophysiological role of Nef-mediated immune function on disease pathogenesis.

1.2. History of clinical evidence of the role of HIV Nef in disease pathogenesis

1.2.1. Simian Immunodeficiency Virus (SIV) studies

Initial evidence for the importance of Nef in disease progression was provided by Kestler et al., (1991) who demonstrated the need for an intact SIV-mac239 *nef* allele for the maintenance of high viral loads and progression towards immunodeficiency in this animal model (4). Deletions in *nef* were associated with blunted simian immunodeficiency disease severity in rhesus macaques. Moreover, when the macaques were infected with defective viruses that contained premature stop codons in *nef*, a decrease in virulence with slow or no disease progression was noted. However, mutations eventually occurred *in vivo* to restore the *nef* open reading frame, supporting strong selective pressure to express functional Nef protein for maximum infectivity *in vivo*.

1.2.2. The Sydney blood bank cohort (SBBC) study

In humans, the role of HIV Nef in pathogenesis is most clearly demonstrated by long term non-progressors (LTNP) infected with *nef*-deleted virus (5, 53, 54). In the Sydney blood bank cohort (SBBC), eight individuals became infected with *nef*-defective HIV after being transfused with blood products from a single blood donor who harbored *nef*-deleted HIV. Six of these patients have since been extensively studied. All of them harbored HIV quasispecies with various inactivating deletions within *nef*; these included 177- and 11-base pairs from *nef*-alone region and 120- and 86-base pairs from *nef-LTR* overlap region (53). The fact that these patients were infected by a *nef* defective virus is a rare occurrence. Additional cases of long-term non-progression of HIV infection

attributed to *nef*-defective virus have also been documented. For instance, in Kirchhoff et al. (1995), the clinical attributes of the infected patient were similar to those of the SBBC, however slight differences in infecting virus was noted; namely, deletions in the region in which the *nef* gene and the *LTR* overlap were distinct in size and position from those of the virus infecting persons in the SBBC (5). Attenuated disease with *nef*-defective strains has also been documented in non-subtype B infections. For example, infection with a circulating recombinant form 01_AE bearing deletions in the *nef-LTR* region was documented in a patient who had a persistently low antibody response and undetectable viral load over the course of 5-year follow-up period (55).

Altogether, the rhesus macaque and human studies provide evidence for the relevance of *nef* in disease progression.

1.3. Nef-mediated CD4 downregulation

The ability of Nef to downregulate CD4 molecules from the surface of infected cells is a well conserved function of *nef* and was the first of Nef's functions to be documented (8, 56). It has since been established that Nef acts by linking mature CD4 to the clathrin adaptor protein-2 (AP2) which in turn recruits other clathrin dependent trafficking components that act in concert to internalize CD4 through clathrin-coated pits (57, 58). Nef-mediated CD4 downregulation prevents superinfection of infected cells (59) and enhances release of virions (60). More recently it has been discovered that Nef-mediated CD4 downregulation also enables infected cells to escape antibody-dependent cell-mediated cytotoxicity (ADCC) (61), specifically by preventing conformational changes induced by CD4-envelope interaction that are needed to expose ADCC epitopes (62). This was initially demonstrated using laboratory adapted *nef* isolates and was subsequently confirmed using primary *nef* alleles (63, 64). The sequence motif essential for Nef-mediated CD4 downregulation has been described to be the well conserved di-leucine motif ($L_{164}L_{165}$)(65–67) through mutagenesis of laboratory adapted strains. Therefore, to determine its physiological relevance, studies of primary *nef* isolates are warranted in order to identify motifs in natural sequences that are involved in downregulation of CD4 molecules from the surface of infected cells.

Various lines of evidence support Nef function as a correlate of HIV pathogenesis. HIV Elite controllers, defined as patients who maintain undetectable viral

loads or <50 copies/ml of blood for a period of at least one year without antiretroviral therapy (68–70), display on average reduced Nef-mediated CD4 downregulation function as compared to chronic progressors (71). In addition, *nef* alleles isolated in acute infection have been shown to exhibit better CD4 downregulation function as compared to those isolated from chronic stage of HIV infection (72). This observation suggests that this function is particularly critical in the early phase of HIV infection.

Nef-mediated CD4 downregulation function also differs by HIV subtype. In a study investigating subtype-based differences in CD4 downregulation function across subtypes A, B, C, and D, subtype C *nef* alleles displayed modest yet significantly lower CD4 downregulation capacity compared to other subtypes (73). This suggests the existence of inherent differences in immune evasion functions of different HIV-1 group M subtypes, and it is tempting to postulate that these differences may in turn contribute to differential pathogenicity observed in different HIV subtypes (74–76).

1.4. Nef-mediated HLA-I downregulation

The downregulation of HLA-I, particularly HLA-A and HLA-B alleles is another well characterized property of Nef (10). Nef-mediated downregulation of HLA-A and HLA-B enables infected cells to evade elimination by cytotoxic CD8+ T lymphocyte responses restricted by alleles belonging to these loci (77). Two mechanistic models of HLA-I downregulation have been proposed. The first model proposes that Nef forms a complex with adaptor protein-1 and the newly synthesized HLA-I molecules in the trans-Golgi network (TGN) and this prevents their translocation from the TGN to the plasma membrane (78). The second model suggests that Nef complexes with Phosphofurin acidic cluster sorting protein 2 (PACS- 2) through which it interacts with Src family kinases to activate a signaling cascade that results in trafficking of HLA-I molecules from the cell surface to endosomes (79–83). This trafficking occurs in adenosine diphosphate ribosylation factor-coated vesicles. The endosomes are then translocated to the TGN where Nef forms a complex with Phosphofurin acidic cluster sorting protein 1 (PACS-1) to retain the internalized HLA-I molecules (84, 85).

The impact of naturally occurring sequence variation on HLA-I downregulation has been previously reviewed by Mwimanzi et al. (86). However, our understanding of the impact of naturally occurring CTL escape mutations in *nef* on disease outcomes

remains incomplete. A subsequent study by Kuang et al. suggested that altered Nef-mediated HLA downregulation function contributed to disease progression (71). Specifically, *nef* sequences isolated from acutely infected persons who went on to become viremic controllers exhibited impaired immune evasion functions and reduced virion infectivity compared to those from acutely infected persons who did not subsequently achieve natural virologic control. Furthermore, in one of the acute controllers who was further investigated, HLA-associated polymorphisms selected as a result of host CTL pressure acted to further reduce Nef-mediated HLA downregulation capacity. This underscores the role played by host immune pressure in selecting HIV mutations that are costly to viral protein function; moreover this observation represented among the first documented evidence of fitness-costly immune-driven mutations in *nef* (71).

HIV subtype-specific differences have also been described for Nef-mediated HLA-I downregulation function (73). The functional hierarchies observed for Nef-mediated HLA downregulation were similar to those for CD4 downregulation; subtype B *nefs* displayed better average function than subtypes A/D, while subtype C displayed the weakest average functions of all four subtypes tested. The overall functional differences for HLA downregulation however were of a greater magnitude than those observed for Nef-mediated CD4 downregulation. This observation is tempting to speculate a potential disposition of host to accelerated clinical disease outcome based on the subtype of the infecting virus.

1.5. Nef-mediated immune evasion as a correlate of reservoir size

One key defining feature of HIV infection is the establishment of viral reservoirs very early in infection that persist long-term, even during suppressive antiretroviral therapy (87). The viral reservoir has been defined as cells or body tissues in which a replication-competent form of the virus stably persists for prolonged periods of time (88). It is now known that a small fraction of latently infected resting memory $CD4^+$ T cells carrying an integrated form of the viral genome represent the major HIV reservoir (89, 90); virus persistence is achieved when the infected $CD4^+$ T cell reverts to a resting state which then enables the integrated virus to escape biochemical decay processes or elimination via immune effector mechanisms (88). The size of an individual's latent

reservoir is important because at least in theory, having a smaller reservoir size would mean a patient is more amenable to cure. Several factors have been implicated in determining viral reservoir size. They include clinical factors such as timing of cART initiation (91–93) and set-point viral load (94) and immunologic factors such as homeostatic proliferation (95), clonal expansion (96, 97), HIV-specific granzyme B responses (98). One additional possible factor that could contribute to determining the size of viral reservoir is immune evasion functions such as those mediated by the HIV Nef accessory protein. However, evidence directly linking Nef mediated immune evasion functions and viral reservoir size in HIV infection are lacking. As HIV latency and eradication research currently represents a priority area, my thesis aims to narrow this knowledge gap: specifically, my thesis focuses on characterizing genetic and phenotypic diversity of primary *nef* isolates from a cohort of early treated HIV infected individuals and how these impacts on viral reservoir size.

1.6. Conclusions

HIV's high rate of replication and mutation, coupled with immune selection pressure, accounts for a substantial portion of the genetic diversity observed in *nef* both within and between hosts (99–103). Recently, efforts have been made to elucidate variation in the nef gene and associated impact on disease outcome.

To further explore biological relevance of these in vitro-defined Nef functions, characterizing the functional integrity of primary isolate *nef* alleles and their associated clinical outcomes is needed. Particularly, more data are needed to determine how genetic and functional variability in Nef-mediated immune evasion impacts on viral pathogenesis, as well as Nef's potential role in the establishment of viral reservoirs. This information may in turn inform the development of new preventive or therapeutic strategies, as well as approaches to limit or prevent the establishment of viral reservoirs towards the ultimate goal of an HIV cure.

