Env Mutations are a Gateway for Acquisition of INSTI Resistance

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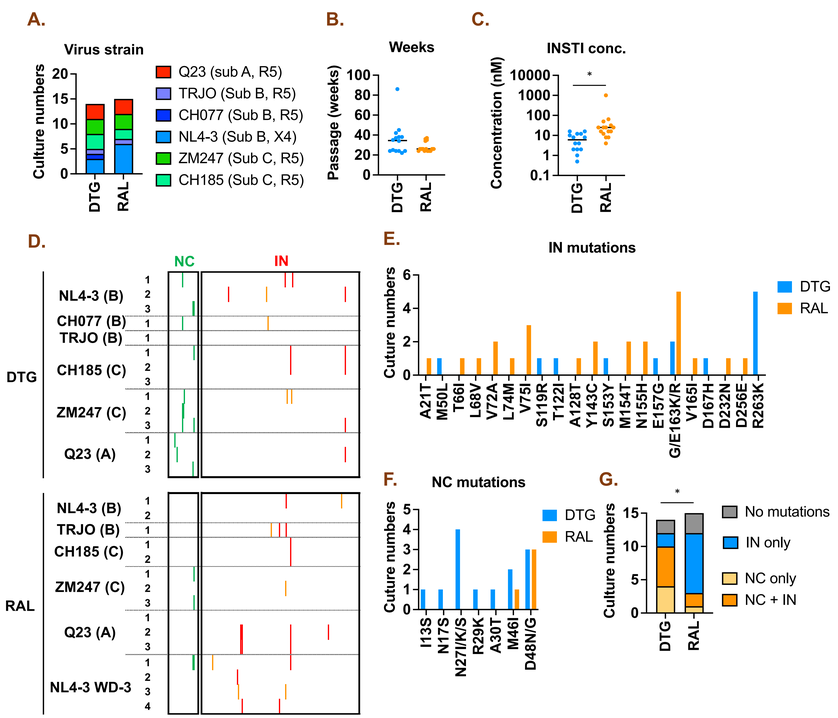
Background

Virological failure (VF) has been observed in people with HIV (PWH) receiving second-generation integrase (IN) strand transfer inhibitors (INSTIs) without IN resistance mutations. To elucidate alternative INSTI resistance mechanisms, **we previously passaged HIV-1 in a T-cell line for ~1 year with escalating concentrations of the INSTI dolutegravir (DTG). HIV-1 developed resistance to DTG by sequentially acquiring mutations in Env, nucleocapsid (NC), and, in some cases, IN.** Importantly, Env mutations not only conferred INSTI resistance but also compensated for infectivity defects caused by IN mutations. This study investigates the role of Env mutations in the acquisition of INSTI resistance mutations in IN and NC.

Methods

Multiple HIV-1 isolates were serially passaged (for ~2 years) in primary PBMCs in the presence of DTG or the first-generation INSTI, raltegravir (RAL). We evaluated the impact of selected mutations on replication kinetics and viral infection via cell-free or cell-cell transmission. In PBMCs, we cultured wild-type (WT) HIV-1_{NL4-3} or the DTG-selected HIV_{NL4-3} Env mutant (12 replicates each) with 2 nM DTG for 20 weeks. Viral DNA was quantified longitudinally, and GagPol regions were sequenced to monitor emerging mutations.

Figure 1: Different IN/NC mutation profiles emerge in the presence of DTG and RAL



Long-term passaging of multiple HIV-1 strains (subtypes) in PBMCs in the presence of DTG or RAL

(A-C) Summary of viral strains, passage duration, and INSTI concentrations used in this study. Independent experiments were conducted using PBMCs from different donors.

