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Full-length sequencing of HIV-1 proviruses in large infected cell clones in PWH on ART reveals a predominance of solo-LTRs

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Background: Standard methods of amplifying and sequencing HIV-1 proviruses do not capture long terminal repeats (LTRs) in their entirety due to technical difficulties related to sequence identity between these elements. Moreover, defective proviruses consisting of a single (solo) LTR, are not detected at all, and thus their frequency and significance are poorly understood. Here, we circumvent these difficulties by performing nested PCR using host or host-virus junction primers in water-in-oil (W/O) emulsion compartments to amplify and sequence HIV-1 proviruses in large T cell clones in their entireties. Our results indicate that the prevalence of solo-LTRs, a product of homologous LTR recombination, may be much higher than previously realized.

Methods:

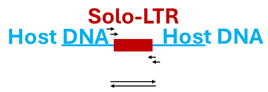
- Designed clone-specific host and/or host-junction primers for outer and inner/nested PCR
  - ✓ Use integration site analysis (ISA) data (Guo, S., et al., PLoS Pathog. 2022) to identify the 10 largest clones for each donor on ART
  - ✓ To minimize off-target priming, outer junction primers pair not more than four 3'-terminal nucleotides to LTR sequence

Clone-specific "Junction-to-Junction" (J-to-J) PCR

- ➢ Utilizes host and/or junction primers and emulsion PCR
- ➢ Captures entire 2-LTR and solo-LTR proviruses from specific clones



Amplicon produced by "inner" primers



Amplicon produced by "inner" primers

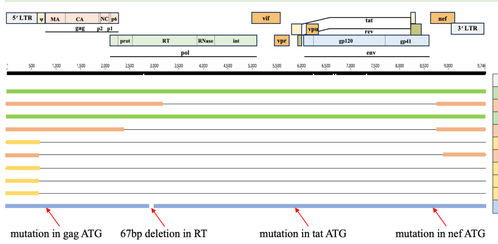
  - ✓ To prevent exonucleolytic primer trimming, four 3'-terminal bridging phosphorothioates are added to each primer
- Outer PCRs are emulsified by droplet PCR (Droplet Generator, BioRad). Droplet generation prior to PCR:
  - ✓ partitions proviral sequences in separate droplets
  - ✓ prevents generation of chimeras between proviruses from different loci
  - ✓ reduces DNA complexity in provirus-positive droplets
- Emulsions are broken, reaction products purified using SPRI-select beads, and subjected to nested/inner clone-specific PCR
- Amplicons fractionated over gDNA tapes (Agilent TapeStation) for length approximation, and sequenced (Sanger, PacBio, Oxford Nanopore).

Fractionation of J-to-J HIV proviral amplicons from 10 largest C-03 clones

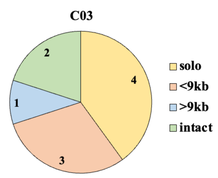
Genomic TS	Amplification of top 10 C-03 clones										
clone	C03_01_ ZNF268	C03_02_ ODF3B	C03_03_ TAB2	C03_04_ ISOC1	C03_06_ GIMAP2	C03_07_ NUP210L	08_ RPRD2	09_ USP16	12_ AFF1	14_ RPL38	1kb extend DNA ladder
19.231 reads total	278	127	120	113	92	89	69	61	55	45	
in/out of gene	in	in	in	in	out	in	in	in	in	in	
provirus orientation relative to the host gene	intron 3 antisense	upstream sense	intron 5 antisense	intron 1 antisense	upstream antisense	intron 30 antisense	intron 4 sense	intron 8 antisense	intron 2 antisense	intron 3 antisense	
DNA ladder	1	2	3	4	5	6	7	8	9	10	11
proviral DNA [bp]	9,767	4,192	9,720	5,637	634	1,472	641	641	634	9,668	

- Novel Method for Integration Site-Specific Full Genome Sequencing:
  - ✓ Junction to junction sequencing of both Solo-LTR and 2-LTR HIV genomes
  - ✓ Non-Biased (internal sequence independent)
- Solo-LTRs Persist in Abundant Infected Cell Clones During Sustained ART

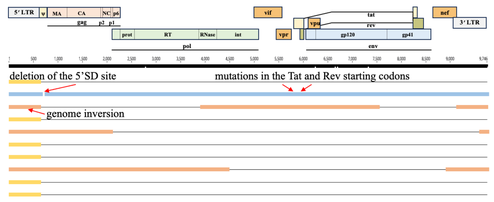
Virogram alignment of HIV-1 proviruses in the C03 donor



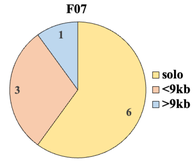
host gene	IS reads	HIV length
ZNF268	278	9,767
ODF3B	127	4,192
TAB2	120	9,719
ISOC1	113	5,637
GIMAP2	92	634
NUP210L	90	1,472
RPRD2	69	641
USP16	61	641
AFF1	55	634
RPL38	45	9,668