1.7. Thesis Overview

This thesis focuses on characterizing genetic and phenotypic diversity of primary *nef* isolates from a cohort of early treated HIV infected individuals and how these impacts on viral reservoir size. The present chapter provides a review of studies that

have evaluated the function of primary *nef* isolates *in vitro*, mainly in the context immune evasion functions and viral pathogenesis. **Chapter 2** comprises a primary research study that explores the hypothesis that naturally-occurring genetic and functional variation in Nef might influence the extent of HIV reservoir seeding in early infection. To test this hypothesis, we employ an in vitro CEM T cell line model that stably expresses CD4 and has been engineered to express HLA-A*02. Using this model, we assessed the effect of expression of the different participant-derived Nef clones on cell surface HLA-A*02 and CD4 levels on CEM T cells. Our analyses revealed HIV subtype and Nef-mediated HLA-I downregulation as significant correlates of HIV reservoir size measured at 48 weeks after initiation of suppressive combination antiretroviral therapy. Lastly, **chapter 3** provides a brief summary of the thesis and provides forward-looking statements.

Altogether, the results identify novel viral factors, namely HIV subtype and Nef-mediated HLA-I downregulation, as significant correlates of reservoir size. Further elucidation of the underlying mechanisms could inform the design of novel therapies or vaccines that can limit or prevent the establishment of HIV reservoirs.

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Chapter 2.

Characterization of Nef-mediated immune evasion functions and identification of correlates of reservoir size in early-treated individuals

This work has been accepted for publication in Omondi FH, Chandrarathna S, Mujib S, Brumme CJ, Jin SW, Sudderuddin H, Miller R, Rahimi A, Laeyendecker O, Bonner P, Yue FY, Benko E, Kovacs CM, Brockman MA, Ostrowski M, Brumme ZL. 2018. ***HIV subtype and Nef-mediated immune evasion function correlate with viral reservoir size in early-treated individuals****. J Virol (Accepted, 30-Nov-2018).*

I performed all functional studies of Nef clones included in this chapter, analyzed this data and co-wrote the manuscript. I also contributed substantially to Nef genotyping, cloning, and sequence validation. M.A.B., M.O. and Z.L.B. conceived and designed experiments. P.B. collected and processed samples. S.W.J. and H.S. assisted with western blots, cloning and flow cytometry. S.C. and A.R. assisted with genotyping, cloning and preliminary functional assays. S.M. provided clinical data for recency estimation, baseline granzyme B responses, CD4+ T cell counts, plasma viral load, baseline and week 48 post-cART proviral DNA levels and replication competent reservoir levels. C.J.B. performed multivariable regression analyses. Z.L.B. assisted with data analysis and co-wrote the manuscript.

The major barrier to achieving HIV cure or remission, where the latter is defined as a state where combination antiretroviral therapies (cART) could be discontinued without the risk of viremia recrudescence (1), is the long-term persistence of latently HIV-infected cells. Primarily comprising long-lived resting memory $CD4^+$ T cells (2-4) that harbor integrated replication competent HIV in a near transcriptionally quiescent state (5-7), these "viral reservoirs" can re-activate at any time to produce infectious virions (7-12). As individuals with smaller latent HIV reservoirs should be more amenable to cure, remission and/or post-treatment virologic control (13-15), the identification of clinical, immunologic and other determinants of reservoir size is a priority.

It is now well-established that early cART initiation limits latent HIV reservoir size (16-20), and that set-point viral load and duration of viral suppression on cART represent additional positive and negative correlates of reservoir size respectively (21). Immunological factors also play a role. Homeostatic proliferation (22) and clonal

expansion (23-28) of latently HIV-infected $CD4^+$ T-cells directly influence latent HIV reservoir size and dynamics, and evidence indicates that initial antiviral immune responses may also modulate reservoir establishment and persistence (29, 30). A recent longitudinal study of an acute infection cohort reported that pre-therapy levels of key cytokines correlated with HIV DNA levels after 96 weeks of cART (29). Moreover, a previous analysis of the cohort presently studied identified baseline HIV-specific granzyme B responses, contributed mainly by Human Leukocyte Antigen (HLA) class I-restricted $CD8^+$ T-cells, as significant negative correlates of reservoir size 48 weeks post-cART as measured in terms of HIV proviral loads as well as levels of replication-competent viral infectious units per million $CD4^+$ T-cells (30).

The observation that early HIV-specific adaptive immune responses correlate with reservoir size (30) prompts the hypothesis that viral factors that facilitate evasion of such responses might also influence reservoir establishment and persistence. The HIV accessory protein Nef represents such a factor. Nef evades host adaptive immunity by downregulating cell-surface HLA-A and -B (31) as well as CD4 (32, 33). The former function allows infected cells to evade HLA-restricted $CD8^+$ T-cell responses (34), while the latter function allows infected cells to evade antibody-dependent cell-mediated cytotoxicity [ADCC] by reducing the capacity of cell-surface Env to transit to its CD4-bound conformation that is required for ADCC epitope exposure (35). As primary *nef* sequences differ in their ability to downregulate CD4 and HLA, the latter in particular (36-41), we hypothesized that individuals harboring *nef* sequences with strong immune evasion function would display larger reservoirs due to Nef-mediated protection of infected cells from immune clearance. Indeed, a role for Nef in maintaining the HIV reservoir is supported by the recent observation that pharmacologic inhibition of Nef promoted $CD8^+$ T-cell-mediated elimination of latently HIV-infected cells *in vitro* (42).

More broadly, given the vast genetic diversity of HIV globally (pandemic group M strains currently comprise 9 subtypes and 96 circulating recombinant forms (43)), modulatory effects of HIV genotype/phenotype variation on latent reservoir size are conceivable. In support of this, a recent study reported that HIV reservoir sizes among virally-suppressed individuals in Uganda (where subtypes A and D predominate) were three times smaller than among individuals in the United States (where subtype B predominates), where differences were not clearly attributable to demographic or clinical characteristics (21). HIV subtype B *nef* sequences display, on average, the highest CD4

and HLA downregulation activities of all major group M subtypes (38), prompting the hypothesis that superior within-host Nef function may play a key role in establishing larger reservoirs in subtype B-infected persons.

Towards identifying novel virologic correlates of HIV reservoir size, we performed HIV subtyping and assessed within-host Nef function among 30 individuals with acute/early (<6 months) infection who took part in a clinical trial comparing standard and intensive cART, and who had previously been assessed in detail for their clinical, immunologic and reservoir size characteristics (20, 30). The original trial identified timing of cART initiation, but not initial treatment regimen, as a significant correlate of HIV reservoir size measured at 48 weeks post-cART (20). A subsequent analysis that pooled all participants regardless of initial regimen identified HIV-specific CD8+ granzyme B responses directed against Tat/Rev, Env, Gag, and Vif, as well as proteome-wide, as additional negative correlates of reservoir size (30). With the present study, we extend these observations to identify HIV subtype and Nef-mediated immune evasion function as additional novel correlates of reservoir size in early-treated individuals, supporting virologic characteristics as critical modulators of the HIV reservoir.

2.1. Methods

2.1.1. Study participants

Study participants, determined to have been infected with HIV for less than 6 months and who were initially naive to cART, were originally recruited from the Maple Leaf Clinic in Toronto, Ontario, Canada as part of a treatment intensification clinical trial (ClinicalTrials.gov Identifier: NCT01154673). The original trial (20) recruited 32 males aged 22-59 who self-identified as men who have sex with men; the present study included 30 of these participants for whom baseline plasma was available for analysis. All participants gave written informed consent and the study was approved by the University of Toronto, St. Michael's Hospital and Simon Fraser University research ethics boards. As described previously (20), acute/early HIV infection was defined by one of the following criteria: (1) positive HIV antibody test (Western blot) with documented negative test in previous 6 months; or (2) positive/weakly positive HIV enzyme-linked immunosorbent assay (ELISA), with indeterminate or evolving Western blot with demonstrated HIV antigenemia (p24) or viremia (HIV viral load ≥ 500

copies/mL); or (3) negative HIV antibodies in the setting of an illness compatible with acute seroconversion with demonstrated p24 antigenemia or plasma viremia; or (4) a compatible clinical history of a recent seroconversion illness within the last 6 months, with a documented high-risk exposure within 6 months, with a negative HIV antibody test within the last year. Blood was collected at baseline and participants were immediately initiated on cART; blood was additionally collected 48 weeks thereafter. Treatment regimen had no differential impact on HIV reservoir size (20); the present study therefore analyzed all participants regardless of regimen.

2.1.2. Determination of recent infection

A published multiassay algorithm (MAA), which identifies infections that have occurred within a mean 141 days of seroconversion, was used to refine HIV infection timing estimates (44). Briefly, baseline sera were tested with the BED-Capture EIA assay (Calypte Biomedical, Lake Oswego, OR) and average normalized optical density (OD-n) was calculated. Antibody avidity of sera was measured using a modified Genetic Systems 1/2+O ELISA kit (Bio-Rad, Hercules, CA). Individuals were classified as having recent infection (MAA+) if their baseline sample exhibited a BED-CEIA normalized optical density of <1.0, an antibody avidity index of <80%, a positive viral load and a CD4 count >200 cells/μl; otherwise, individuals were classified as MAA-.