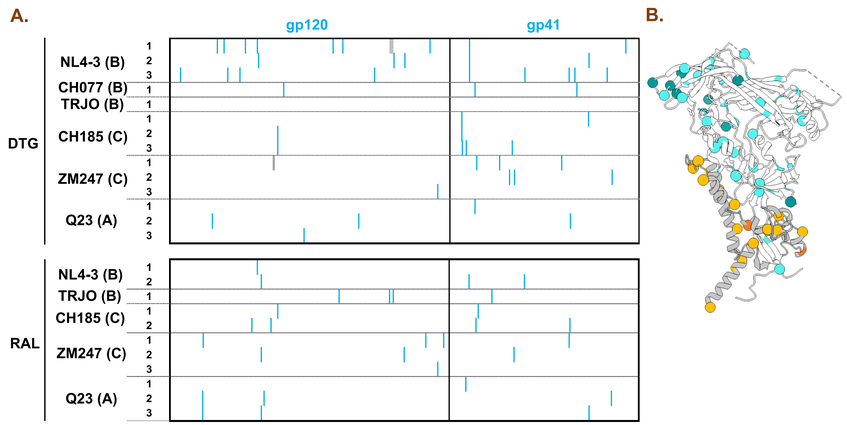
(D) Schematic representation of the identified IN or NC mutations in the presence of DTG or RAL in PBMC; identified mutations in each culture are shown in the color bar [NC: green, IN: red (resistance-associated mutations; RAM) or orange (non-RAM)].

(E-F) Details of selected IN and NC mutations in the presence of INSTIs

(F) Number of cultures acquiring NC, IN, or combined NC+IN mutations. The frequency of NC mutations (NC only or NC + IN) was compared by Fisher's exact test (*P < 0.05)

- **HIV-1 can exploit non-canonical resistance pathways to evade second-generation INSTIs**
- **Env mutations facilitate the acquisition of resistance mutations in NC and IN in the presence of INSTIs**

Figure 2: HIV-1 acquires Env mutations independent of viral strain



Long-term passaging of multiple HIV-1 strains (subtypes) in PBMCs in the presence of DTG or RAL

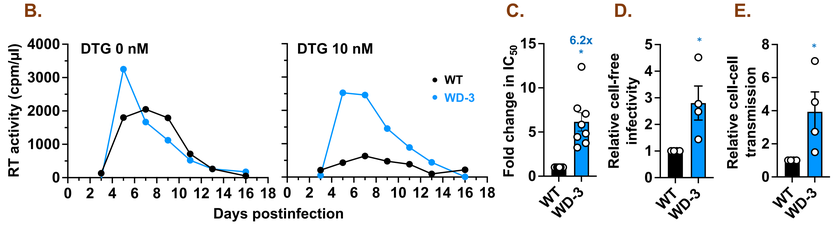
(A) Schematic representation of the identified Env mutations in the presence of DTG or RAL in PBMC; identified point mutations or deletions in each culture are shown in blue or gray bar, respectively. Independent experiments were conducted using PBMCs from different donors.

(B) Selected Env mutations identified in SupT1 cells (Hikichi et al., Sci Adv 2024) and PBMCs under INSTI pressure; gp120 and gp41 mutations are shown in cyan and orange, respectively, with recurrent mutations identified in both cell types highlighted in darker colors.

Figure 3: Env mutations confer resistance to INSTIs in primary cells

A.

	gp120								gp41	
	C1		V1/V2		V3		V4	C4	HR1	CT
	87	99	138	160	298	316	400-405	475	547	832
WT NL4-3	V	D	T	N	R	A	WSTEG	M	D	V
PBMC culture (16 nM, P31)	E	N	I	Y	K	T	Δ	I	G	A
Clone: WD-3	E	N	I	Y	K	T	Δ	I	G	V



(A) The Env-coding region derived from PBMC culture was cloned into the parental NL4-3 strain. WD-3 is derived from viruses replicating in PBMC in the presence of 16 nM DTG (culture 1 (Fig. 2), week 31).

(B) Replication kinetics of WD-3 in PBMC in the absence or presence of DTG. PBMCs were infected with the indicated viruses.

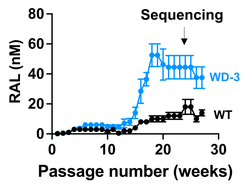
(C) Fold change in IC_{50} values was calculated on the basis of the area under the curve (AUC) of the replication kinetics. Data were obtained from eight different donors (mean +/- SE, *P <0.05, unpaired t-test).

(D) Cell-free infectivity in primary $CD4^+$ T cells using HIV-1 nanoLuc reporter virus bearing the indicated Env (mean +/- SE, *P <0.05, unpaired t-test).

