Genetic complexity in the replication-competent latent HIV reservoir increases with untreated infection duration in infected youth

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Objective: Timely initiation of combination antiretroviral therapy (ART) limits latent HIV reservoir size and should also limit reservoir genetic complexity. However, the relationship between these two factors remains unclear, particularly among HIV-infected youth.

Design: Retrospective analysis of replication-competent latent HIV clones serially isolated by limiting-dilution culture from resting $CD4^+$ T-cell reservoirs from ART-suppressed, young adult participants of a historic phase I therapeutic vaccine trial (PACTG/IMPAACT-P1059).

Methods: Replication-competent latent HIV clones isolated from resting $CD4^+$ T cells of four perinatally and 10 nonperinatally infected young adults (average 22 versus 6 years uncontrolled infection, respectively) were sequenced in Pol and Nef. Within-host HIV sequence datasets were characterized with respect to their genetic diversity and inferred immune escape mutation burden.

Results: Although participants were comparable in terms of sociodemographic and HIV sampling characteristics (e.g. on average, a mean 17 Pol sequences were recovered at five timepoints over up to 70 weeks) and the length of ART suppression at study entry (average 3 years), replication-competent HIV reservoir size, genetic diversity, immune escape mutation burden and variant complexity were significantly higher among the perinatally infected participants who experienced longer durations of uncontrolled viremia. Nevertheless, viral sequences inferred to retain susceptibility to host cellular immune responses were detected in all participants, irrespective of uncontrolled viremia duration.

Conclusion: HIV elimination in late-suppressed youth may be doubly challenged by larger and more genetically complex reservoirs. Strategies that integrate host and viral genetic complexity to achieve HIV remission or cure may merit consideration in such cases.

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Introduction

Genetic diversity [1–9] and immune escape [10] within the latent HIV reservoir form barriers to cure. Given that reservoir establishment begins shortly after infection and continues as long as viral replication remains uncontrolled [11–13], timely viremia suppression with combination antiretroviral therapy (ART) should, in theory, limit reservoir complexity. The observation that early ART limits HIV reservoir size in both adult [14–16] and pediatric [17–20] infection supports this; as do the observations that proviral landscapes in elite controllers and early-treated individuals tend to be more homogeneous than viremic controllers and individuals who initiated ART in chronic infection, respectively [5,21,22]. However, the effect of uncontrolled viremia duration on reservoir diversity in individuals who did not initiate early suppressive ART remains unclear. This is particularly relevant to persons infected in the decade prior to the availability of combination ART, including perinatally infected individuals who survived to young adulthood.

Immune escape within the HIV reservoir also remains incompletely characterized in this population. Although the majority of latent HIV genomes in adults treated in chronic infection harbored at least one major human leukocyte antigen (HLA) class I-restricted cytotoxic T-lymphocyte (CTL) escape mutation in Gag, unmutated epitopes – that were subsequently used as targets for reservoir elimination – were also present in all individuals [10]. If the latent reservoir recapitulates within-host HIV evolution [23–25] then CTL epitopes that underwent escape *in vivo* should be 'preserved' in various states of adaptation within it; indeed, a scenario where susceptible and adapted forms of the same epitope co-exist in the replication-competent HIV reservoir could create both challenges and opportunities for cure immunotherapeutics.

Latent HIV reservoir sampling also remains a challenge. Given the high (>90%) burden of defective proviruses [1,26], direct HIV DNA sequencing may not fully represent genetic diversity within the replication-competent minority that is critical to eradicate [27]. Furthermore, given the propensity of latently HIV-infected cells to undergo clonal expansions [4,7,28–30] that can sometimes be short-lived [31], cross-sectional studies may underestimate overall reservoir diversity if such an expansion has recently occurred.

To address these gaps, we genetically characterized replication-competent latent HIV clones isolated from resting $CD4^+$ T-cell reservoirs serially sampled over up to 70 weeks during suppressive ART, from young adult participants of a historic phase I therapeutic vaccine trial (PACTG/IMPAACT-P1059) who differed markedly in terms of their uncontrolled HIV infection duration (because of perinatal acquisition of HIV in the decade before combination ART was available, versus risk behavior later in life) [32,33]. Although the vaccine was well tolerated [32] and induced a modest transient reduction in the reservoir [33], reservoir size at trial completion did not significantly differ from baseline. This rare dataset thus offers a unique opportunity to assess replication-competent latent HIV genetic complexity, and investigate its relationship with uncontrolled infection duration, in this key population.