2.1.3. Reservoir size measurements

The frequency of $CD4^+$ T-cells carrying HIV proviral DNA and harboring replication competent HIV were enumerated by real-time PCR and quantitative coculture assays, respectively, as previously described (20, 30). Briefly, for proviral DNA quantification, genomic DNA was isolated from 2×10^6 purified $CD4^+$ T cells (Qiagen, Valencia, CA), and 1μg of DNA was used as template in real-time PCR reactions (7500 real-time PCR system; Applied Biosystems, Foster City, CA) utilizing both HIV-specific (Long-Terminal Repeat) and host specific (Ribonuclease P) primers/probes in a 50 μl total reaction volume (TaqMan gene Expression master mix; Applied Biosystems). All PCR reactions were performed in triplicate; the detection limit of the assay is 2.6 copies of HIV DNA. For replication-competent HIV quantification, highly enriched (>97% pure) $CD4^+$ T cells were seeded to tissue culture plates, and irradiated PBMCs (8×10^6) from HIV-negative donors were added to each well along with anti-CD3 antibody and

incubated overnight in media containing recombinant interleukin-2 (20 units/ml). $CD8^+$-depleted and anti-CD3-stimulated PBMC blasts (1×10^6) from HIV-negative donors were added to each well the following day and on day 7. HIV p24 levels in culture supernatants were quantified by ELISA between days 14 and 21, and the numbers of infectious units per million (IUPM) cells were determined as previously described (45).

2.1.4. Immunologic and Immunogenetic assays

Total HIV-specific granzyme B responses were determined by ELISpot at baseline as previously described (30). Briefly, cryopreserved peripheral blood mononuclear cells (PBMC) were plated at either 1 or 2 $\times 10^5$ cells/well into 96-well plates pre-coated with monoclonal granzyme B coating antibody (Mabtech, Cincinatti, OH) and stimulated with pools of 15-mer peptides with an 11-amino-acid overlap spanning the entire HIV proteome (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) for 48 hours. Negative-control wells contained only cells in medium with 2, 4, or 12µl of DMSO; positive-control wells contained 1µg/ml of staphylococcal enterotoxin B (Sigma, St. Louis, MO). After incubation, biotinylated antibody, streptavidin-alkaline phosphatase (both from Mabtech ELISpot kits) and alkaline phosphatase color development solution (Bio-Rad, Hercules, CA) were added sequentially, with thorough washes before and after each step. All samples were tested in duplicate. Image analysis was conducted using an ImmunoSpot series 3A analyzer (Cellular Technology, Ltd., Cleveland, OH). Human Leukocyte Antigen (HLA) class I typing was performed at allele-level resolution by PCR sequence-specific primer typing (AllSet+ Gold HLA ABC Low-Resolution kit; One Lambda) as per the manufacturer's protocol. Ambiguous types for two participants were resolved by sequence-based typing as described in (46).

2.1.5. Nef isolation and cloning

HIV-1 *nef* was amplified from plasma-derived HIV RNA in a one-step RT-PCR reaction featuring a high-fidelity polymerase (Invitrogen Superscript®III One-step RT-PCR Platinum®Taq HiFi) using HIV-specific primers optimized to amplify all major HIV-1 subtypes: Forward 5'-TAGCAGTAGCTGRGKGRACAGATAG-3' (HXB2 nucleotides 8683-8707) and Reverse 5'-TACAGGCAAAAAGCAGCTGCTTATATGYAG-3' (HXB2 9536-9507). A nested PCR was then performed using a high-fidelity enzyme (Roche

Expand HiFi™) and primers that contained AscI (forward) and SacII (reverse) restriction enzyme sites for cloning: Forward 5' – AGAGCACC**GGCGCGCC***TCCACATACCTASAAGAATMAGACARG*-3' (Asc I site bolded, HXB2 nucleotides 8746-8772 italicized) and Reverse 5'-GCCT**CCGCGG**ATCGAT*CAGGCCACRCCTCCCTGGAAASKCCC*-3' (SacII site bolded, HXB2 nucleotides 9474-9449 italicized). Amplicons were then cloned into pSELECT (InvivoGen), an expression vector containing separate CMV and composite human EF1/HTLV promoters allowing simultaneous expression of GFP and *nef*, respectively. Briefly, a modified pSELECT-GFPzeo plasmid containing a linker bearing AscI and SacII restriction sites was digested with AscI and SacII enzymes and gel-purified (GeneJet gel extraction kit, Thermo Scientific). Participant-derived *nef* amplicons were digested with AscI and SacII, ligated into cut pSELECT-GFPzeo (T4 ligase; Thermofisher), transformed into chemically-competent *E. coli* (*E. Cloni* 10G DUOs; Lucigen) and plated onto zeocin-containing Luria-Bertani (LB) agar plates and incubated for 16-18 hours. A minimum of three colonies per participant were subsequently propagated overnight in zeocin-containing LB broth, after which plasmid DNA was purified (OMEGA E.Z.N.A. plasmid mini Kit; Thermofisher). The presence of an insert was verified by restriction enzyme digest followed by agarose gel electrophoresis.

Sequencing of bulk *nef* PCR products as well as clones containing correctly-sized inserts was performed on an ABI3130xl automated DNA analyzer using the ABI Prism Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems). Chromatograms were edited in Sequencher 5.0 (GeneCodes). Nucleotide sequence alignments were performed using HIVAlign (options: MAFFT (47); codon alignment) hosted on the Los Alamos HIV sequence database (LANL) webserver (48) and manually edited using Aliview (49). Maximum Likelihood phylogenies were constructed using PhyML (50) under a general time-reversible (GTR) model of codon substitution (51). HIV subtyping was performed using the recombinant identification program (RIP) hosted on the LANL webserver (52). Deposition of Nef clone sequences into GenBank is in progress and accession numbers will be provided prior to publication.

2.1.6. Nef-mediated CD4 and HLA-I downregulation assays

Three Nef clones per participant were assessed for their CD4 and HLA-I downregulation capacity using a published assay that involves transfection of *nef*

plasmid DNA into a CEM-derived CD4+ T-cell line engineered to express HLA-A*02 (CEM-A*02) (38, 39, 53, 54). The *nef* allele from HIV-1 subtype B reference strain SF2, cloned into pSELECT-GFPzeo (Nef_{SF2}), was used as the positive control while empty pSELECT-GFPzeo served as a negative control. Briefly, 4 µg of participant-derived or control *nef* plasmid DNA was delivered into 500,000 CEM-A*02 cells by electroporation in 96-well plates (where positive and negative controls are included in each row) and cells were incubated for 20-24 hours. Cells were then stained with APC-labeled anti-CD4 and PE-labeled anti-HLA-A*02 antibodies (BD Biosciences) and cell surface expression of these molecules was measured using flow cytometry. Receptor downregulation functions of participant-derived Nef clones were normalized to those of the positive control Nef_{SF2} using the following equation, where MFI (GFP+) and MFI (GFP-) refer to the median fluorescence intensity of CD4 (or HLA-I) expression in the Nef-expressing and Nef non-expressing gates, respectively:

$$\frac{1 - \frac{\mathrm{MFI}_{\mathrm{Participant}}\ (\mathrm{GFP}^{+})}{\mathrm{MFI}_{\mathrm{Participant}}\ (\mathrm{GFP}^{-})}}{1 - \frac{\mathrm{MFI}_{\mathrm{SF2}}\ (\mathrm{GFP}^{+})}{\mathrm{MFI}_{\mathrm{SF2}}\ (\mathrm{GFP}^{-})}}$$

As such, normalized MFI values of 100% indicate downregulation capacity equivalent to that of Nef_{SF2}, whereas values of <100% and >100% indicate downregulation capacities inferior or superior to Nef_{SF2} respectively. All participant-derived Nef clones were assayed in a minimum of triplicate in independent experiments and results are represented as the mean of these measurements.

2.1.7. Western blot

Steady-state Nef protein levels were measured by Western blot for selected clones exhibiting maximal function for HLA downregulation. A total of 2.5×10^6 CEM cells were transfected with 10 µg of participant-derived or control plasmid DNA and cell pellets were harvested 24 hours later. Cells were lysed with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris HCl, 150 mM NaCl) containing a protease inhibitor cocktail (P8340; Sigma). Following centrifugation, the resultant supernatants were subjected to SDS-PAGE and proteins were electro-blotted onto polyvinylidene difluoride (PVDF) membrane. Nef was detected using sheep polyclonal anti-HIV-1 Nef serum

(1:2,000 dilution) (NIH AIDS Research and Reference Reagent Program, USA) primary antibody, followed by horseradish peroxidase (HRP)-conjugated donkey anti-sheep IgG (1:35,000 dilution) (GE Healthcare). Blots were visualized using ImageQuant™ LAS 4000 chemiluminescent imager (GE Healthcare).

2.1.8. Statistical analysis

In univariable analyses, Spearman's correlation was used to assess the relationship between continuous variables (*e.g.* Nef CD4 and HLA-I downregulation capacities, HIV clinical parameters, reservoir size measurements) while the Mann-Whitney U test was used for binary variables (*e.g.* presence versus absence of protective HLA alleles; MAA+ versus MAA-; HIV subtype B versus not). To identify correlates of reservoir size 48 weeks post-cART, multivariable models were constructed by linear regression using a stepwise selection procedure. Variables investigated in primary and sensitivity analyses were all those with $p<0.05$ in univariate analyses; these included Nef-mediated HLA downregulation function (per normalized % functional increment), HIV-1 subtype (reference group subtype B), baseline $\log_{10}$ plasma viral load (per $\log_{10}$ increment), baseline CD4 count (per cell/mm^3 increment), baseline $\log_{10}$ proviral DNA (per $\log_{10}$ increment), total HIV-specific Granzyme B responses (per spot forming cell/10^6 PBMC increment) and very early cART initiation (MAA+ vs. MAA- [reference group]). Statistical tests were performed in Prism v5.0 and multivariable models were constructed in R (version 3.5.0) using the "MASS" package.

