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

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Abstract

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 4 perinatally- and 10 non-perinatally-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: While participants were comparable in terms of sociodemographic and HIV sampling characteristics (*e.g.* on average, a mean 17 Pol sequences were recovered at 5 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

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susceptibility to host cellular immune responses were detected in all participants, irrespective of uncontrolled viremia duration.

Conclusions: 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.

Keywords

HIV; latent reservoir; replication-competent; genetic diversity; immune escape; young adults; youth

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. While 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

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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 (due to 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

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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 <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 [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

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Replication-competent latent HIV sequences were serially sampled from resting CD4+T-cells collected from 14 PACTG/IMPAACT-P1059 participants during suppressive ART (Figure 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 vs 0.39,

$p=0.014$), however the groups did not otherwise differ in terms of age, gender 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 $pVL < 50$ 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 <6.5 copies/ml for both groups (Figure 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 5 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, followup duration or % unique sequences (Figure 1A), though note only Nef sequences were obtained for participants 6 and 19, and only Pol for participant 17 (Figures 1B–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 (Figures 1B–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 to nonperinatally-infected participants for both Pol (mean 0.21 vs. 0.004 nucleotide substitutions/site, $p<0.0001$) (Figure 1D) and Nef (mean 0.023 vs. 0.012 nucleotide substitutions/site, $p=0.033$, Figure 1E). Replication-competent HIV reservoir diversity was also significantly higher in perinatally- compared to 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 $R=0.75$; $p=0.0032$; Figure 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 Figure 2A). For each sequence, we calculated the %

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 Figure 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 Figure 2A). In Pol, the percentage of HLA-adapted sites was overall higher in perinatally- compared to nonperinatally-infected participants (mean 48% vs. 32%, $p=0.043$; Figure 2B), as was the percentage of optimally-described Pol CTL epitopes exhibiting within-host amino acid variation (median 75% vs. 0%, $p=0.032$; Figure 2C). Similar trends were observed for Nef (Figures 2D–E).

