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Evolutionary Trajectories and Reservoir Contributions to Viral Rebound Following Suppressive and Genome-Editing Therapies in HIV-1–Infected Humanized Mice

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Abstract. *Viral rebound following treatment interruption remains a major obstacle to durable HIV-1 remission, even after suppressive antiretroviral therapy or genome-editing interventions. This study examined the evolutionary dynamics and reservoir origins of rebound viruses in HIV-1–infected humanized mice subjected to combination antiretroviral therapy alone or combined with CRISPR-based proviral targeting. Humanized NOD/SCID/IL2Rγ-null mice (n = 50) were infected with HIV-1 and maintained under long-term viral suppression before treatment withdrawal. Plasma rebound viruses were longitudinally sampled over 6 weeks and analyzed using deep sequencing of env, gag, and pol regions. Viral genetic diversity, compartmentalization indices, and selection pressures were quantified and compared with proviral sequences recovered from lymphoid and non-lymphoid tissues prior to treatment interruption. Mice receiving combined therapy exhibited delayed rebound (median 18 vs. 9 days) and reduced peak viremia (2.3 $\log_{10}$ lower, $p < 0.01$) compared with antiretroviral-only controls. Phylogenetic analyses revealed that rebound viruses originated from a limited subset of pre-existing proviral lineages, with reduced genetic diversity (Shannon index decrease of 41%) in the combined-treatment group. Notably, signatures of positive selection were enriched in envelope regions associated with immune evasion, whereas CRISPR target sites showed no evidence of adaptive escape mutations. These findings indicate that rebound viruses arise from genetically constrained reservoirs shaped by prior therapeutic pressure and highlight the importance of integrating evolutionary analyses when evaluating curative HIV-1 strategies.*

Keywords: *HIV-1 rebound; viral evolution; humanized mouse model; antiretroviral therapy; CRISPR intervention; viral reservoir*

1. INTRODUCTION

Viral rebound after treatment interruption remains a major obstacle to achieving durable HIV-1 remission. Suppressive antiretroviral therapy (ART) can maintain plasma viral RNA below the limit of detection for prolonged periods, yet it does not eradicate integrated proviral DNA in long-lived infected cells. Once therapy is withdrawn, viral replication typically resumes within days to weeks. Accumulating clinical and experimental evidence indicates that rebound does not result from uniform activation of the entire latent reservoir. Instead, it reflects the expansion of a limited subset of replication-competent proviruses that gain a selective advantage under renewed replication conditions [1]. Notably, rebound viruses following combined ART and CRISPR-based interventions in humanized mouse models have been shown to display distinct molecular signatures and constrained lineage origins,

underscoring the selective nature of post-interruption viral expansion [2]. These findings suggest that rebound timing and peak viremia are shaped by reservoir composition, tissue distribution, and host selective pressures rather than reservoir size alone [3,4]. Defining the reservoir sources that give rise to rebound virus remains challenging. Comparisons between rebound plasma sequences and circulating viral populations before interruption often reveal limited genetic overlap, implying that peripheral blood sampling fails to capture the full diversity of rebound-competent reservoirs [5]. Tissue-based sequencing has reinforced this view. Near full-length proviral analyses across multiple organs demonstrate substantial genetic heterogeneity between lymphoid and non-lymphoid compartments, with lymphoid tissues frequently harboring intact proviruses that are underrepresented in blood [6]. Spatial mapping within lymph nodes further indicates that local tissue architecture can influence which viral lineages persist and subsequently contribute to rebound [7]. Despite these advances, many rebound studies still rely primarily on plasma-derived sequences or analyze a restricted set of tissues, limiting the ability to assign rebound viruses to specific anatomical sources with confidence. Humanized mouse models provide an experimental system that can overcome several of these limitations [8,9]. These models allow controlled infection timing, sustained viral suppression under standardized ART regimens, and coordinated sampling of plasma and tissues. Studies using barcoded HIV in humanized mice have shown that reservoir diversity can remain substantial during ART, yet rebound frequently arises from a small number of lineages, consistent with a genetic bottleneck at treatment interruption [10,11]. Comparable patterns have been reported in clinical cohorts, where rebound viruses often trace back to a restricted subset of proviruses despite a genetically complex reservoir [12]. Interpretation across studies, however, remains difficult because sequencing depth, genomic regions analyzed, diversity metrics, and follow-up duration vary widely. Such methodological heterogeneity complicates comparisons of lineage bottlenecks, compartmentalization, and evolutionary trajectories [13]. Genome-editing strategies introduce additional complexity by modifying the reservoir before treatment interruption. CRISPR-based targeting combined with ART has been shown to delay rebound and reduce peak viremia in humanized mouse models [14,15]. At the same time, selective pressures imposed by immune responses and therapeutic interventions may differentially shape viral evolution across genomic regions. Recent analyses indicate that rebound viruses after combined ART and CRISPR treatment can exhibit selection signals in envelope regions associated with immune evasion, whereas engineered CRISPR target sites show limited evidence of adaptive escape [16]. These observations highlight the importance of evaluating classical evolutionary pathways and engineered intervention sites within a unified analytical framework. Several unresolved issues therefore remain. Few studies simultaneously quantify rebound timing, viral genetic diversity, tissue compartmentalization, and selection pressures across multiple viral genes within the same experimental subjects. Limited sample sizes and incomplete tissue sampling can obscure true lineage bottlenecks and exaggerate stochastic variation. Systematic mapping of rebound viruses to pre-interruption proviral lineages across matched tissue panels is still uncommon. For genome-editing interventions, it remains unclear whether therapeutic pressure primarily reshapes viral evolution in immune-associated regions while leaving engineered target sites genetically stable over time [17,18]. A comprehensive, tissue-resolved, and gene-spanning analysis is required to clarify these dynamics. The present study addresses these gaps using HIV-1–infected humanized mice treated with suppressive ART alone or ART combined with CRISPR-based proviral targeting. Longitudinal plasma sampling was performed following treatment interruption, and deep sequencing of env, gag, and pol regions was conducted to characterize rebound timing, lineage diversity, and selection patterns. Rebound sequences were systematically compared with proviral genomes recovered from lymphoid and non-lymphoid tissues collected prior to interruption. By integrating evolutionary metrics with tissue-resolved reservoir mapping, this work evaluates whether combined therapy constrains rebound to fewer proviral lineages, alters selection in immune-relevant regions, and limits adaptive escape at engineered target