2.2. Results

2.2.1. Participant characteristics and Nef clonal isolation

Participants included 30 initially cART-naive men with acute/early (<6 months) HIV infection aged 22-59, who took part in a clinical trial comparing standard and intensive cART (20). Blood was collected at baseline (cART initiation) as well as 48 weeks post-cART for virologic, immunologic, clinical and reservoir size assessments, where the latter were measured in terms of proviral loads, as well as levels of replication-competent infectious viral units, per million $CD4^+$ T-cells (20). The original trial identified earlier cART initiation and baseline HIV-specific CD8+ granzyme B responses, but not initial treatment regimen, as significant correlates of smaller HIV

reservoir size 48 weeks post-cART (20, 30). As such, for the present study we analyzed all participants together regardless of treatment regimen. At baseline, pre-treatment median plasma viral load (pVL) of participants was 4.2 (interquartile range [IQR] 3.7-4.9) log_{10} copies HIV RNA/ml, median CD4 count was 445 (IQR 358-690) cells/mm^3 and 20 of 30 (67%) participants were estimated to be within 141 days of HIV seroconversion using a published multiassay algorithm (MAA+) (44).

For each participant, three *nef* sequences were isolated from baseline plasma HIV RNA, cloned into a GFP-reporter plasmid using previously described methods (36, 38, 54), and confirmed to cluster with their respective bulk plasma HIV RNA *nef* sequence (**Figure 1**). For 27 of 30 (90%) individuals, all three Nef clones were unique at the amino acid level, while for the remaining 3 individuals (5286, 5305 and 5306) all isolated clones were identical at the amino acid level. Within-host *nef* diversity correlated significantly with timing of cART initiation. Nef clones from participants who initiated cART very early (within 141 days of seroconversion; "MAA+ group") differed at a median of only two residues (IQR 1–4), while those who initiated cART slightly later, but still within 6 months of infection ("MAA- group"), differed at a median of 6 residues (IQR 3–8) (Mann-Whitney $p = 0.008$). This is consistent with sexually-acquired HIV infections being generally established by a single transmitted/founder virus (55, 56) followed by within-host diversification of this strain (57-61) until cART is initiated.

Of the 30 participants, 25 (83%) harbored HIV subtype B while 5 (17%) harbored non-B subtypes (2 CRF01_AE and 3 subtype G) (**Figure 1**), a distribution that is consistent with recent HIV molecular epidemiologic trends in Canada (62). Of note are the presence of two participant pairs, one each in subtypes B and G, who harbored highly similar viral strains, identifying them as putative transmission pairs (HIV genetic similarity was confirmed via Gag sequencing; not shown). All but three of the 90 isolated Nef clones were genetically intact: two clones for participant 5284 harbored internal stop codons while one clone for participant 5318 lacked the terminal stop codon (**Figure 2**).

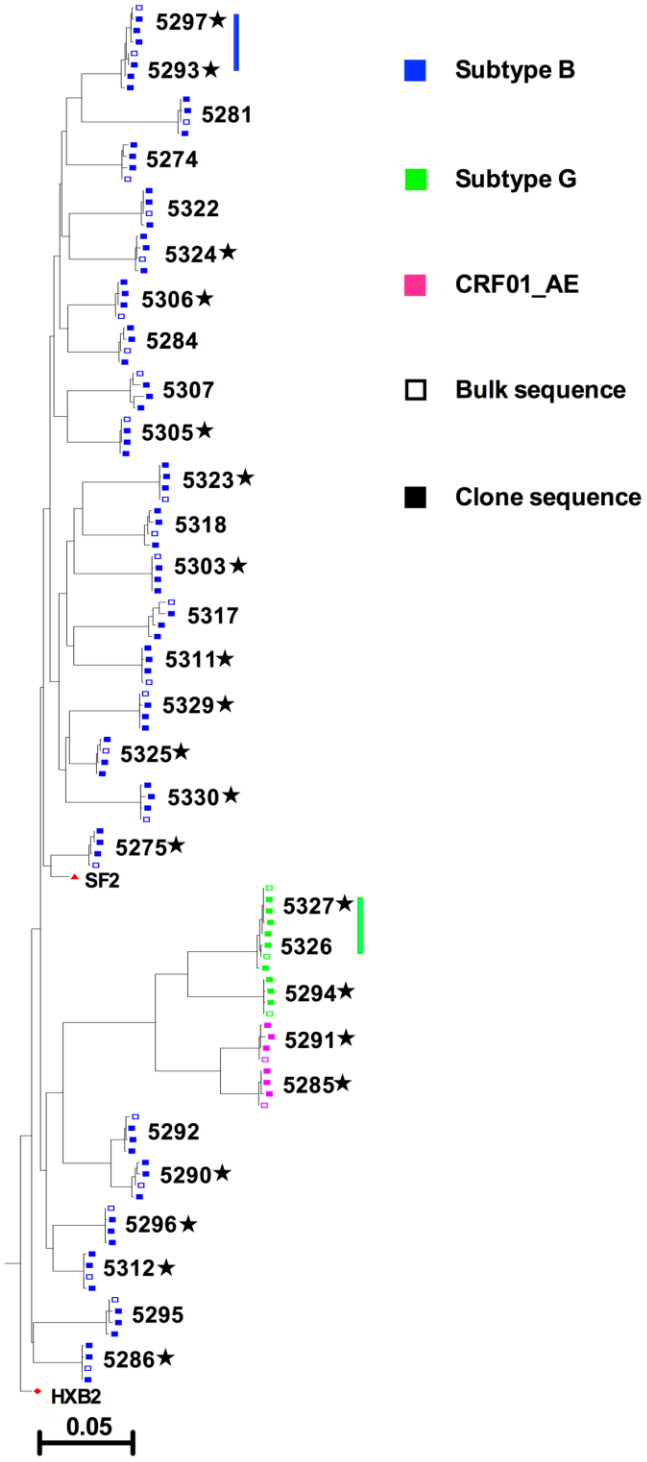

Figure 1. Maximum Likelihood phylogeny relating participant bulk and clonal plasma HIV RNA Nef sequences.

HIV *nef* isolates are colored by subtype: B (blue), G (green) and CRF01_AE (pink); bulk and clonal sequences are indicated by open and closed squares respectively. Participants who initiated cART very early (within an estimated 141 days of seroconversion) as determined by a multi-assay algorithm (MAA+) are marked by a star. Two participant pairs who share highly

genetically similar viruses, one in subtype B and the other in subtype G, are indicated by vertical lines. Phylogeny is rooted on the HIV subtype B reference strain HXB2. Scale in estimated nucleotide substitutions per site.

2.2.2. Functional assessment of participant-derived Nef clones

The ability of each Nef clone to downregulate cell-surface CD4 and HLA class I was assayed using an *in vitro* flow cytometry assay, as described previously (38, 39, 53, 54). The function of each clone was normalized to that of the HIV subtype B Nef_{SF2} reference strain, which displays strong CD4 and HLA downregulation activities (36, 38, 39), such that a normalized value of 100% indicates downregulation capacity equivalent to Nef_{SF2} whereas values of <100% and >100% indicate downregulation capacities inferior or superior to Nef_{SF2} respectively (for more details see methods). Representative raw data for negative control (empty vector), positive control (Nef_{SF2}) and participant-derived *nef* sequences, along with their corresponding Nef_{SF2}-normalized functions, are shown in **Figure 2A**. Independent replicate measurements of the same Nef clone were highly consistent (**Figures 2B, 2C**): median inter-replicate (*i.e.* within-clone) standard deviations (SD) were 3.3% (IQR 2.0-5.2%) for CD4 and 5.6% (IQR 3.9-8.2%) for HLA downregulation. As expected, within-host Nef function was highly consistent for the three participants for whom identical Nef clones were independently isolated, but more variable for the other 27 participants for whom all Nef clones were unique. Specifically, the median SD of within-host (*i.e.* between clone) CD4 downregulation activities was 0.8% for the three individuals for whom identical Nef clones were independently isolated vs 3.3% for those for whom all isolated Nef groups were unique (Mann-Whitney p=0.03), while the corresponding values for HLA downregulation were 3.6% vs 12.7%, respectively, (p=0.06). Moreover, among the Nef clones that displayed the poorest HLA downregulation function were those isolated from the subtype G putative transmission pair (5326 and 5327), identifying their shared strain as partially attenuated for this function. Together, these observations demonstrate that our Nef functional assays are both sensitive and reliable.

Overall, the range of Nef-mediated CD4 downregulation (**Figure 2B**) was relatively narrow both within and between hosts. The median CD4 downregulation function of *all* participant-derived Nef clones was 96% (IQR 89-99%); in fact, only 12 Nef clones (including the 3 with major genetic defects) exhibited normalized CD4 downregulation functions below 80% compared to Nef_{SF2}. Within-host variation in Nef-

mediated CD4 downregulation function was similarly limited (median standard deviation 2.5% [IQR 1.5-16.6%]); in fact, this distribution was not significantly different than that of the *inter-replicate* standard deviations reported above (Mann-Whitney, p=0.8). Overall, these results are consistent with CD4 downregulation function being highly conserved among primary Nef isolates (38, 39, 53, 54). In contrast, Nef-mediated HLA downregulation varied more widely both within and between hosts. The median HLA downregulation function of *all* participant-derived Nef clones was 72% (IQR 48-89%), while the median standard deviation of within-host Nef clones was 9.5% (IQR 3.5-25.4%) (**Figure 2C**), where the latter distribution *was* significantly higher than the median *inter-replicate* standard deviations reported above (Mann-Whitney, p=0.02).