(E) Cell-cell transmission in primary $CD4^+$ T cells. Data were obtained from at least four different donors (mean +/- SE, *P <0.05, unpaired t-test).

Figure 4: Env mutations facilitate the acquisition of IN and NC resistance mutations

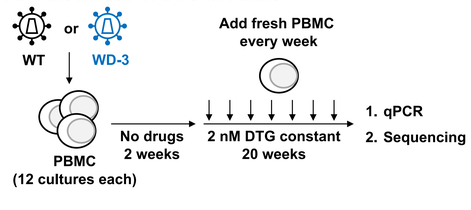
RAL dose escalation in PBMC



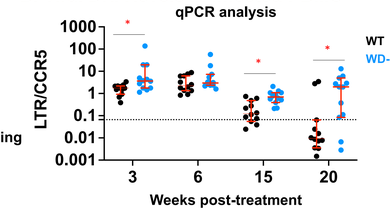
		Conc.	NC	IN	Env
WT	1	16 nM	None	**N155H**, D56E	N160K, E648K
	2	8 nM	None	V165I	D167G, D547G
	3	8 nM	None	None	N160K, N637K
	4	8 nM	None	None	N160K, D547G
WD-3	1	50 nM	**M46I, D48N**	A21T, **G163R**	F175L, N186K, S432G
	2	25 nM	None	**T66I**	V127I
	3	25 nM	None	M154I, L68V, **Y143H**	F175L, S168R
	4	50 nM	None	**V75I**	F175L, N186K, I439T, L785M

Long-term passaging of WT NL4-3 or WD-3 in PBMCs (four cultures each) with increasing RAL concentrations; NC, IN, and Env regions were sequenced using genomic DNA in the infected PBMC at 26 weeks postinfection. Mutations are bolded if listed in the Stanford HIVDB or reported to be synergistic with INSTI resistance-associated mutations (RAMs) (e.g., V75I). The NC mutations were identified as RAMs based on Hikichi et al. (2024, 2025).

A. Fixed dose of DTG in PBMC



B. Viral DNA levels in infected PBMC



C. Sequencing analysis of NC/IN-coding region (20 weeks post-DTG treatment)

	Culture #	NC	IN
WT	1	No mutations	D41N
	2	No mutations	D256N
	3–12	Under detection limit by qPCR	

	Culture #	NC	IN
WD-3	1	No mutations	**S153F**
	2	No mutations	**G163R**
	3	No mutations	I135T/**R263K**
	4	**D48N**	No mutations
	5	**D48N**	A38S/V165I/**S153Y**
	6	No mutations	V150I
	7	No mutations	M154I
	8–10	No mutations	
	11–12	Under detection limit by qPCR	

No virus detection by qPCR
No mutations in NC or IN
Non-INSTI resistance associated mutations

IN resistance-associated mutations
NC mutations
NC + IN resistance-associated mutations

WT vs WD-3
***P* = 0.0373**

(A) Long-term passaging of WT NL4-3 or WD-3 in PBMCs (12 cultures each) in the presence of 2 nM DTG

(B) Viral DNA levels in the infected PBMCs were measured by qPCR at the indicated time points by qPCR experiments Each dot indicates the viral DNA level in the individual PBMC culture

(C) Sequence analysis of NC/IN-coding regions at 20 weeks post-DTG treatment. The INSTI resistance-associated mutations are highlighted in bold if listed in the Stanford HIVDB. Fisher's exact test was performed to compare the frequency of IN/NC resistance-associated mutations at 20 weeks post-DTG treatment.

Conclusion

- **HIV-1 sequentially acquires mutations in Env, NC, and IN in the presence of DTG across different strains and subtypes.**
- **Env mutations accumulate in structurally and functionally conserved domains, such as the gp120–gp41 interface.**
- **Env mutations reduce INSTI susceptibility by enhancing infectivity through cell-free infection and/or cell-to-cell transmission.**
- **Env mutations also facilitate the subsequent acquisition of resistance mutations in NC and IN, leading to higher-level INSTI resistance.**

References

- Hikichi et al., *Science Advances*, 2024 and 2025