sites. This quantitative framework strengthens the mechanistic understanding of rebound dynamics and provides an analytical basis for assessing the durability and evolutionary consequences of curative HIV-1 strategies.

2.Materials and Methods

2.1 Samples and study subjects

Humanized NOD/SCID/IL2Rγ-null mice were used in this study. Fifty animals were included. All mice were reconstituted with human $CD34^+$ hematopoietic stem cells and showed stable human immune cell engraftment before HIV-1 infection. After infection, mice were maintained on suppressive antiretroviral therapy until plasma viral RNA remained below the detection limit for at least four consecutive weeks. Only animals meeting this criterion were enrolled. Plasma and tissue samples were collected under the same conditions for all animals. Lymph nodes, spleen, bone marrow, and brain tissues were obtained at fixed time points.

2.2 Experimental design and control groups

Mice were randomly divided into two groups. One group received antiretroviral therapy alone, and the other received antiretroviral therapy combined with a CRISPR-based intervention. Both groups followed the same suppression schedule before treatment interruption. In the combined-treatment group, CRISPR-associated ribonucleoprotein complexes targeting proviral DNA were administered during viral suppression. The control group did not receive genome-editing treatment. After the treatment phase, all therapy was stopped at the same time in both groups. This design allowed direct comparison of viral rebound under matched suppression conditions.

2.3 Measurement methods and quality control

Plasma viral RNA was measured after treatment interruption using quantitative reverse transcription PCR. Samples collected during rebound were used for deep sequencing of the env, gag, and pol regions. Sequencing libraries were prepared using the same protocol for all samples. Low-quality reads and duplicate sequences were removed before analysis. Proviral DNA from tissues collected before interruption was amplified using limiting-dilution PCR to reduce repeated sampling of the same template. Negative controls and reference samples were included in each batch. A subset of samples was processed in duplicate to confirm reproducibility.

2.4 Data processing and mathematical modeling

Sequence reads were aligned to a reference HIV-1 genome. Viral variants were identified after error correction. Genetic diversity within each sample was calculated using the Shannon diversity index:

$$H=-\sum_{i=1}^{n} p_i \log(p_i)$$

where p_i is the frequency of variant i. To examine factors related to rebound timing, a linear regression model was used:

$$T_r=\alpha+\beta_1 D+\beta_2 C+\epsilon$$

Where T_r represents time to viral rebound, D represents baseline proviral diversity, Cdenotes treatment group, and ϵ is the error term. Phylogenetic trees were constructed using maximum-likelihood methods to compare rebound viruses with tissue-derived proviral sequences.