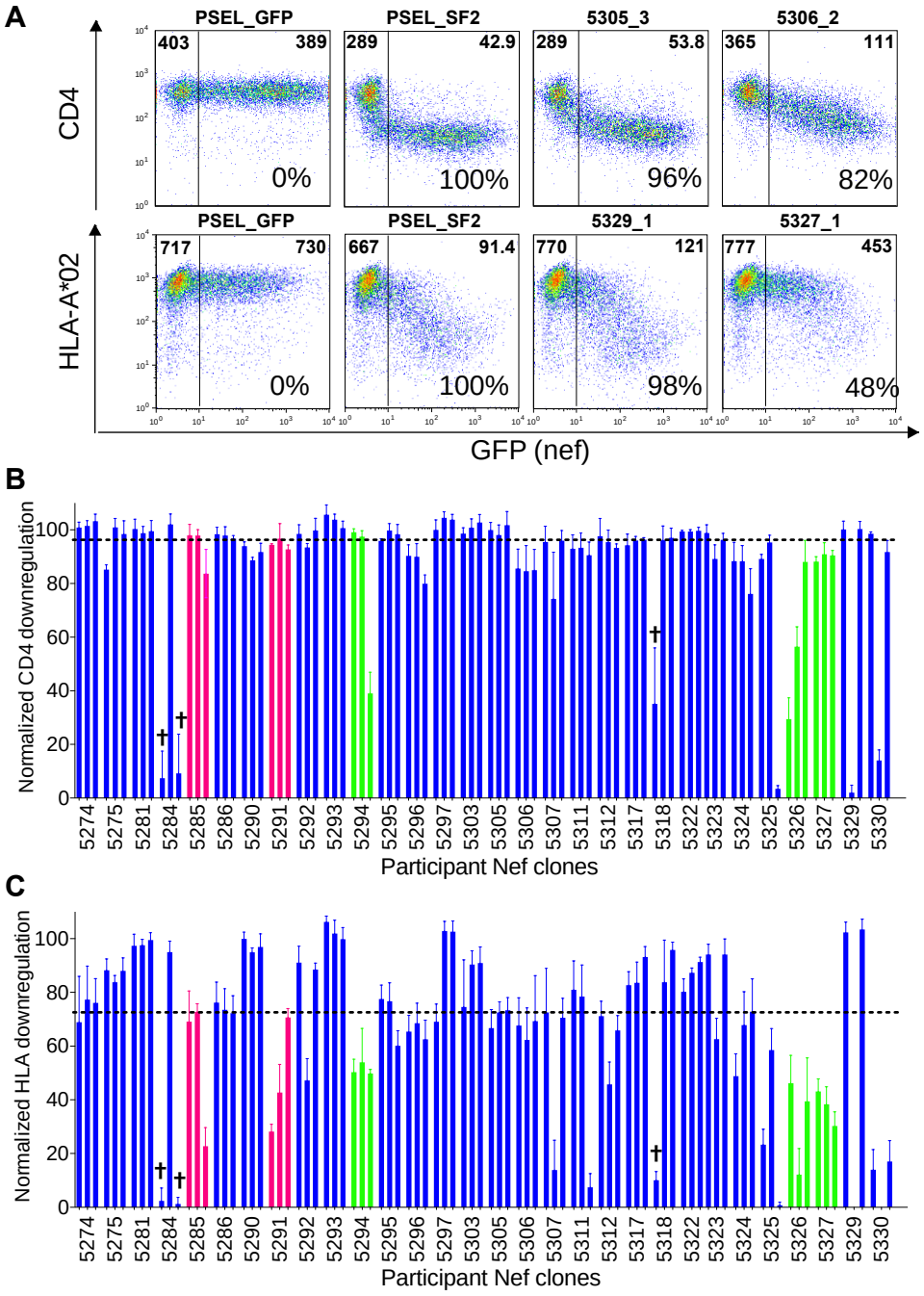

Figure 2. CD4 and HLA-I downregulation functions of participant Nef clones. *Panel A.* Representative flow plots demonstrate surface CD4 and HLA-I expression following transfection of CEM cells with empty vector (PSEL_GFP), positive control Nef (PSEL_SF2) and example participant Nef clones. Median fluorescence intensity (MFI) of receptor staining in the GFP^- (untransfected) and GFP^+ (Nef-transfected) cells are displayed in the upper left and right corners of each gate, respectively. The normalized *in vitro* function of each Nef control and participant clone, relative to Nef_{SF2}, is indicated as a percentage in the bottom right corner. *Panel B.* The normalized CD4 downregulation function of each participant Nef clone is shown, expressed as the mean (box height) and standard deviation (error bars) of a minimum of triplicate independent experiments. Clones are colored by HIV subtype, as described in Figure 1. Defective

Nef clones are indicated by a cross; these clones encoded either internal stop codons or lacked a stop codon. The dotted horizontal line indicates the population median. *Panel C.* The normalized HLA-I downregulation function of each participant Nef clone is shown, with all labeling as described for panel B.

Within-host Nef clone function, particularly for HLA downregulation, varied widely (*e.g.* whereas 5281's Nef clones ranged from 97.3 to 99.4% function, 5311's ranged from 7 to 81% despite being genetically intact). Given that we cannot rule out PCR or cloning errors as the cause of our minority of genetically and/or functionally defective clones, we elected to represent each participant's Nef CD4 and HLA-I downregulation function as the *maximal* value observed within each host. Sensitivity analyses were also performed using the *median* of all within-host clones tested. For 16 of 30 (53%) participants, the maximum within-host CD4 and HLA downregulation values were derived from different Nef clones, whereas for the remainder of participants the same clone displayed the greatest function for both activities. Overall, maximal within-host CD4 and HLA downregulation function correlated positively (Spearman's R=0.56, p=0.0013) (**Figure 3A)**. Steady-state Nef protein expression was also confirmed by Western Blot for all but one of the maximally HLA-downregulating clones (representative data in **Figure 3B**), where the latter may be attributable to suboptimal primary antibody binding to this particular protein sequence since this clone did not display functional impairments.

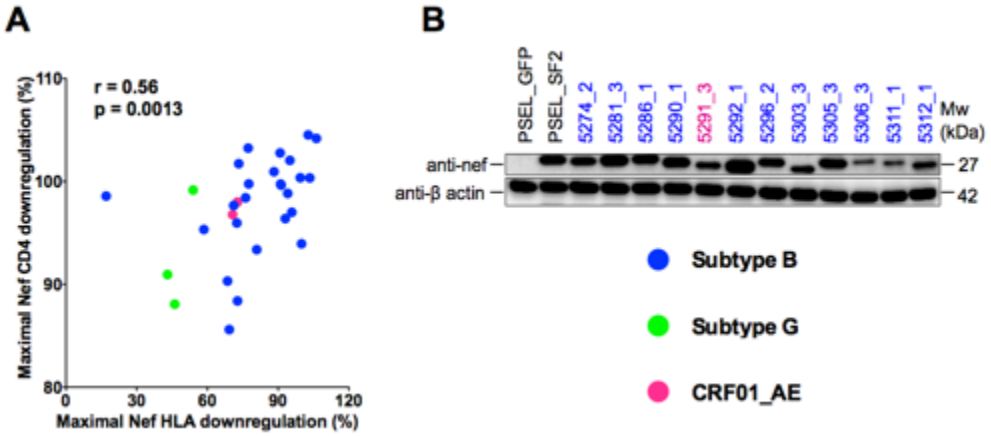

Figure 3. Relationship between Nef-mediated CD4 and HLA-I downregulation functions, and Nef steady-state protein expression.

Panel A. Spearman's correlation between maximal within-host Nef-mediated HLA-I and CD4 downregulation function. Nef isolates are colored according to HIV subtype. *Panel B.* Western blot analyses of Nef and cellular β-actin is shown for assay controls and 12 representative

participant clones that exhibited maximal intra-individual HLA downregulation activity. Sample identifiers are colored according to HIV subtype.

2.2.3. Validating established, and identifying novel, univariable correlates of reservoir size

We next moved to our main objective of identifying correlates of reservoir size at 48 weeks post-cART, at which point plasma viremia had been suppressed to undetectable levels for a median of 40 (IQR 32-44.5) weeks in study participants. Reservoir size was measured in peripheral blood $CD4^+$ T-cells in terms of total proviral burden (quantified by PCR and expressed in log_{10} HIV DNA copies per million $CD4^+$ T cells) and by the levels of replication competent virus (measured by quantitative HIV coculture and expressed as infectious units per million $CD4^+$ T cells [IUPM]). As these two measurements correlated robustly (Spearman's R=0.6, p=0.001), and reservoir size is more routinely estimated by proviral DNA quantification rather than laborious virus culture, the former was selected as the primary reservoir size measurement in our analyses.

We first verified that our study was sufficiently powered to identify established correlates of HIV reservoir size by confirming these relationships in our data (**Figure 4**). As previously reported (30, 63), a very strong correlate of log_{10} proviral DNA levels 48 weeks post-cART in peripheral blood $CD4^+$ T-cells was log_{10} proviral DNA levels at baseline (Spearman's R=0.84, p<0.0001; **Figure 4A**). In addition, log_{10} proviral DNA levels at 48 weeks correlated positively with baseline log_{10} pVL (Spearman's R=0.45, p=0.014), negatively with baseline CD4 counts (Spearman's R=-0.54, p=0.0019) and negatively with baseline total HIV-specific Granzyme B responses (Spearman's R=-0.57, p=0.001; **Figures 4B-D**) (30, 64-66). Also consistent with previous studies of the present (20, 30) and other (18, 67, 68) cohorts, early cART was also significantly associated with a smaller reservoir (median 2.27 [IQR 2.14-2.50] versus 2.91 [IQR 2.42-3.22] log_{10} copies HIV DNA/million cells in MAA+ vs. MAA- participants respectively, p=0.0045; **Figure 4E**). Carriage of HLA-B*27 and/or B*57 was not associated with reservoir size (p=0.7), though only three participants expressed one of these protective alleles.