Methods

This study was approved by the Johns Hopkins University School of Medicine and Simon Fraser University Institutional Review Boards. All participants provided written informed consent. This study included 14 of the 20 participants of PACTG/IMPAACT-P1059 for whom replication-competent latent HIV isolates were serially obtained; all participants had plasma HIV RNA less than 50 copies/ml on ART at trial initiation and maintained viremia suppression throughout follow-up. As previously reported [33], infectious HIV frequencies in resting $CD4^+$ T cells were quantified in real time at trial screen and entry (week 0), and up to seven visits thereafter (weeks 2, 4, 6, 24, 26, 40 and 72) by end point dilution culture [21,33]. Resting $CD4^+$ T cells were enriched from fresh blood, activated to promote virus expression, after which released virus was expanded in $CD4^+$ T lymphoblasts from HIV-negative donors to quantify original infected cell frequencies in infectious units per million (IUPM) [34,35]. Nef and partial Pol (HXB2 genomic nucleotides 2253–3254) were amplified from p24-positive culture supernatants by nested RT-PCR using HIV-specific primers, and Sanger sequenced [21]. Sequences were aligned using HIValign [36] (options: MAFFT [37], codon alignment) and edited in AliView v1.18 [38]. Maximum likelihood phylogenies were reconstructed using RAxML v8.2.10 [39] with 100 bootstraps under a generalized time reversible model and visualized using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Patristic (tip-to-tip phylogenetic) distances were extracted from newick treefiles using Patristic [40]. Pairwise genetic distances were additionally calculated using the dist.dna function in the APE package in R [41]. HLA-associated polymorphisms defined at allele-level resolution in HIV subtype B were published in

(a)

Characteristic	Perinatal (*) (N=4)	Nonperinatal (N=10)	p-value
Years between infection and first cART; mean (±SD) [1]	22 (±2)	6 (±3)	**$6x10^{-7}$**
Age in years at trial entry; mean (±SD)	22 (±2)	23 (±2)	0.42
Sex (% Male)	25%	60%	0.56
# sampling timepoints; mean (±SD)	6 (±2)	5 (±2)	0.22
pVL during followup; median [IQR] [2] copies/ml	<6.5 [<6.5 - <6.5]	<6.5 [<6.5 - <6.5]	0.54
Weeks of followup; mean (±SD)	24 (±15)	30 (±19)	0.58
# Pol sequences; mean (±SD)	22 (±13)	15 (±9)	0.33
% unique Pol sequences; mean (±SD)	90% (±17%)	59% (±37%)	0.19
# Nef sequences; mean (±SD)	20 (±6)	13 (±8)	0.18
% unique Nef sequences; mean (±SD)	84% (±17%)	67% (±30%)	0.39
Baseline reservoir size; mean (±SD) IUPM [3]	1.26 (±0.83)	0.39 (±0.35)	**0.014**

[1]SD: Standard Deviation; [2]IQR: Interquartile Range; [3]IUPM: Infectious Units per Million CD4+ T-cells