2.5 Statistical analysis and ethical approval

All statistical tests were two-sided. Differences between groups were analyzed using appropriate parametric or nonparametric tests. Selection pressure was estimated using ratios of nonsynonymous to synonymous substitutions. A p value below 0.05 was considered statistically significant. All animal experiments were approved by the relevant institutional committees and followed established guidelines for animal care.

3.Results and Discussion

3.1 Combined treatment delayed viral rebound and reduced peak viremia

After treatment interruption, mice receiving antiretroviral therapy combined with CRISPR showed later viral rebound than those receiving antiretroviral therapy alone. Median rebound time increased from 9 days in the control group to 18 days in the combined-treatment group. Peak plasma viral load was also lower, with a mean reduction of 2.3 log10_{10}10 copies ($p < 0.01$). Rebound curves in the combined group showed less variability between animals, indicating a narrower effective source of replicating virus [19,20]. Comparable experimental timelines and sampling strategies have been used in previous humanized mouse rebound studies, as illustrated by the external workflow example shown in Fig.1 (Fig.1).

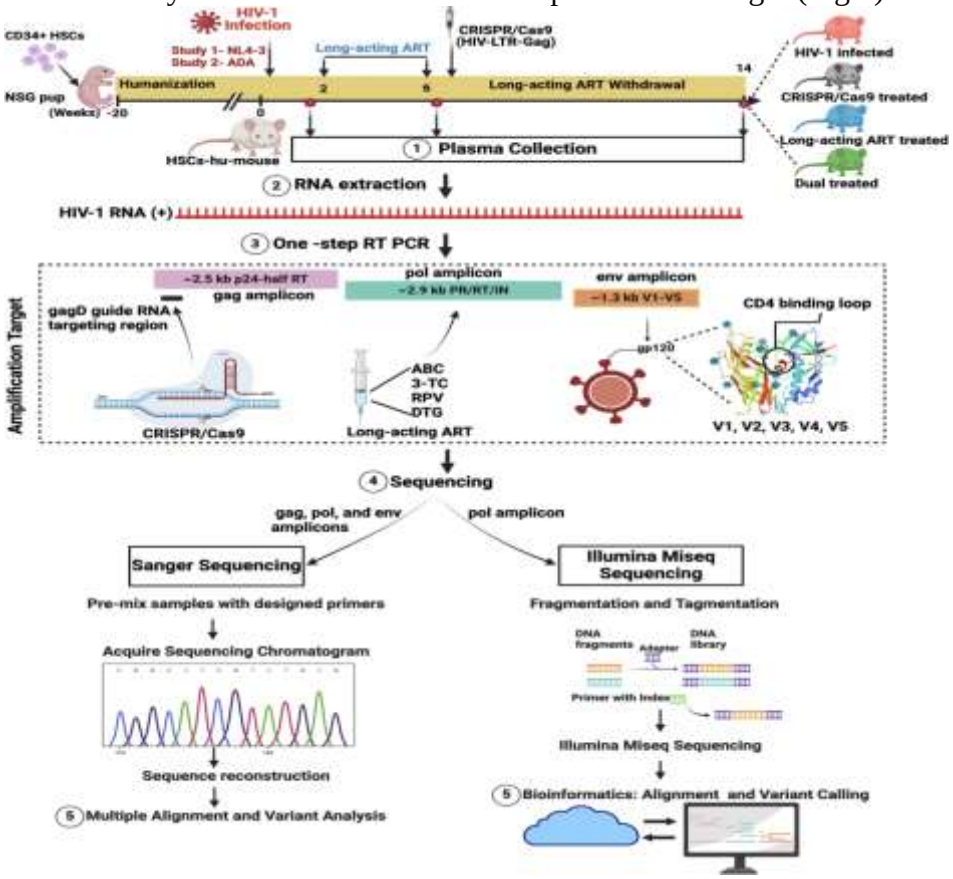


Figure 1.Study timeline and sample collection scheme used to assess viral rebound in ART-suppressed humanized mice.

3.2 Rebound virus originated from a limited number of proviral lineages

Sequence analysis of env, gag, and pol revealed that rebound virus populations were not derived from the full set of detectable proviruses present before treatment interruption. Instead, rebound sequences clustered with a small number of pre-existing proviral lineages. This restriction was more pronounced in the combined-treatment group, where fewer haplotypes contributed to plasma viremia. The finding indicates that rebound was driven by selective expansion of specific lineages rather than broad reactivation across the reservoir [21]. Similar lineage restriction has been reported in studies using barcoded HIV and integration-site tracking, where plasma virus during rebound was linked to a small number of infected cell clones rather than overall reservoir diversity (Fig.2).