No significant relationship was observed between maximal (nor median) Nef-mediated CD4 downregulation and log_{10} proviral DNA levels on cART (Spearman's R=0.27, p=0.15; **Figure 4F,** and data not shown). However, a significant positive

association was observed between maximal within-host Nef-mediated HLA downregulation function and HIV reservoir size (Spearman's R=0.61, p=0.0004; **Figure 4G**). This relationship persisted when *median* within-host Nef-mediated HLA downregulation was evaluated (Spearman's R=0.4, p=0.027; data not shown). Maximal within-host Nef-mediated HLA downregulation also correlated positively with replication-competent reservoir size on cART (Spearman's R=0.36, p=0.056; **Figure 4H**). Furthermore, Nef-mediated HLA downregulation function correlated inversely with total HIV-specific Granzyme B responses (Spearman's R=-0.37, p=0.04; **Figure 4I**), suggesting that the ability of an individual's autologous Nef to subvert antiviral cellular immune responses via HLA class I downregulation may influence the development of such responses *in vivo*.

We next stratified our dataset based on HIV subtype and observed that subtype B infected participants harbored significantly larger reservoirs than those harboring non-B infections (median 2.45 [IQR 2.27-2.89] vs 1.72 [IQR 0.96-2.10] $\log_{10}$ copies HIV DNA/million $CD4^+$ T- cells respectively, p=0.0008; **Figure 4J**). This relationship persisted even after we excluded one member of each of the two putative transmission pairs (p=0.002; data not shown), indicating that it is not simply driven by the relatively attenuated Nef-mediated HLA downregulation function observed in the subtype G pair. Subtype B infected participants also harbored larger replication-competent reservoirs in the setting of cART (p=0.032; **Figure 4K**).

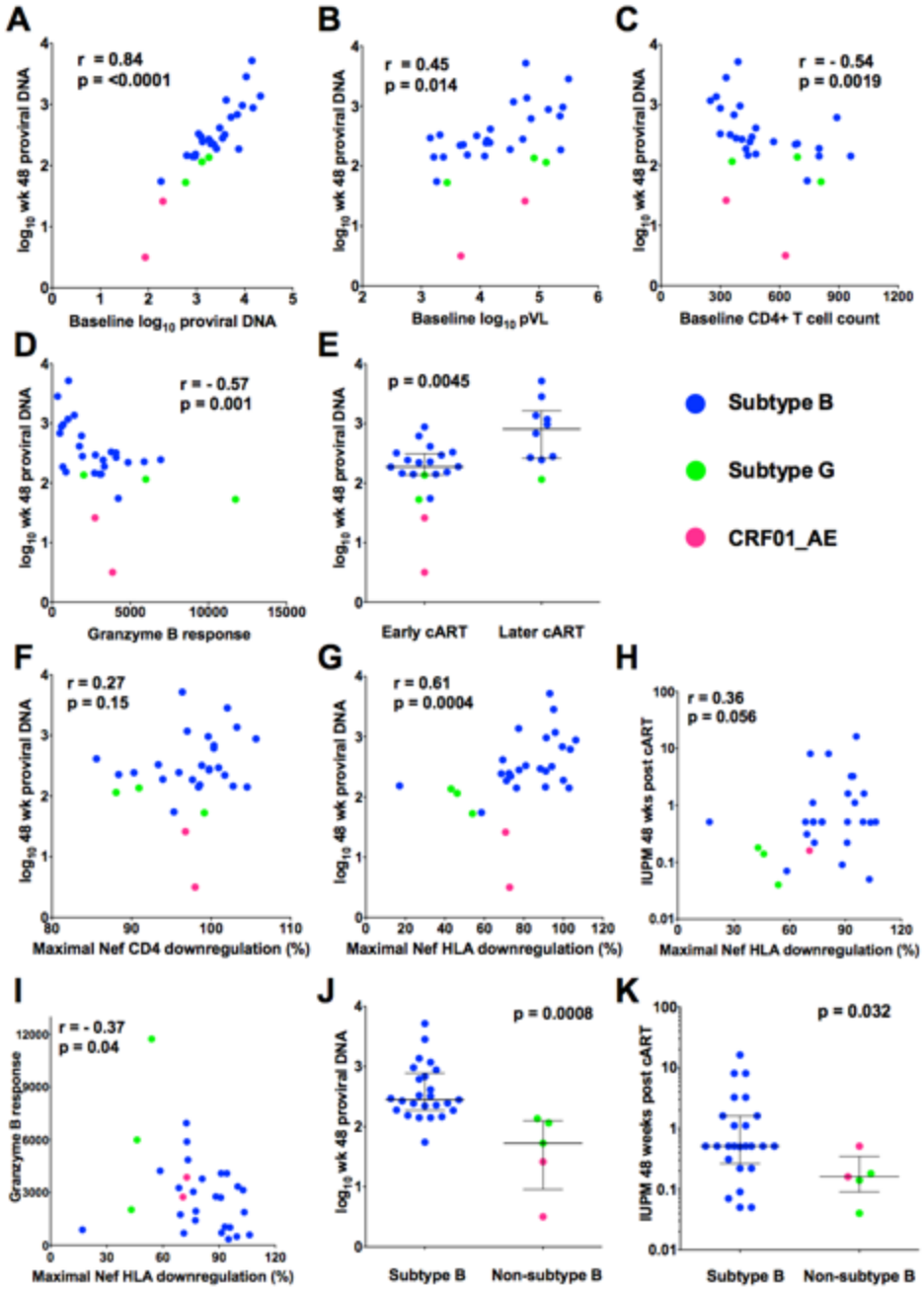

Figure 4. Established and novel correlates of HIV reservoir size.

Panels A-G. Correlations are displayed between log_{10} proviral DNA levels measured 48 weeks post-cART and either baseline proviral DNA levels (A), baseline log_{10} plasma viral load (B), baseline CD4+ T cell counts (C), baseline granzyme B responses (D), timing of cART (E), baseline maximal Nef-mediated CD4 downregulation (F), or baseline maximal Nef-mediated HLA-I downregulation (G). *Panel H*: Correlation between Nef-mediated HLA-I downregulation and replication HIV reservoir size measured 48 weeks post-cART is shown. *Panel I:* Correlation between Nef-mediated HLA-I downregulation and baseline HIV-specific Granzyme B responses is shown. *Panel J:* Week 48 proviral DNA levels are shown, stratified by HIV subtype. *Panel K:* Week 48 replication competent reservoir sizes, assessed as infectious units per million CD4+ T cells (IUPM), are shown, stratified by HIV subtype. Statistical significance was assessed using

Spearman's correlation or Mann-Whitney U tests. Nef isolates in all panels are colored according to HIV subtype.

2.2.4. Stratification by HIV subtype and multivariable analyses

Having identified seven univariable correlates of reservoir size on cART (baseline log_{10} HIV DNA levels, pVL, $CD4^+$ T cell counts, total HIV-specific granzyme B responses and Nef-mediated HLA downregulation function, plus timing of cART initiation and viral subtype), we next wished to tease apart their individual contributions to this outcome variable. In doing so, it became apparent that the subtype B and non-B infected participants differed significantly with respect to some key parameters (**Table 1**).

Table 1. Comparison of immunological and virological parameters between subtype B and non-Subtype B infections

Characteristic		Subtype B (N=25)	non-Subtype B (N=5)	p-value
Baseline	Plasma viral load, log_{10} copies/ml	4.2 (3.7 - 4.8)	4.8 (3.6 - 5.0)	0.8
	$CD4^+$ T-cell count, cells/mm^3	440 (360 - 685)	630 (345 - 750)	0.7
	Proviral DNA, log_{10} copies/10^6 $CD4^+$ T cells	3.4 (3.1 - 3.9)	2.8 (2.1 - 3.2)	0.01
	Replication competent HIV, log_{10} IUPM[a]/10^6 $CD4^+$ T cells	1.2 (0.9 - 2.0)	1.2 (0.2 - 1.9)	0.7
	Very early cART[b]; % MAA+[c]	80%	20%	0.6
	Granzyme B response, spot forming cells/10^6 PBMC[d]	2710 (946 - 3930)	3863 (2380 - 8860)	0.095
	Maximal normalized Nef-mediated HLA downregulation	88 (73 - 95)	54 (45 - 72)	0.006
	Maximal normalized Nef-mediated CD4 downregulation	99 (96 - 101)	97 (90 - 99)	0.1
post-cART (week 48)	Proviral DNA log_{10} copies/10^6 $CD4^+$ T cells	2.5 (2.3 - 2.9)	1.7 (1.0 - 2.1)	0.0008
	Replication competent HIV, log_{10} IUPM/10^6 $CD4^+$ T cells	-0.3 (-0.6 - 0.2)	-0.8 (-1.1 - (-0.5))	0.03

All results are reported as median and interquartile range (IQR) except where indicated
[a] IUPM: Infectious Units per Million $CD4^+$ T cells
[b] cART: combination antiretroviral therapy
[c] MAA+: Multiassay Algorithm + (participants who started cART within an estimated 141 days after infection)
[d] PBMC: Peripheral Blood Mononuclear Cells

Specifically, subtype B-infected participants had significantly higher proviral DNA levels at baseline and 48 weeks post-cART (all $p<0.05$), and displayed significantly

better Nef-mediated HLA downregulation than non-subtype B-infected participants (median 88 [IQR 73-95] vs 54 [IQR 45-72], p=0.006), consistent with previous reports of superior function of subtype B Nef isolates (38). Furthermore, a multivariable linear regression model constructed using stepwise selection, that incorporated HIV subtype, baseline log_{10} pVL, timing of cART and Nef-mediated HLA downregulation identified HIV subtype as the strongest independent correlate of reservoir size (after adjusting for other variables, B infection was associated with an estimated 0.98 log_{10} higher proviral DNA levels than non-B infection; **Table 2**). Baseline log_{10} pVL and timing of cART explained an additional proportion of reservoir size variation, the former significantly so.

