PHENOTYPIC AND FUNCTIONAL HETEROGENEITY CONTRIBUTE TO THE PERSISTENCE OF THE CLONAL CD4 RESERVOIR

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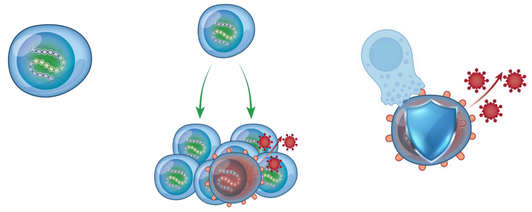
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BACKGROUND

Latency

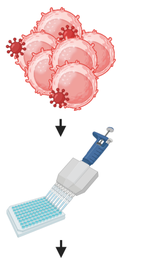
Clonal expansion

Resistance to killing/death



Mechanisms of HIV reservoir persistence

METHODS



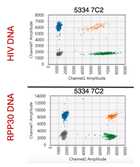
$CD4^+$ memory T cells isolated from PWH

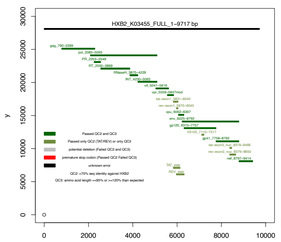
Expand with irradiated feeder cells, anti-CD3/ CD28, & 5x ARVs

Screen by p24 ELISA & serial dilute for subcloning

Screen by p24 ELISA post subcloning

1:2 ratio intact HIV : human gene





Donor	Gene	Chromosome	Orientation
5104	ATP2B4	1	anti-sense
OM5334	ZNF721	19	anti-sense
603	ZNF486	19	sense
JM	ZNF561	19	anti-sense

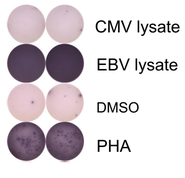


Figure 1: Clonal populations were shown to harbor a single intact provirus with a unique integration site.
(A) IPDA analysis of a pure clonal population
(B) FLIPSEQ analysis of integrated provirus in clonal populations indicates intact provirus
(C) Integration sites were unique to each clonal population
(D) Interferon-γ ELISPOT analysis of ARC ZNF721 reveals EBV specificity