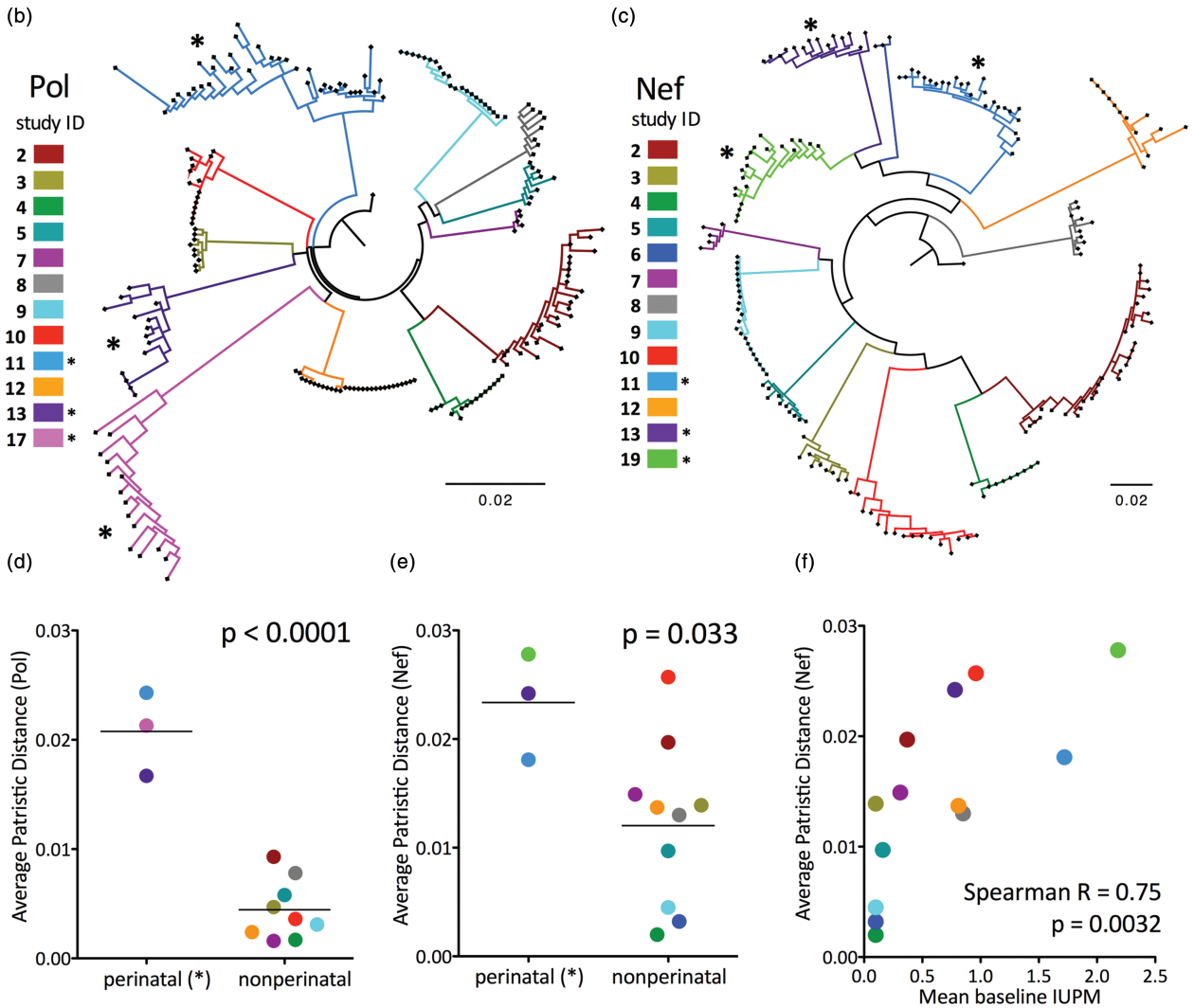

Fig. 1. Genetic diversity within the replication-competent latent HIV reservoir increases with untreated infection duration. (a) Participant clinical, immunogenetic, and HIV reservoir dataset characteristics. Throughout all figures, perinatally infected participants (those with longer uncontrolled infection duration) are denoted by asterisks (*). (b) Maximum-likelihood phylogeny relating within-host HIV Pol sequences, colored by participant. All within-host sequence datasets formed monophyletic clades with 100% bootstrap support. Scale in expected nucleotide substitutions per site. (c) Same as (b), but for Nef. All within-host datasets formed monophyletic clades with 100% bootstrap support except those of participants 11 (99%) and 19 (89%). Differences in overall topologies between Pol and Nef trees are attributable to low bootstrap support for the deeper branches

Ref. [42]. HLA-restricted optimal CTL epitopes were defined using the Los Alamos HIV Molecular Immunology Database with recent updates ([43] and C. Brander, personal communication).

Results

Replication-competent latent HIV sequences were serially sampled from resting $CD4^+$ T cells collected from 14 PACTG/IMPAACT-P1059 participants during suppressive ART **(**Fig. 1a**)**. Participants were stratified into those who acquired HIV perinatally ($N = 4$, in whom mean time between infection and combination ART initiation, henceforth referred to as estimated uncontrolled infection duration, was an estimated 22 years) and those who acquired HIV in adolescence through risk behavior ($N = 10$; mean uncontrolled infection duration 6 years). Consistent with earlier ART limiting reservoir size [14–20], the former had significantly larger reservoirs than the latter (mean baseline IUPM 1.26 versus 0.39, $P = 0.014$); however, the groups did not otherwise differ in terms of age, sex or duration of viremia control on ART at study entry (overall median 3.3; range 0.6–6.4 years) [33].

