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GENETIC DIVERSITY AND EVOLUTION

HIV RNA Rebound in Seminal Plasma after Antiretroviral Treatment Interruption

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ABSTRACT If strategies currently in development succeed in eradicating HIV reservoirs in peripheral blood and lymphoid tissues, residual sources of virus may remain in anatomic compartments. Paired blood and semen samples were collected from 12 individuals enrolled in a randomized, double-blind, placebo-controlled therapeutic vaccine clinical trial in people with HIV (PWH) who began antiretroviral therapy (ART) during acute or early infection (ClinicalTrials registration no. NCT01859325). After the week 56 visit (postintervention), all participants interrupted ART. At the first available time points after viral rebound, we sequenced HIV-1 *env* (C2-V3), *gag* (p24), and *pol* (reverse transcriptase) regions amplified from cell-free HIV RNA in blood and seminal plasma using the MiSeq Illumina platform. Comprehensive sequence and phylogenetic analyses were performed to evaluate viral population structure, compartmentalization, and viral diversity in blood and seminal plasma. Compared to that in blood, HIV RNA rebound in semen occurred significantly later (median of 66 versus 42 days post-ART interruption, $P < 0.01$) and reached lower levels (median 164 versus 16,090 copies/ml, $P < 0.01$). Three of five participants with available sequencing data presented compartmentalized viral rebound between blood and semen in one HIV coding region. Despite early ART initiation, HIV RNA molecular diversity was higher in semen than in blood in all three coding regions for most participants. Higher HIV RNA molecular diversity in the genital tract (compared to that in blood plasma) and evidence of compartmentalization illustrate the distinct evolutionary dynamics between these two compartments after ART interruption. Future research should evaluate whether the genital compartment might contribute to viral rebound in some PWH interrupting ART.

IMPORTANCE To cure HIV, we likely need to target the reservoirs in all anatomic compartments. Here, we used sophisticated statistical and phylogenetic methods to analyze blood and semen samples collected from 12 persons with HIV who began antiretroviral therapy (ART) during very early HIV infection and who interrupted their ART as part of a clinical trial. First, we found that HIV RNA rebound in semen occurred significantly later and reached lower levels than in blood. Second, we found that the virus in semen was genetically different in some participants compared to that in blood. Finally, we found increased HIV RNA molecular diversity in semen compared to that in blood in almost all study participants. These data suggest that the HIV RNA populations emerging from the genital compartment after ART interruption might not be the same as those emerging from blood plasma. Future research should evaluate whether the genital compartment might contribute to viral rebound in some people with HIV (PWH) interrupting ART.

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Antiretroviral therapy (ART) can suppress viral replication to undetectable levels in most people with HIV (1). However, ART cannot eradicate latently infected cells (2), and HIV RNA rebound occurs following treatment interruption (3), even when ART is initiated during the earliest phase of HIV infection in an attempt to limit the size of the HIV reservoir and improve immune reconstitution (4, 5). Rebounding virus might originate from a variety of sources, including the DNA compartment in peripheral blood mononuclear cells (PBMC), RNA or DNA compartments in lymphoid tissues, or possibly other anatomical compartments that harbor replication-competent HIV-infected cells or HIV particles (6–9). In most people with HIV (PWH), the establishment of viral reservoirs in multiple tissues and anatomic compartments occurs within the first few weeks of HIV infection, and this likely includes the genital tract (10, 11). The HIV population during the early stages of infection is typically homogenous (12), but compartmentalization can occur as a consequence of tissue-specific genetic differentiation and restricted viral migration between anatomic sites (13–17). Compartmentalized viral evolution is frequently a consequence of discordant selective pressures and gives rise to tissue-adapted variants, which subsequently contribute to disease pathogenesis and viral transmission (18). Phylogenetic methods needed to quantify this restriction of gene flow between compartments are numerous and well developed (13). Our group and others have used similar methods to describe the presence of compartmentalized HIV RNA populations in the cerebrospinal fluid (CSF) supernatant, mostly using samples from ART-naive individuals (19–21) and from chronically infected individuals following ART interruption (22).

While direct assessment of viral variants sampled from the genital tissue (e.g., testis and prostate) in living individuals is rarely feasible, HIV RNA collected from the seminal plasma can be an informative surrogate (23).

In this study, we leveraged a unique cohort of 12 participants enrolled in a randomized, double-blind, placebo-controlled clinical trial of HIV-MAG DNA vaccine prime and rVSVN4CT1gag booster vaccine in PWH who began ART during acute or early infection (ClinicalTrials registration no. NCT01859325) (24). Comprehensive sequence and phylogenetic analyses were performed to characterize the HIV RNA population rebounding in semen after interruption of ART compared to that in blood. This is important, since compartmentalized HIV reservoirs within the genital tract may be particularly difficult to target with viral eradication strategies, due to limited drug penetration, compartmentalization, tissue-specific viral adaptation, and the presence of unique cellular targets.