RESERVOIR CLONES SHOW POOR LATENCY REVERSAL DESPITE ROBUST PROLIFERATION

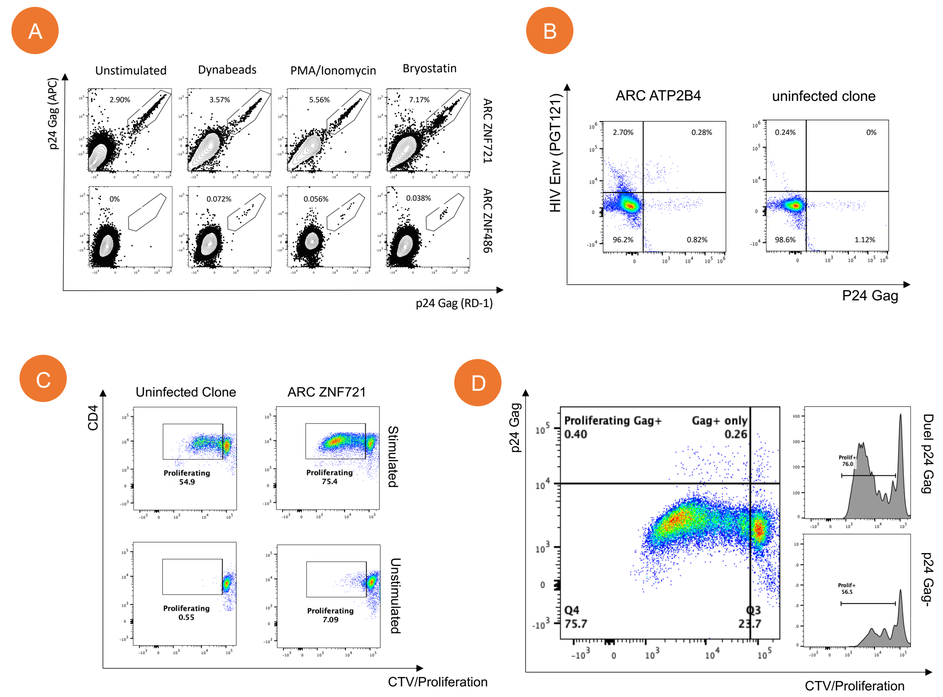


Figure 2: Latency Reversal and Proliferation in HIV-Infected Clonal Populations
(A) Latency Reversal Panel: While the unstimulated HIV-infected clone expressed low levels of p24 Gag, significant latency reversal was achieved with bryostatin stimulation.
(B) Protein Expression Post-Stimulation: HIV-infected clones expressed p24 Gag and Env proteins 72 hours post-stimulation with irradiated feeders and CD3/CD28 antibodies, indicating active viral protein production.
(C) Proliferation of ARC ZNF721: The HIV infected clone showed robust proliferation, even in the absence of stimulation by irradiated feeders and soluble CD3/CD28, as compared to an autologous uninfected clone.
(D) Proliferation was combined with expression of Gag: HIV infected clonal proliferation upon stimulation with irradiated feeder cells and soluble CD3/CD28 induced latency reversal as indicated by p24 Gag expression.

IMMUNOPHENOTYPING OF HIV INFECTED CLONES REVEALS DINSTINC CYTOTOXIC PHENOTYPE

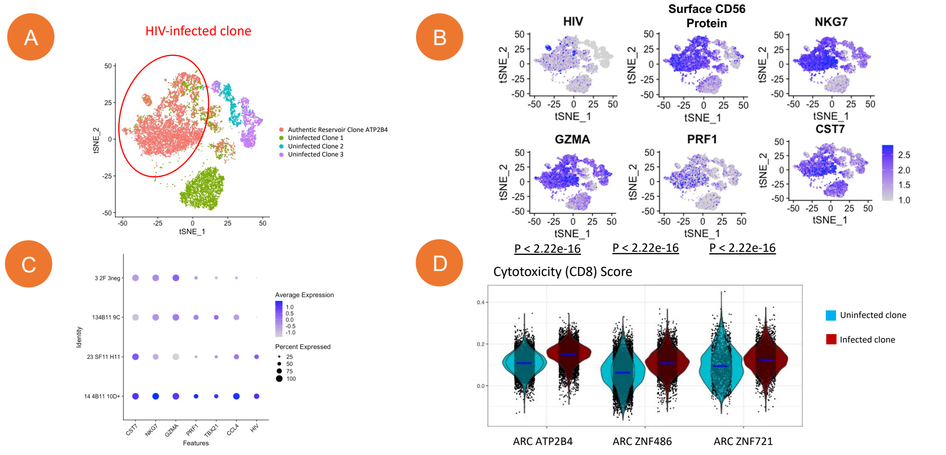


Figure 3: Immunophenotyping of HIV Infected Clones Reveals a Distinct Cytotoxic Phenotype
(A) tSNE Clustering Analysis: Representative tSNE plot illustrating spatial clustering of HIV infected clones compared to autologous, uninfected clones from donor 5104. This visual highlights clear phenotypic distinctions associated with HIV infection.
(B) Cytotoxic T Cell Phenotype: CITE-Seq analysis shows HIV infected clones display a robust cytotoxic T cell phenotype across donors, marked by upregulation of cytotoxicity-associated markers, suggesting an active immune response.
(C) Ex-Treg Signature: Infected clones exhibit higher expression of genes linked to an ex-Treg phenotype as described by Freuchet et al. (*Nat Immunol*, 2023).
(D) Gene Expression Analysis: Single-cell expression of a CD8 cytotoxic gene module (Szabo et al., 2019) subtracted from control features in uninfected clones (blue, n=10 clones) and HIV infected clones (red, n=3 clones) across three donors.