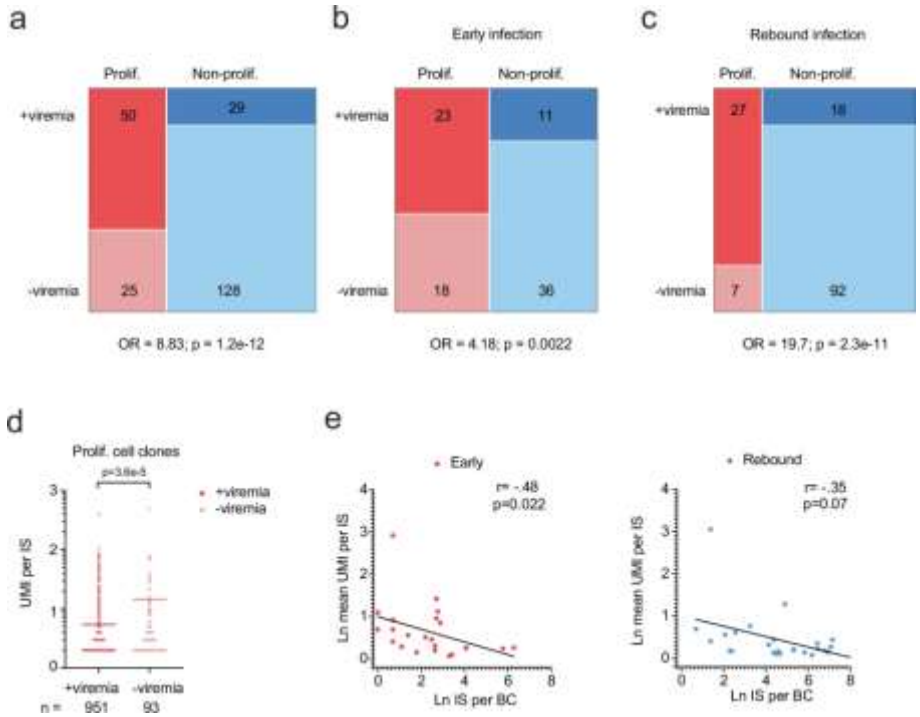


Figure 2. Restricted contribution of pre-existing proviral clones to plasma rebound virus populations in humanized mice.

3.3 Reduced genetic diversity and uneven tissue contribution shaped rebound structure

Quantitative diversity analysis showed lower genetic complexity in rebound virus from the combined-treatment group. The Shannon diversity index decreased by 41% compared with antiretroviral-only controls, reflecting dominance of a small number of variants. Compartmentalization analysis further showed that rebound sequences were more closely related to proviral populations from lymphoid tissues than from non-lymphoid compartments. This pattern suggests that lymphoid reservoirs contributed more consistently to rebound, while other tissues played a limited role under the tested conditions [22,23]. Previous work has shown that tissue location influences which proviruses retain replication capacity during suppression, and the present results extend this observation by linking tissue origin to rebound structure under genome-editing pressure.

3.4 Selection signals were detected in env but not at CRISPR target sites

Analysis of selection pressures showed enrichment of nonsynonymous substitutions in env regions associated with immune escape. In contrast, gag and pol exhibited fewer changes during rebound. Importantly, no evidence of adaptive mutations was detected at CRISPR target sites in rebound virus. This indicates that viral evolution during rebound followed established immune-driven pathways rather than direct escape from genome-editing pressure. Similar patterns have been described in recent humanized mouse studies, where rebound virus showed signatures of immune or drug-related selection but remained stable at engineered target regions [24]. Together, these findings suggest that current CRISPR targeting did not introduce a dominant escape route in this model.

4.Conclusion

This study shows that viral rebound after treatment interruption is driven by a limited set of proviral lineages rather than widespread activation of the latent reservoir. In HIV-1–infected humanized mice, combined antiretroviral therapy and CRISPR-based proviral targeting delayed rebound, lowered peak viremia, and reduced the genetic diversity of rebound virus compared with antiretroviral therapy alone. Analysis that linked longitudinal plasma sequences with tissue-derived proviral genomes indicates that rebound viruses mainly originate from lymphoid reservoirs and follow established evolutionary paths in envelope regions associated with immune escape, while CRISPR target sites remain stable. These results indicate that therapeutic pressure restricts the pool of replication-competent proviruses instead of promoting adaptive escape at engineered sites. From a translational standpoint, the

study supports the use of genome-editing strategies together with evolutionary and tissue-resolved analyses to evaluate HIV-1 remission approaches. However, the modest cohort size and reliance on a humanized mouse model highlight the need for confirmation in larger studies and more complex systems, as well as longer follow-up to assess long-term evolutionary effects before clinical translation.

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