RESULTS

Participants, samples, and clinical laboratory tests. Participants in the genital substudy ($N = 12$) were all cisgender men with HIV, with a median age of 42 years (range, 24 to 54 years), who had started therapy within a median of 25 days after the estimated date of infection and had been receiving ART for a median of 3.5 years (interquartile range [IQR], 2 to 13 years). Characteristics of the study participants are summarized in Table 1. Four study participants were in the placebo arm (ID1 to -4), while the other 8 were in the active vaccine arm (ID5 to -12).

HIV RNA dynamics in blood and semen. Among the 12 participants included in the genital substudy, the median time from ART interruption to viral rebound (defined as first detectable viral load) in blood plasma was 42 days (range, 12 to 83 days) with a median peak HIV RNA load of 16,090 copies/ml (range, 160 to >50,000 copies/ml). The median time from ART interruption to viral rebound in semen was 66 days (range, 16 to 134 days), with a median peak viral load of 164 copies/ml (range, 70 to 3,500 copies/ml). Two participants (ID3 and ID9) experienced small blips in semen before viral rebound in blood. Interestingly, most study participants did not sustain HIV RNA rebound in semen (see Table 2).

TABLE 1 Participants' characteristics

Characteristics	Value ($N = 12$)
Age (yrs) (median [range])	42 (24–54)
Gender: cisgender male (n [%])	12 (100)
Ethnicity: white (n [%])	6 (50)
Viral clade B (n [%])	9 (75)
Time from EDI to ART (days) (median [range])	25 (3–73)
ART exposure (yrs) (median [range])	3.5 (2–13)
$CD4^+$ T cell counts/μl (at study entry) (median [range])	470 (300–790)
$CD4^+$ T cell counts/μl (at the time of ART interruption) (median [range])	510 (390–1430)

HIV RNA population characteristics in blood and semen. To characterize the cell-free HIV RNA population, we successfully sequenced partial *env*, *gag*, and/or *pol* from seminal and blood plasma in 5 participants (ID3 and ID4 in the placebo group and ID5, ID10, and ID11 in the vaccine group). For two participants, we also obtained sequences from one additional longitudinal time point (ID4 and ID5). In blood, we obtained an average of 156,672 reads (range, 69,870 to 798,639) and 9 haplotypes (range, 4 to 20) per sample. In semen, we obtained an average of 252,565 reads (range, 91,028 to 764,568) and 7 haplotypes (range, 1 to 26). Detailed characteristics of the number of reads/haplotypes for each sample are provided in Table 3.

Although not statistically significant, HIV RNA in semen had a higher entropy than the HIV RNA population in blood (*env*, $P = 0.25$; *gag*, $P = 0.07$; *pol*, $P = 0.08$). Specifically, the mean and 0.95 confidence intervals (CI95%) of entropy in seminal plasma were 0.0505 ± 0.0253 (*env*), 0.0335 ± 0.0155 (*gag*), and 0.0515 ± 0.0215 (*pol*) compared to 0.0396 ± 0.0304 (*env*), 0.0231 ± 0.0061 (*gag*), and 0.0375 ± 0.0078 (*pol*) in blood (Fig. 1 and Table 4).

The mean diversity was not significantly different between the first and the second sampled time points (when available). When looking at individual participants, all but one participant (ID11) with available sequence data presented higher HIV RNA diversity in semen in at least one coding region (Table 5).

Phylogenies and population structure of the rebounding HIV RNA populations. All sequences clustered by study participant where combined in a single phylogeny, which shows that there was no interparticipant contamination (Fig. 2).

Using a conservative combination of tree-based (Simmonds association index and Slatkin-Maddison tests) and distance-based (fixation index [F_{ST}] test) analyses and after adjusting for the haplotype frequencies, we found evidence of significant viral compartmentalization in at least two of the three measures for 3 of 5 participants in one coding region (ID4 *pol*, ID5 *gag*, and ID10 *pol*, $P < 0.05$) (Fig. 3). Phylogenetic trees illustrating different HIV RNA populations in blood and semen are shown in Fig. 4. Tree topologies showed intermingled sequences from blood and seminal plasma, confirming the lack of viral compartmentalization for most participants and regions. Phylogenetic trees for *pol* D4 and *pol* D10 confirm the presence of a monophyletic clade originating from semen, but a definitive evaluation is limited due to the short length of the sequences and the overall limited diversity in our data set.