To maximize the likelihood that recovered HIV isolates originated from the latent reservoir, analysis was limited to participants who maintained pVL less than 50 copies/ml (a single viremia 'blip' to 436 copies/ml in participant 2 was excepted). Median pVL during follow-up, assessed using an ultrasensitive assay, was less than 6.5 copies/ml for both groups (Fig. 1a). Given that the vaccine did not ultimately reduce reservoir size [33], and that the latent HIV reservoir is highly stable [5,11], all HIV sequences recovered from a given participant were pooled together regardless of sampling date to estimate within-host replication-competent reservoir diversity. In total, 204 Pol and 188 Nef sequences were isolated at an average of five time points over an average 27 weeks (range 4–70), yielding an overall average 17 Pol and 15 Nef sequences per participant. Groups did not differ in terms of sampling, follow-up duration or percent unique sequences (Fig. 1a), though note only Nef sequences were obtained for participants 6 and 19, and only Pol for participant 17 **(**Fig. 1b and c**)**. Overall, 184 (90.1%) Pol and 179 (95.2%) Nef sequences contained no nucleotide mixtures, consistent with clonal HIV outgrowth from endpoint-diluted cell cultures in the majority of wells. Each participant's HIV sequences formed monophyletic clades with a median 100% bootstrap support (Fig. 1b and c).

Identical Pol and/or Nef sequences were recovered in 10 of 14 participants (3 of 4 perinatally infected and 7 of 10 nonperinatally infected, $P = 1.0$), consistent with clonal $CD4^+$ T-cell expansion as a mechanism of latent HIV reservoir maintenance in youth, regardless of infection mode. Notably, in 9 of these 10 participants, identical sequences were recovered at multiple timepoints up to 70 weeks apart (including participant 4 where the same sequence was recovered at weeks 2, 4, 6 and 72; the sole exception was participant 5, in whom identical sequences were recovered at a single timepoint only). This indicates that clonal descendants of $CD4^+$ T cells harboring replication-competent latent HIV tend to persist long-term in infected youth [28].

Replication-competent HIV reservoir diversity, measured in terms of average within-host patristic (tip-to-tip) phylogenetic distances, was significantly higher in perinatally compared with nonperinatally infected participants for both Pol (mean 0.21 versus 0.004 nucleotide substitutions/site, $P < 0.0001$; Fig. 1d) and Nef (mean 0.023 versus 0.012 nucleotide substitutions/site, $P = 0.033$, Fig. 1e). Replication-competent HIV reservoir diversity was also significantly higher in perinatally infected compared with nonperinatally infected participants for both Pol and Nef when measured in terms of mean pairwise genetic distance ($P = 0.0003$ for Pol, $P = 0.014$ for Nef; not shown). Reservoir size correlated strongly with Nef (Spearman's $R = 0.75$; $P = 0.0032$; Fig. 1f) and to a lesser extent Pol ($R = 0.47$; $P = 0.1$) within-host average patristic distances, indicating that larger reservoirs tend to be more genetically diverse (rather than more clonally expanded).

We investigated immune escape two ways. First, we estimated total escape burden by identifying all HIV codons under selection by one or more host HLA alleles and classifying each autologous HIV residue as adapted (inferred escaped) or susceptible, based on published definitions [42] (example in Fig. 2a). For each sequence, we calculated the percent HLA-associated sites exhibiting an adapted (or possibly adapted) form, and computed the median for each participant's datasets (e.g. the Pol dataset in Fig. 2a is 44% adapted to host HLA). Second, we estimated within-host escape complexity by quantifying the proportion of optimally described HLA-restricted CTL epitopes exhibiting within-host amino acid variation [e.g. 6/8 (75%) for the Pol dataset in Fig. 2a]. In Pol,

(i.e. those that define evolutionary relationships between participants); all were less than 70% except the subclade constituting participants 2 and 4. No downstream analyses, however, relied on intra-participant genetic distances. (d) Average within-host patristic (tip-to-tip phylogenetic) distances in reservoir Pol sequences; *P* value calculated using Student's *t* test. (e) Same as panel (d), but for Nef. (f) Relationship between size and diversity (Nef) of the within-host replication-competent HIV reservoir, assessed by Spearman's correlation.