HIV INFECTED CLONES EXHIBITED HETEROGENOUS CTL RESISTANCE

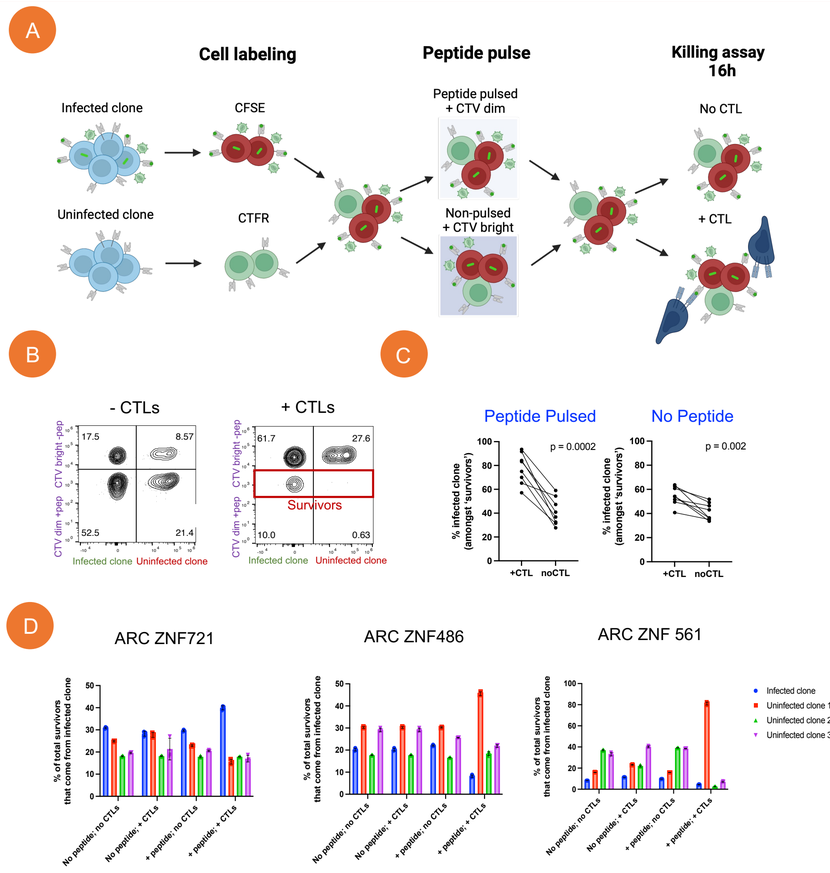


Figure 4: CTL Resistance and Susceptibility in HIV-Infected Clones Compared to Uninfected Clones
(A) CTL Resistance Assay Schematic: The CTL resistance assay assessed clone susceptibility by labeling infected clones with CFSE and uninfected clones with CTFR. Populations were peptide-pulsed, stained with CTV bright and CTV dim, recombined, and exposed to CTLs for 16 hours. Previous research has shown that cytotoxic infected cells are resistant to killing (Herrera et al. *bioRxiv,* 2024)
(B) CTL-Mediated Killing Susceptibility: The 603 ZNF486 infected clone was more susceptible to CTL killing than three autologous uninfected clones, showing differential CTL targeting.
(C) Resistance to CTL Killing: The 5014 ATP2B4 showed resistance to CTL killing in a peptide pulsed CTL resistance assay.
(D) Variation in CTL Resistance Across Donors: Resistance to CTL killing varied across clones from different donors, suggesting diverse immune escape mechanisms within the viral reservoir.

CONCLUSIONS

	Clone #1	Clone #2	Clone #3	Clone #4
	5104 ATP2B4	OM5334 ZNF721	603 ZNF486	JM ZNF561
Latency	✓	✓✓	✓✓✓✓	n/a
CTL resistance	✓✓✓	✓✓	✓	✓✓
Proliferation	✓	✓✓✓✓	✓✓✓	✓✓

We have developed a method to isolate pure, clonal populations of the HIV reservoir from individuals living with HIV. This approach enables us to comprehensively characterize and immunophenotype these clonal populations. Additionally, we have functionally analyzed these clones, shedding light on potential mechanisms underlying their persistence *in vivo*. These clonal populations offer a unique opportunity to study the HIV reservoir *in vitro*, providing valuable insights into the dynamics of viral persistence

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