DISCUSSION

A successful HIV eradication strategy must target all known mechanisms of viral persistence (25). One such mechanism is the establishment of genetically distinct proviral populations in different tissues and anatomical compartments (14), including the genital tract and the central nervous system. In this study, we prospectively collected serial semen samples during structured ART interruption from a unique cohort of 12 PWH enrolled in a randomized, double-blind, placebo-controlled clinical trial of a therapeutic HIV vaccine (24). All study participants began ART during acute or early HIV infection and with sustained viral suppression thereafter (24). The results of the parent trial were previously reported (24): compared to placebo, the vaccination had no significant effect on the kinetics or magnitude of viral rebound after interrup-

TABLE 2 Viral loads in blood and semen after interruption of antiretroviral therapy

Participant ID	Intervention	No. of wks	HIV RNA (copies/ml)[a]		On ART
			Semen HIV RNA	Blood HIV RNA	
ID1	Placebo	0	NA	<40	Yes
		2	<40	<40	No
		5	<40	<20	No
		6	<40	2,532	No
		7	309	15,955	No
		10	<40	14,385	No
		12	<40	6,212	No
		14	<40	5,795	No
ID2	Placebo	0	<40	<40	Yes
		2	<40	<40	No
		4	<40	<40	No
		12	832	103,410	No
ID3	Placebo	0	<40	<40	Yes
		2	<40	<40	No
		4	88	<40	No
		6	<40	393	No
		9	**194**	**3,677**	No
		10	238	91	No
		12	<40	68	No
		14	<40	<40	No
		18	<40	<40	No
ID4	Placebo	0	<40	<40	Yes
		2	<40	<40	No
		4	<40	2,230	No
		6	**234**	**173,502**	No
		10	**125**	**503**	No
		12	<40	460	No
ID5	Vaccine	0	<40	<40	Yes
		3	<40	<20	No
		4	<40	1,527	No
		5	**794**	**83,710**	No
		6	**3,475**	**5 145,797**	No
		10	<40	264	Yes
		14	<40	<40	Yes
ID6	Vaccine	0	<40	<40	Yes
		2	<40	144	No
		4	<40	5,721	No
		9	63	2,598	Yes
		10	68	361	Yes
		14	<40	<40	Yes
		17	<40	<40	Yes
ID7	Vaccine	0	<40	<40	Yes
		2	<40	<40	No
		4	<40	<40	No
		6	<40	<40	No
		9	<40	80	No
		11	<40	287	No
		12	<40	2,391	No
		14	<40	21,997	No
		18	<40	10,450	Yes
ID8	Vaccine	0	<40	<40	Yes
		2	<40	<40	No
		4	<40	<40	No
		9	<40	78	No
		10	<40	112	No
		14	<40	158	No
		15	92	71	No
		18	<40	<40	Yes
		21	<40	<40	Yes
ID9	Vaccine	0	<40	<40	Yes
		2	134	<40	No
		4	<40	1,470	No
		5	<40	310	No
		10	<40	154	No
		11	<40	654	No
		14	<40	1,530	No
		21	<40	105	No
		24	<40	101	No
ID10	Vaccine	0	<40	<40	Yes
		3	<40	<40	No

(Continued on next page)

TABLE 2 (Continued)

Participant ID	Intervention	No. of wks	HIV RNA (copies/ml)[a]		On ART
			Semen HIV RNA	Blood HIV RNA	
		4	<40	<40	No
		6	<40	3,283	No
		11	<40	2,856	No
		13	**94**	**2,358**	No
		15	<40	2,938	No
		17	<40	2,392	No
		22	<40	<40	Yes
ID11	Vaccine	0	<40	<40	Yes
		7	<40	<40	No
		9	<40	548,703	No
		11	52	806	No
		14	<40	488	No
		15	<40	389	No
		17	<40	9,713	No
		19	**120**	**2,932**	No
		27	<40	222	Yes
ID12	Vaccine	0	<40	<40	Yes
		2	<40	1,620	No
		5	<40	16,224	No

[a]Boldface font indicates time points with available sequencing data. NA, not available; ART, antiretroviral therapy; No. of wks, number of weeks postinterruption of ART.

tion of ART and no impact on the size of the HIV reservoir in the $CD4^+$ T cell compartment. Notably, almost one-third of participants in the placebo arm exhibited sustained suppression of viremia (<400 copies/ml) after treatment interruption, which was consistent with that recently reported in a retrospective analysis of transient viral control in a subgroup of participants in the Short Pulse AntiRetroviral Therapy at Acute Seroconversion (SPARTAC) trial (26). Consistently, the magnitude of HIV RNA rebound in seminal plasma was small, and none of the participants presented a sustained