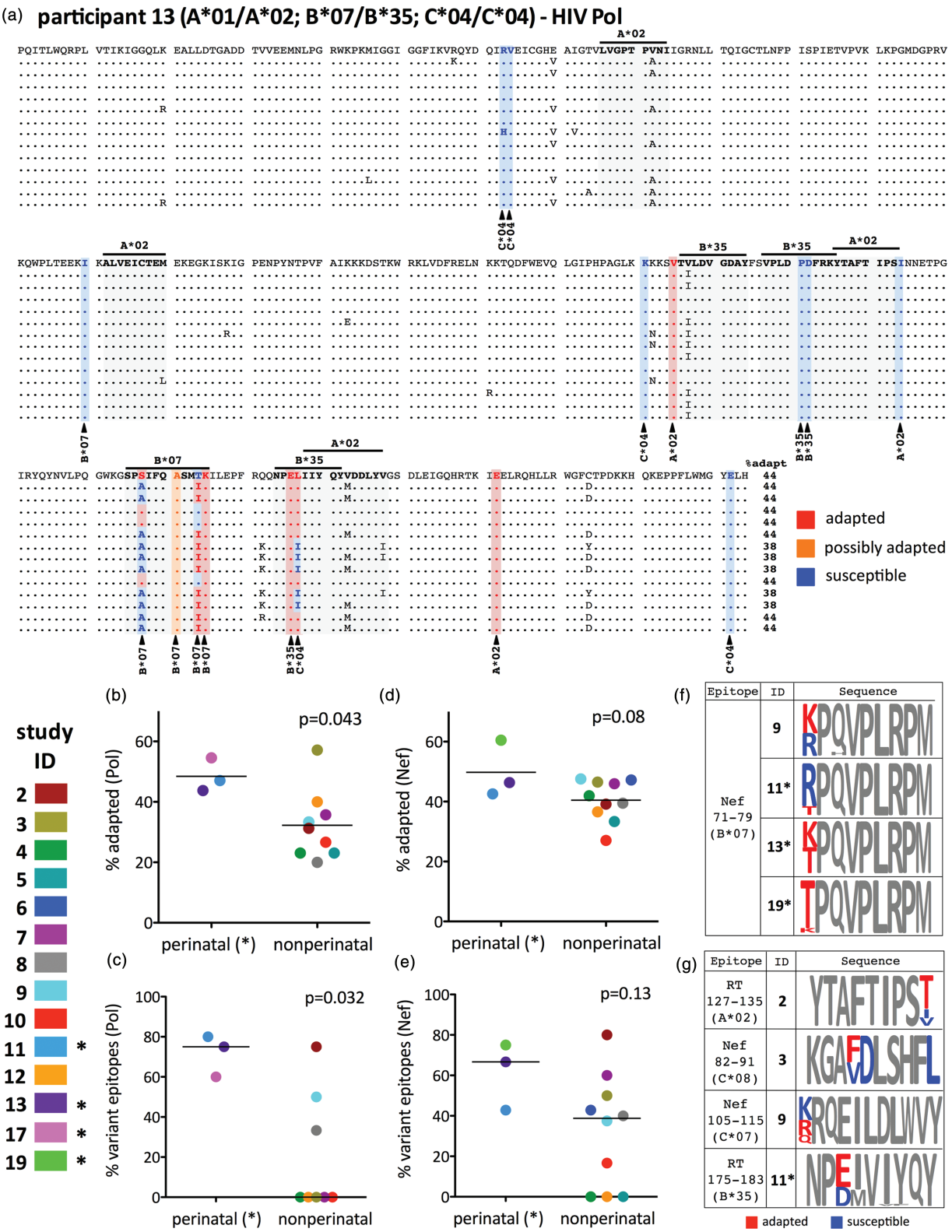

Fig. 2. Immune escape burden within the replication-competent latent HIV reservoir is complex, and generally increases with untreated infection duration. (a) Pol sequence alignment for participant 13 (perinatally infected). The reference sequence (top) was arbitrarily chosen from among those recovered from CD4$^+$ T cells sampled at the earliest timepoint. Sites of HLA-driven adaptation in Pol (defined in [42]) are highlighted, with red, orange and blue denoting adapted (inferred escaped), possibly adapted and susceptible forms, respectively. Optimally described CTL epitopes restricted by host HLA alleles are shaded in grey. The proportion of HLA-associated sites exhibiting adapted or possibly adapted forms is reported after each sequence. Note the three codons (257, 264 and 277 in

the percentage of HLA-adapted sites was overall higher in perinatally infected compared with nonperinatally infected participants (mean 48 versus 32%, $P = 0.043$; Fig. 2b), as was the percentage of optimally described Pol CTL epitopes exhibiting within-host amino acid variation (median 75 versus 0%, $P = 0.032$; Fig. 2c). Similar trends were observed for Nef (Fig. 2d and e).