Table 2. Contribution of Nef-mediated HLA downregulation, baseline plasma viral load, very early cART (MAA+) and, HIV subtype on log10 proviral DNA levels 48 weeks post-cART: final multivariable model

Variable	Final model	
	Estimate[a]	p-value
Nef-mediated HLA downregulation	-	-
Very early cART[b] (MAA+[c])	-0.32	0.066
Baseline log_{10} plasma viral load	0.30	0.011 *
HIV Subtype B	0.98	0.00001***

[a] β Estimates are expressed as follows: Nef-mediated HLA downregulation (per % function increment), and baseline plasma viral load (per log_{10} increment). For timing of cART initiation, starting cART later than 141 days post-infection (MAA-) is the reference group. For HIV subtype, non-subtype B is the reference group. Adjusted $r^2 = 0.65$, $p = 1.1 \times 10^{-6}$.

[b] cART: combination antiretroviral therapy

[c] MAA+: Multiassay Algorithm; participants who started cART within 141 days after infection

Given the strong relationship between HIV subtype and reservoir size in our cohort (**Table 1**), we next confirmed that our previously-identified correlates of reservoir size remained significant when restricting to subtype B infections (N=25). They did in all cases (**Figures 5A-F**). From strongest to weakest, correlates of log_{10} proviral DNA in subtype B infections at 48 weeks post-cART were: baseline log_{10} proviral DNA (Spearman's R=0.81, p<0.0001), baseline CD4 count (R=-0.71, p<0.0001), baseline pVL (R=0.65, p=0.0005), time of cART initiation (median 2.4 vs. 3.0 log_{10} copies in earlier vs later treated persons, p=0.003), baseline HIV-specific Granzyme B responses (R=-0.53, p=0.0068), and maximal within-host Nef-mediated HLA downregulation function (R=0.44, p=0.026).

Determining the contributions of individual variables to a given outcome is challenging when these variables are highly intercorrelated. In the present study, 10 of the 15 possible pairwise relationships between univariable correlates of reservoir size were statistically significant (**Figure 5G**). Baseline $\log_{10}$ proviral DNA, baseline pVL and baseline HIV-specific Granzyme B responses correlated most strongly with one another: Spearman's R= 0.86 (proviral/pVL), -0.71 (pVL/granzyme) and -0.62 (proviral/granzyme), all $p<0.001$. Nef-mediated HLA downregulation, however, was the least interconnected variable. The only other factor it significantly correlated with was baseline $\log_{10}$ proviral DNA ($R=0.4$, $p=0.045$), consistent with a role of Nef in modulating infected cell levels in untreated HIV infection.

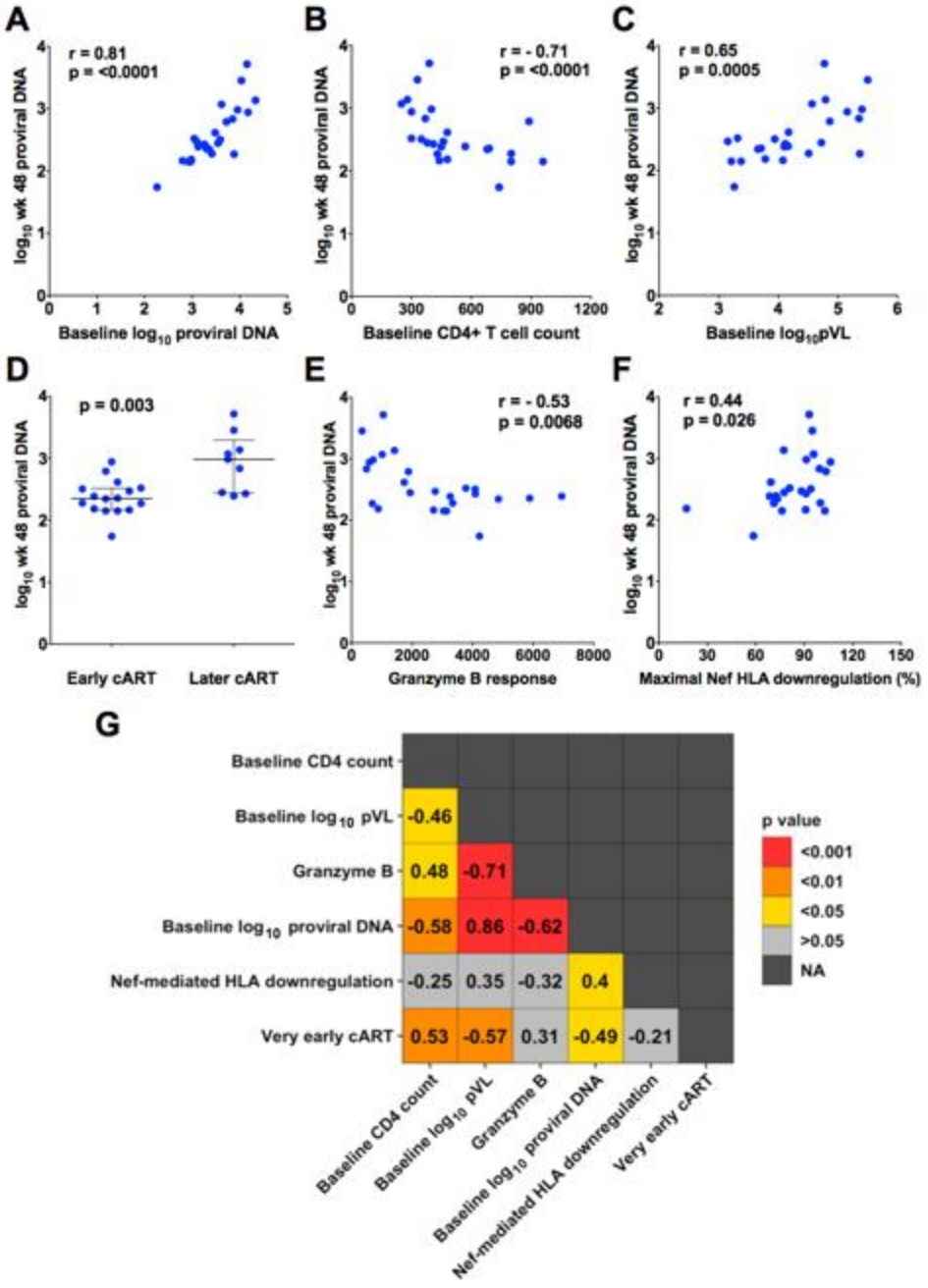

Figure 5. Confirmation of identified correlates in HIV subtype B infections only.

Panels A-F: Correlations are shown between log_{10} proviral DNA levels measured 48 weeks post-cART and either baseline proviral DNA levels (A), baseline $CD4^+$ T cell counts (B), baseline log_{10} plasma viral load levels (C), timing of cART (D), baseline granzyme B responses (E) or maximal baseline Nef-mediated HLA downregulation function (F), where all analyses are restricted to subtype B infections only. *Panel G:* A heatmap is shown, summarizing Spearman's rho values (reported in boxes) and p-values (depicted as colors) for the 15 possible pairwise comparisons between correlates of reservoir size identified in this study.

To identify which factors represented independent correlates of reservoir size in subtype B infection, and in particular whether Nef-mediated HLA downregulation was among them, we constructed multivariable linear regression models using stepwise selection. To minimize the risk of overfitting, we followed the minimum criterion of five degrees of freedom per included variable (69, 70) (*i.e.* models restricted to the 25 subtype B infections feature 20 degrees of freedom and could thus contain a maximum of 4 variables) and we ensured that models would only incorporate one each of the three most highly intercorrelated variables (**Figure 5G**). Given our specific interest in Nef, we also excluded baseline $\log_{10}$ proviral DNA from the models due to its significant relationship with Nef-mediated HLA downregulation.

This left us with two models: the first which incorporated Nef, baseline $\log_{10}$pVL, baseline CD4 count and early cART (**Table 3**) and the second which incorporated Nef, baseline granzyme B, baseline CD4 count and early cART (**Table 4**). Notably, both final models retained Nef-mediated HLA downregulation as a variable and were highly statistically significant overall (adjusted R^2=0.54, p=4.4×10^{-4} for Model 1; adjusted R^2=0.58, p=8.7×10^{-5} for Model 2). Specifically, the final **model 1** retained all four input variables, with none individually significant, indicating that all four contribute to reservoir size to a comparable extent and together explain 54% of the variance in reservoir size (**Table 3**). Specifically, after adjustment for other parameters, **model 1** estimated that each 10% increase in Nef-mediated downregulation led to an 0.06 $\log_{10}$ increase in reservoir size on cART.

Table 3: Model 1: Contribution of Nef-mediated HLA downregulation, baseline plasma viral load, baseline CD4 T-cell count and timing of therapy initiation on log10 proviral DNA levels 48 weeks post-cART

Variable	Final model Estimate[a]	p-value
Nef-mediated HLA-I downregulation	0.006	0.11
Baseline CD4+ T cell count	-0.0006	0.14
Very early cART[b] (MAA+[c])	-0.25	0.13
Baseline $\log_{10}$ plasma viral load	0.19	0.10

[a] β Estimates are expressed as follows: Nef-mediated HLA downregulation (per % function increment), baseline plasma viral load (per $\log_{10}$ increment), baseline CD4+ T cell count (per cell/mm^3 increment). For timing of cART initiation, starting cART later than 141 days post-infection (MAA-) is the reference group. Adjusted r^2 = 0.54, p = 4.4×10^{-4}.
[b] cART: combination antiretroviral therapy
[c] MAA+: Multiassay Algorithm; participants who started cART within 141 days after infection

The final **model 2** retained three of the four input variables: Nef function, granzyme B, and early cART, where the latter two remained significant and these three variables together explained 58% of the variance in reservoir size. Consistent with the first model, **model 2** estimated that each 10% increase in Nef-mediated downregulation function led to an 0.065 $\log_{10}$ increase in reservoir size after adjustment for other parameters. Taken together, these observations support Nef function as a novel contributor to reservoir size in multivariable models that explain >50% of the variance in reservoir size.