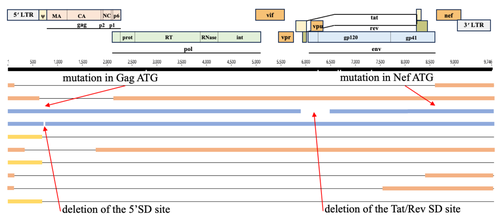
Virogram alignment of HIV-1 proviruses in the F07 donor



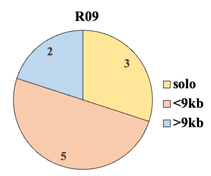
host gene	IS reads	HIV length
EVI2B	419	635
CLEC2B	384	9,681
PTPN4	229	5,118
NUP62	144	637
ATRX	105	2,227
PDE7B	94	634
SMURF1	79	635
ERN1	77	5,395
TSC22D2	67	635
TRMT11	62	634



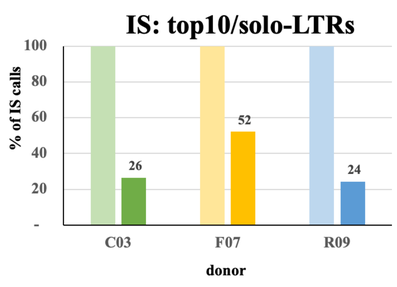
Virogram alignments of HIV-1 proviruses in the R09 donor



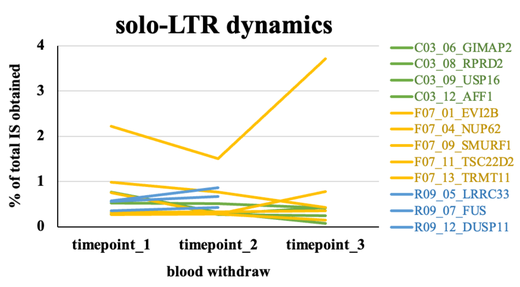
host gene	IS reads	HIV length
HLA-B	294	1,294
HCG27	204	8,325
ITGAL	184	9,070
LIN54	152	9,701
LRRC33	149	625
SIK3	132	8,329
FUS	122	625
THADA	98	1,294
BTRC	83	1,967
DUSP11	78	624



Solo-LTRs are 24-52% of the top 10 largest HIV-1 infected T cell clones



With exception of EVI2B, solo-LTR clones are stable



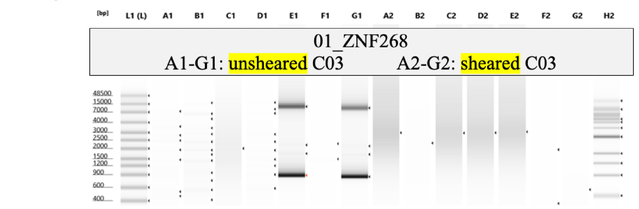
Study Participants:

PID	age	sex	race	years on ART	current ART regimen	CD4+ T cells (cells/mm^3)	plasma HIV-1 RNA at referral (cps/mL)	HIV-1 DNA (cps/10^6 PBMC)
C03	44	male	white	12	DRV/ETV/DTG	416	62	2,505
F07	62	male	African American	22.9	ABC/3TC/EFV	1023	52	1,603
R09	77	male	white	11.1	TDF/FTC/EFV	380	197	1,533

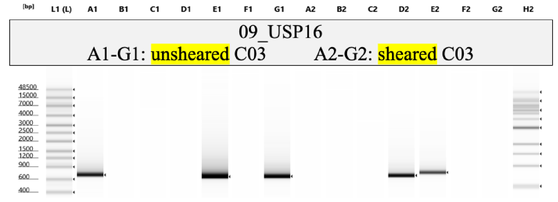
Validating Solo-LTR clones

- Recombination between 5' and 3' LTRs during PCR can create solo-LTR artifacts indistinguishable from amplicons derived from genuine solo-LTRs
- To make this distinction, a new aliquot of gDNA is sheared (avg. ~1600 bp) prior to re-screening by dPCR1
  - ✓ Due to shearing, 2-LTR proviruses are almost all fragmented, thus preventing their amplification by PCR
  - ✓ LTR-containing proviral fragments are partitioned into different droplets, thus preventing inter-LTR recombination and formation of pseudo solo-LTR artifacts
  - ✓ gDNA shearing do not prevent amplification of genuine solo-LTRs, since the entirety of the amplicon, including flanking host DNA, is compartmentalized
- Neither the 2-LTR provirus nor the recombinant pseudo solo-LTR artifacts amplify from sheared DNA
- **Genuine solo-LTRs (~634 bp) amplify irrespective of gDNA shearing**

Amplicon derived from intact C03 HIV provirus integrated into ZNF268 (and associated pseudo solo-LTR artifact)



Amplicon derived from genuine solo-LTR C03 HIV provirus integrated into USP16



Summary:

- Solo-LTRs may represent a large % of the proviral burden.
- These studies are consistent with our recent observations that solo LTRs are prevalent among the largest infected T cell clones in children with HIV (CWH) (Botha JC, et al., mBio. 2023).
- **The biological/virological impact of persistent solo-LTRs should be evaluated in future studies.**

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