Notably, however, despite uncontrolled infection durations of >20 years and overall high adaptation levels in some participants, no reservoir was completely adapted to host HLA (Figures 2B, 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 (Figure 2F). Four participants (9, 11, 13, 19) expressed HLA-B*07, all of whom exhibited high Nef adaptation. However, while 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: >60% and >30% of participants harbored at least one Pol or Nef epitope respectively where this occurred (examples in Figure 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]. While 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 pre- and post-vaccine 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 immunotherapeutic HIV cure strategies. While, 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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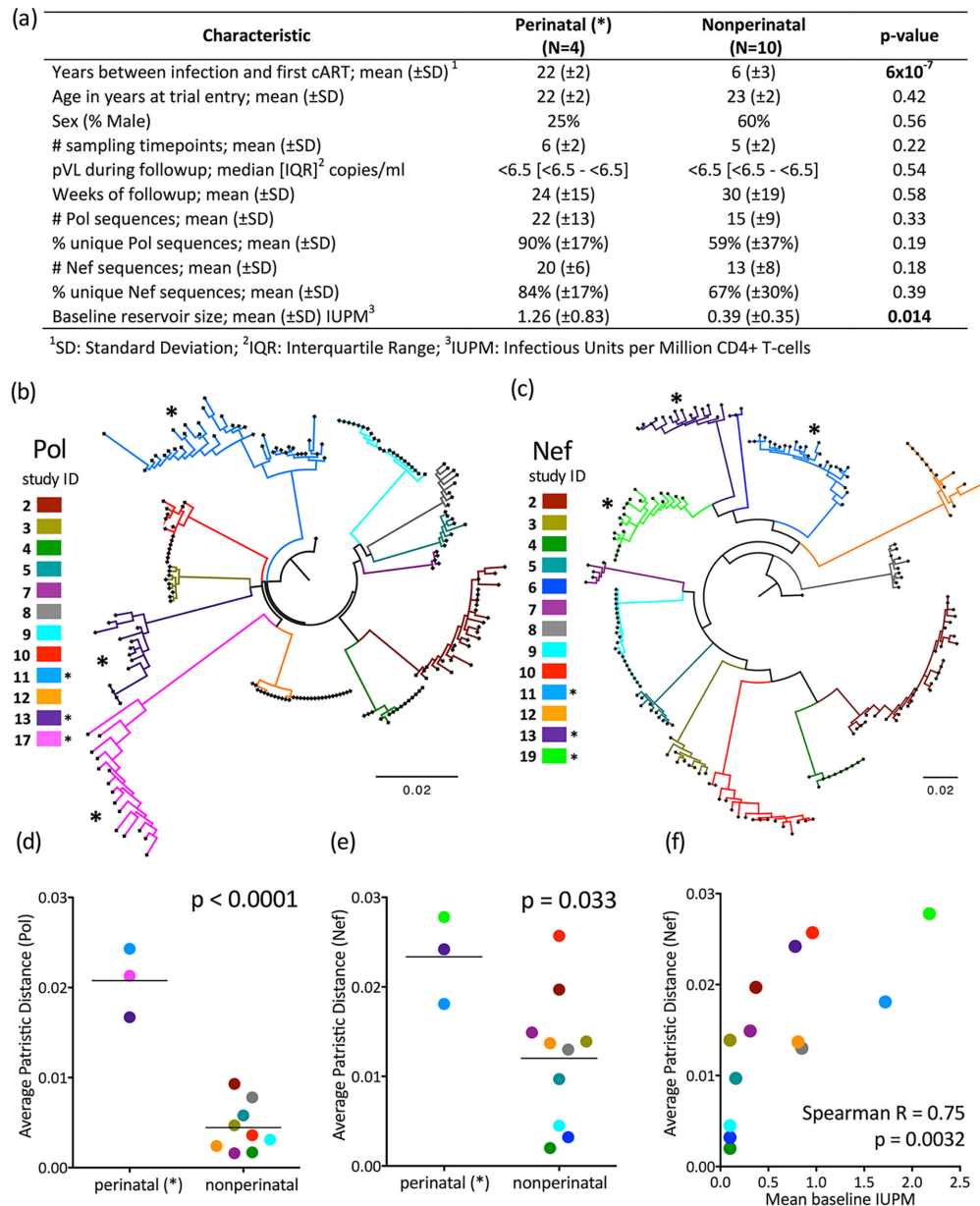


Figure 1: Genetic diversity within the replication-competent latent HIV reservoir increases with untreated infection duration.

Panel 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 (*). *Panel 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. *Panel 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 (*i.e.* those that define evolutionary relationships *between* participants); all were <70% except the subclade

comprising participants 2 and 4. No downstream analyses however relied on intra-participant genetic distances. *Panel D*: Average within-host patristic (tip-to-tip phylogenetic) distances in reservoir Pol sequences; p-value calculated using Student's T-test. *Panel E*: Same as panel D, but for Nef. *Panel F*: Relationship between size and diversity (Nef) of the within-host replication-competent HIV reservoir, assessed by Spearman's correlation.