Table 4: Model 2: Contribution of Nef-mediated HLA downregulation, baseline CD4+ T-cell count, timing of therapy initiation and HIV-specific granzyme B responses on log10 proviral DNA levels 48 weeks post-cART

Variable	Final Model Estimatea	p-value
Nef-mediated HLA-I downregulation	0.0065	0.06
Baseline CD4+ T cell count	-	-
Very early cARTb (MAA+c)	-0.42	0.003 **
Granzyme B response	-0.0001	0.009 **

a β Estimates are expressed as follows: Nef-mediated HLA downregulation (per % function increment), baseline CD4+ T cell count (per cell/mm3 increment), Granzyme B production (per spot forming cell/106 PBMCs increment). For timing of cART initiation, starting cART later than 141 days post-infection (MAA-) is the reference group. Adjusted r2 = 0.58, p = 8.7×10-5

b cART: combination antiretroviral therapy

c MAA+: Multiassay Algorithm; participants who started cART within 141 days after infection

2.3. Discussion

Our results identify HIV subtype and Nef-mediated HLA downregulation function as univariable and multivariable correlates of viral reservoir size measured approximately one year following initiation of suppressive cART in persons who initiated treatment within 6 months of infection. These observations highlight virologic characteristics - both genetic and functional - as potentially novel determinants of HIV reservoir establishment and persistence. The identification of HIV subtype as a statistically significant multivariable correlate of reservoir size is particularly notable given the modest number of non-B infections in the cohort (5 of 30). Taken together with recent reports of smaller reservoir sizes among individuals infected with non-B subtypes (21), the relationship between infecting HIV subtype and reservoir size merits further

study in larger cohorts. It will be particularly important to establish that viral subtype is not simply associated with a non-viral feature that influences HIV reservoir size (*e.g.* a host genetic factor that differs in frequency between populations in which HIV subtype distribution also differs). Furthermore, it will be important to quantify what portion of the effect of HIV subtype on viral reservoir size is attributable to subtype-dependent differences in Nef-mediated HLA downregulation function (38), which were strongly apparent in the present cohort (**Figure 4J**), versus what portions are attributable to other genetic and/or phenotypic viral features.

Similarly, our identification of Nef-mediated HLA downregulation as a correlate of reservoir size, even after stratification by HIV subtype and adjustment for established immunologic and clinical correlates, also merits further investigation. In particular, it will be important to establish whether Nef-mediated HLA downregulation influences HIV reservoir size on cART primarily through Nef's ability to modulate *pre-cART* $\log_{10}$ proviral DNA levels (*e.g.* by allowing infected cells to evade CTL-mediated clearance during active viremia, thereby increasing the absolute number of infected cells that subsequently enter, and persist in, a latent state), or whether Nef-mediated HLA downregulation function additionally influences reservoir dynamics during suppressive cART (*e.g.* by shielding latently-infected cells spontaneously reactivating from latency from detection by HLA-restricted HIV-specific CTL).

Results of our study suggest that Nef influences reservoir size, at least in part, by influencing HIV proviral DNA levels during untreated infection. Nef-mediated HLA downregulation correlated significantly with pre-cART proviral DNA levels (**Figure 5G**); moreover, an exploratory multivariable model that incorporated $\log_{10}$ proviral DNA, baseline CD4, early cART and Nef-mediated HLA downregulation (noting that the latter three variables correlated significantly with the former one) identified baseline $\log_{10}$ proviral DNA as the single most significant predictor of reservoir size on cART ($p=1.2\times10^{-6}$) (overall model adjusted $R^2 = 0.74$, $p=1.2\times10^{-7}$), with early cART also surviving multivariable correction with p=0.026. Though we did not directly investigate mechanism in the present study, two lines of evidence support the notion that Nef influences pretreatment proviral DNA levels by allowing infected cells to evade HLA-mediated immune responses. First, Nef-mediated HLA downregulation correlates negatively with HIV-specific granzyme B responses (**Figure 4I**). Furthermore, we have previously demonstrated *in vitro* that the extent of Nef-mediated HLA downregulation on

target cells presenting the relevant HLA-bound peptide significantly inversely correlates with their ability to be recognized by effector T-cells expressing a T-cell receptor specific to this peptide/HLA combination (54, 71). Whether Nef function *additionally* influences the subsequent persistence of latently-infected cells during cART is less clear. In fact, our results do not directly support this (as evidenced by the above observation that Nef function did not remain a significant correlate of reservoir size on cART after adjusting for baseline proviral DNA levels, and the exploratory observation that Nef function did not correlate with the absolute level of decline of proviral DNA levels during cART; data not shown). Nevertheless, given the very strong relationship between baseline and post-cART proviral DNA levels (**Figure 4A**), and the fact that reservoir size declines very slowly on cART (72-74), it would require larger cohorts with substantially longer follow-up times than the present study (48 weeks only) to adequately assess Nef's additional possible modulatory effects on reservoir decay dynamics during suppressive cART. The observation that pharmacologic inhibition of Nef promoted $CD8^+$ T-cell-mediated elimination of latently HIV-infected cells *in vitro* (42) supports this notion; and given our study's modest size and follow-up time, we cannot conclusively rule this out. One caveat is that future studies, if undertaken using observational cohorts, would additionally need to control for duration of cART suppression - a variable that we did not need to consider since ours was a post-hoc analysis of a clinical trial where cART duration was the same for all participants. Indeed, the latter point, as well as the fact that all participants were treated within 6 months of HIV infection, likely underlies our ability to identify correlates of reservoir size with a relatively modest number of participants.

In conclusion, the present study identifies Nef-mediated HLA downregulation function and infecting HIV subtype, which are known regulators of HIV infectivity and pathogenesis (36, 39, 75-80), as potential modulators of HIV reservoir size. Further research on the extent to which these and other virologic characteristics - both genetic and functional - influence HIV reservoir establishment and persistence is merited.

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Chapter 3.

Thesis summary

Human Immunodeficiency Virus rapidly adapts to its host environment, giving rise to the extensive HIV genetic diversity of modern strains (1–5). HIV genetic diversity can in turn lead to functional diversity of circulating strains, thereby potentially influencing pathogenesis (6–11). The Nef protein has been particularly well-studied in this regard (12–15). While sequence-phenotype relationships in Nef were initially largely defined *in vitro* using laboratory-adapted *nef* alleles, the impact of naturally-occurring sequence variation on HIV pathogenesis can only be understood by characterizing the dynamic range of function of naturally circulating *nef* sequences. Clinical, immunological and host genetic determinants of disease progression have been described (16–20); however, there is comparatively limited knowledge on the influence of viral factors on HIV pathogenesis.

The ability of HIV to establish viral reservoirs very early in infection that persist long-term, even during suppressive antiretroviral therapy (21) is one critical defining feature of the virus, but the impact of sequence-phenotype relationships in natural HIV sequences on the establishment and/or maintenance of HIV latency is even less well understood. Several factors that influence HIV reservoir size have been previously described (22–29) and recently, a report linking HIV-specific granzyme B responses produced by CD8+ T cells was published (29). Based on this observation and given that CD8+ T cell responses are HLA-I restricted, we tested the hypothesis that naturally-occurring genetic and functional variation in Nef might influence the extent of HIV reservoir seeding in early infection. We employed an in vitro CEM T cell line model stably expressing CD4 and that was engineered to express HLA-A*02 and assessed the effect of the different participant-derived Nef clones on cell surface expression levels of HLA-A*02 and CD4 on CEM T cells. From our analyses we identified HIV subtype and Nef-mediated HLA-I downregulation as significant correlates of HIV reservoir size measured at 48 weeks after initiation of suppressive combination antiretroviral therapy. And because we additionally identified previously known clinical and immunological correlates of reservoir size in our cohort, we further performed multivariate analyses to

determine if Nef-mediated immune evasion function particularly HLA-I downregulation was maintained as a correlate of reservoir size. Our results supported Nef-mediated HLA-I downregulation as a key correlate of reservoir size, although the association did not reach a statistical significance in the final multivariate model. This is the first study that identifies an association between Nef-mediated immune evasion function and HIV reservoir size.

In conclusion, this thesis identifies novel viral factors - Nef function and viral subtype - that could potentially determine establishment and size of the HIV reservoir. This knowledge is an important contribution to HIV research as it highlights viral (genetic) factors as key determinants of HIV reservoir size. This opens up avenues for further research that could inform therapeutic or vaccine design strategies geared towards elimination of viral reservoirs or prevention of its establishment. Further studies are warranted to elucidate definitive mechanisms directly linking Nef function to the establishment of viral reservoir. Particularly, given that our sample size was relatively small, it would be important to verify these study findings in larger cohorts especially with more representation of non-B subtypes. In addition, given the geographical distribution of HIV subtypes, understanding whether host immunogenetics could be a contributing factor to viral establishment and persistence warrants further studies. Recent report by Usami et al. and Rosa et al. showed that Nef antagonizes host restriction factors, SERINC 3 and SERINC 5, thereby enhancing HIV infectivity (30, 31). It would be interesting to study the role of natural sequence variation in *nef* and how this impact on this its activity and whether there exists any association with disease progression.

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