TABLE 3 Number of reads and haplotypes for each sample from 5 study participants with available sequencing data

Participant ID	Intervention	Compartment	Region	No. of reads[a]		No. of haplotypes[a]	
				TP1	TP2	TP1	TP2
ID3	Placebo	Blood	ENV	265,903		9	
			GAG	475,530		4	
			POL	197,201		28	
		Semen	ENV	112,448		2	
			GAG	NA		NA	
			POL	764,568		1	
ID4	Placebo	Blood	ENV	68,840	84,619	11	4
			GAG	263,382	316,046	6	3
			POL	92,861	103,091	9	7
		Semen	ENV	84,847	37,308	6	5
			GAG	398,760	245,348	2	5
			POL	75,182	43,460	23	26
ID5	Vaccine	Blood	ENV	146,747	181,407	6	4
			GAG	427,147	371,492	3	3
			POL	NA	NA	NA	NA
		Semen	ENV	31,103	73,285	8	7
			GAG	270,242	362,771	3	4
			POL	NA	NA	NA	NA
ID10	Vaccine	Blood	ENV	114,756		6	
			GAG	410,215		9	
			POL	141,114		10	
		Semen	ENV	NA		NA	
			GAG	105,728		3	
			POL	566,952		15	
ID11	Vaccine	Blood	ENV	121,687		9	
			GAG	344,835		6	
			POL	69,870		6	
		Semen	ENV	NA		NA	
		Semen	GAG	91,028		4	
		Semen	POL	NA		NA	

[a]NA, not available (coverage too low); TP, time point.

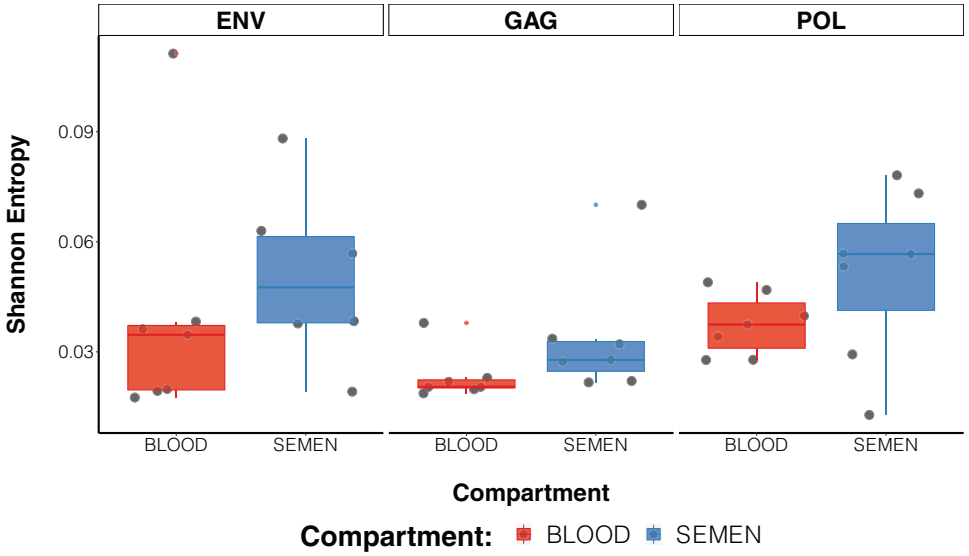

FIG 1 Molecular diversity measures in blood and semen. Viral diversity was assessed by measuring the Shannon entropy index from all cleaned reads mapped to HIV partial *gag*, *pol*, and *env* regions. Comparison between entropy measures across participants was performed using a pairwise Wilcoxon adjusted test using the conservative Bonferroni correction method implemented in R package stats.

rebound. Overall, the median time from ART interruption to viral rebound in semen was significantly longer than in blood (66 versus 42 days, respectively), although two participants experienced small blips in semen before viral rebound in blood (see Table 2).

Furthermore, in our study of PWH who started ART during the earliest phase of infection (median of 24 days after the estimated date of infection), we found that 3 of 5 participants with available sequencing data presented a compartmentalized HIV RNA rebound within the genital compartment after ART interruption, even when sampled a few days/week after viral rebound (Fig. 3). This pattern implies that at least some of the rebounding virus within the seminal plasma might originate from a genital source and might represent a barrier to sterilizing cure. This is in line with a recent publication from Ganor et al. (9), which demonstrated using penile tissues from PWH under suppressive combination ART, that urethral macrophages contain integrated HIV-1 DNA, RNA, proteins, and intact virions in virus-containing compartment-like structures, whereas viral components remain undetectable in urethral T cells. Urethral HIV-1 reservoirs might be established during early sexual transmission, resulting in rapid infection of resident macrophages, which subsequently become latent and form inducible reservoirs that can be reactivated with lipopolysaccharide (LPS), as recently reported (27).