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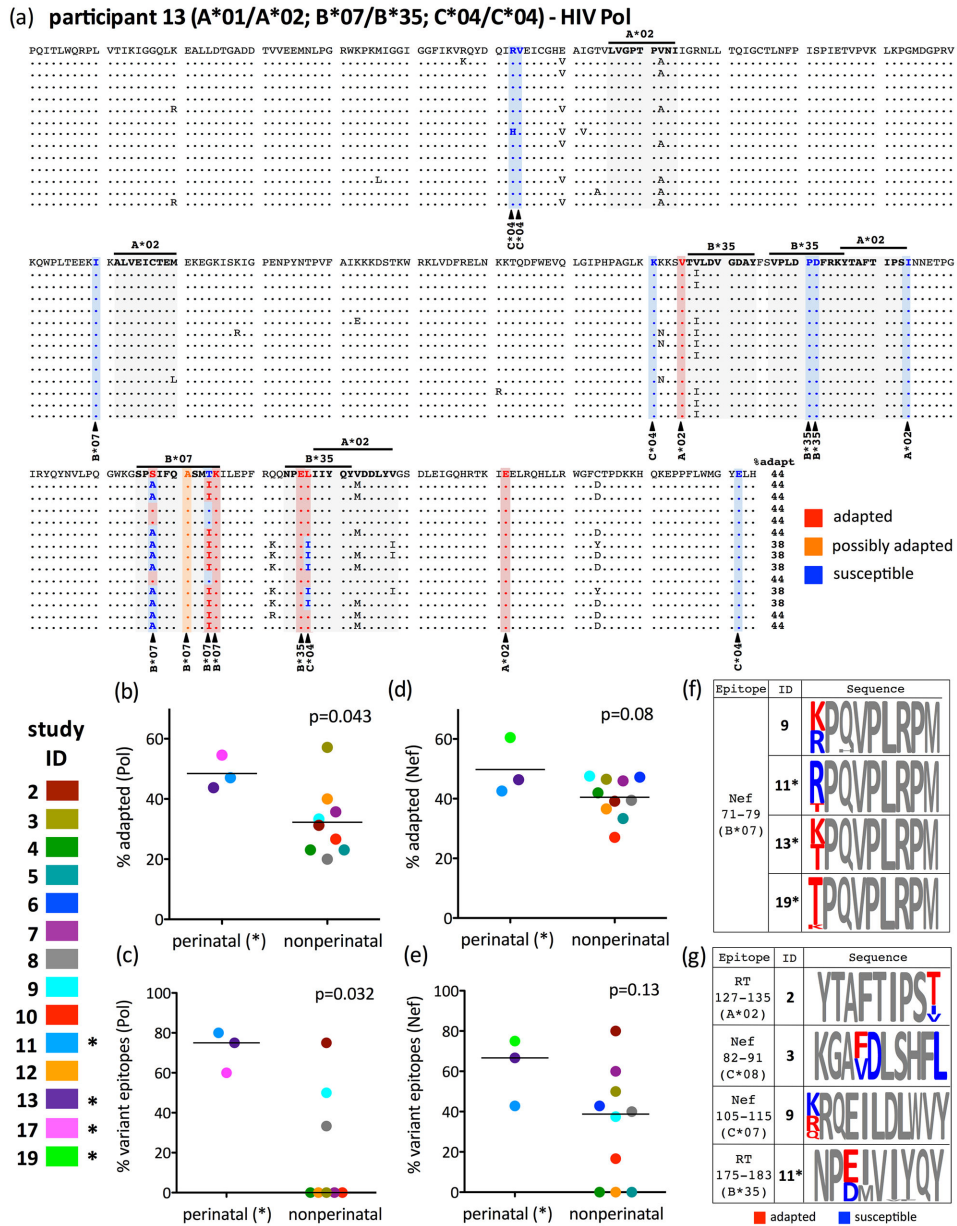


Figure 2: Immune escape burden within the replication-competent latent HIV reservoir is complex, and generally increases with untreated infection duration.
Panel A: Pol sequence alignment for participant 13 (non-perinatally infected). The reference sequence (top) was arbitrarily chosen from among those 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 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. *Panel 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. *Panel C*: Percent of HLA-matched optimal CTL epitopes in Pol exhibiting within-host sequence variation, stratified by group. As the data for non-perinatally infected participants are non-normally distributed, the p-value is calculated using the Mann-Whitney U-Test. *Panels D, E*: Same as panels B and C, but for Nef. *Panel 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. *Panel G*: Additional examples of HLA-restricted optimal epitopes in Pol and Nef where adapted and susceptible forms co-exist within an individual's reservoir.