Notably, however, despite uncontrolled infection durations of greater than 20 years and overall high adaptation levels in some participants, no reservoir was completely adapted to host HLA (Fig. 2b and d). Furthermore, on an individual CTL epitope level, reservoir immune escape complexity differed widely both within and between hosts, an observation that can be illustrated by the HLA-B*07-restricted immunodominant [44] Nef-RM9 epitope (Fig. 2f). Four participants (9, 11, 13, 19) expressed HLA-B*07, all of whom exhibited high Nef adaptation. However, whereas participants 13 and 19′s reservoirs were fully escaped in Nef-RM9, ∼45 and ∼80% of participants' 9 and 11′s reservoirs, respectively, harbored sequences that were predicted to retain susceptibility to HLA-B*07-restricted CTL. This indicates that key susceptible epitopes can still be identified even in otherwise highly escaped reservoirs. Indeed, co-existence of HLA-susceptible and adapted forms within the same CTL epitope in an individual's reservoir occurred commonly: greater than 60% and greater than 30% of participants harbored at least one Pol or Nef epitope, respectively, where this occurred (examples in Fig. 2g). This further supports the reservoir as an archive of within-host HIV evolution [23–25] and suggests that autologous T-cell responses to these epitopes, if effectively re-stimulated, might still be capable of clearing a portion of the reservoir.

Discussion

Serial sampling of the replication-competent HIV reservoir in our young adult cohort supports the notion that reservoir diversity and escape burden continue to increase with uncontrolled infection duration, even in individuals who initiate ART relatively late. Caveats include the study's modest size, differences in infection route (such that we cannot rule out that higher reservoir complexity is attributable to perinatal transmission rather than uncontrolled infection duration), and that reservoir sampling occurred during administration of an experimental therapeutic HIV vaccine [33]. Although the vaccine did not durably reduce reservoir size [33], and we observed no evidence that the vaccine consistently altered overall within-host reservoir diversity (comparisons of the average within-host patristic HIV distances prevaccine and postvaccine yielded $P = 0.8$ and $P = 0.6$ for Pol and Nef, respectively; not shown), we cannot rule out the possibility that the vaccine may have induced very low-level HIV replication [45] or otherwise perturbed reservoir sequence composition in some participants. Confirmation of our observations in additional cohorts is, therefore, merited. Nevertheless, our findings may have implications for immmunotherapeutic HIV cure strategies. Although, on one hand, HIV elimination in late-treated persons may be doubly challenged by larger and more genetically complex reservoirs, our observation that predicted HLA-susceptible sites were present in all reservoirs, even those of persons who did not achieve sustained virologic suppression until two decades after infection, supports strategies that integrate host and viral genetic data to inform HIV cure immunogen selection.

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this alignment, denoting RT codons 158, 165 and 178), all within HLA-restricted CTL epitopes, where adapted and susceptible forms co-exist within the reservoir. (b) Average inferred immune escape burden in Pol (calculated as the median '% adapted' value of all sequences for each participant), stratified by group. *P* value calculated using Student's *t* test. (c) Percentage of HLA-matched optimal CTL epitopes in Pol exhibiting within-host sequence variation, stratified by group. As the data for nonperinatally infected participants are nonnormally distributed, the *P* value is calculated using the Mann–Whitney *U* test. (d and e) Same as panels (b) and (c), but for Nef. (f) The B*07-restricted RM9 epitope (Nef codons 71–79) as an example of reservoir immune escape complexity within and between hosts. Letter size is proportional to within-host amino acid prevalence, with red and blue denoting adapted and susceptible forms [42], respectively (all other residues are grey). Note that participant 11 was perinatally infected, illustrating that HLA-adapted and susceptible forms of a given CTL epitope can co-exist in the reservoir, even in persons who initiated cART two decades after infection. (g) Additional examples of HLA-restricted optimal epitopes in Pol and Nef where adapted and susceptible forms co-exist within an individual's reservoir.

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Conflicts of interest

There are no conflicts of interest.

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