Importantly, the phylogenies for different viral genes sampled from the same individuals did not show identical dynamics. This observation could result from an independent rebound in the genital tract, for which the archived viruses in blood and semen had homogenized *env* but different *rt* genes, e.g., due to a past recombination event. Previous studies have suggested complex intrahost recombination dynamics (28, 29) and demonstrated how selection can affect HIV sequences in different compartments (30, 31), indicating that such eventualities may be common. These findings are comparable to a previous report of rebound in CSF, where unique rebounding species were found (22).

A recent French study of a therapeutic HIV vaccine found a robust early rebound in semen following ART interruption (32), with no evidence of genetic compartmentalization in 10 participants focusing on partial C2/V3 *env* (454 Roche sequencing platform). While the *env* gene has the greatest amount of molecular diversity and evolution of all coding regions (33–36), here we sequenced 3 partial coding regions (*env*, *gag*, and *pol*) and found evidence of significant viral compartmentalization in *pol* ($n = 2$) and *gag*

TABLE 4 Characteristics of each sampled compartment (Shannon entropy) from 5 study participants with available sequencing data

Participant ID	Intervention	Time Point	Compartment	Region	Entropy		
					Mean (IQR)	Maximum	Median
ID3	Placebo	TP1	Blood	ENV	0.0194 (0–0.0050)	0.5623	0
				GAG	0.0205 (0–0.0238)	0.1220	0.0207
				POL	0.0278 (0–0.0397)	0.4276	0.0189
			Semen	ENV	0.0882 (0–0.0448)	1.0968	0
				GAG	NA	NA	NA
				POL	0.0128 (0–0.0194)	0.4362	0
ID4	Placebo	TP1	Blood	ENV	0.0382 (0–0.0216)	0.7181	0
				GAG	0.0198 (0–0.0252)	0.2060	0.0211
				POL	0.0469 (0–0.0457)	0.5586	0.0213
			Semen	ENV	0.0384 (0–0.0298)	0.8426	0
				GAG	0.0273 (0–0.0390)	0.1899	0.0215
				POL	0.0732 (0–0.0584)	0.7959	0.0251
		TP2	Blood	ENV	0.0361 (0–0.0247)	0.6365	0
				GAG	0.0187 (0–0.0250)	0.0922	0.0213
				POL	0.0374 (0–0.0436)	0.4714	0.0200
			Semen	ENV	0.0568 (0–0.0441)	0.9433	0
				GAG	0.0320 (0–0.0449)	0.2293	0.0223
				POL	0.0781 (0–0.0532)	1.1047	0.0238
ID5	Vaccine	TP1	Blood	ENV	0.0347 (0–0.0255)	0.7298	0
				GAG	0.0229 (0–0.0383)	0.1246	0.0213
				POL	NA	NA	NA
			Semen	ENV	0.0630 (0–0.0436)	1.0397	0
				GAG	0.0278 (0–0.0412)	0.1713	0.0206
				POL	NA	NA	NA
		TP2	Blood	ENV	0.0175 (0–0.0203)	0.7851	0
				GAG	0.0205 (0–0.0341)	0.1068	0.0208
				POL	NA	NA	NA
			Semen	ENV	0.0377 (0–0.0256)	0.6365	0
				GAG	0.0336 (0–0.0528)	0.2255	0.0235
				POL	NA	NA	NA
ID10	Vaccine	TP1	Blood	ENV	0.0199 (0–0.0234)	0.3368	0
				GAG	0.0379 (0–0.0414)	0.5690	0.0210
				POL	0.0398 (0–0.0444)	0.4888	0.0205
			Semen	ENV	NA	NA	NA
				POL	0.0191 (0–0.0237)	0.2021	0
				GAG	0.0293 (0–0.0196)	0.5462	0
ID11	Vaccine	TP1	Blood	ENV	0.1113 (0–0.0460)	1.1438	0
				GAG	0.0218 (0–0.0375)	0.2331	0.0211
				POL	0.0490 (0–0.0553)	0.4313	0.0234
			Semen	ENV	NA	NA	NA
				GAG	0.0217 (0–0.0386)	0.0965	0.0212
				POL	NA	NA	NA

($n = 1$). Therefore, focusing on one partial coding region (*env*) may have incorrectly inferred that some viral populations were not compartmentalized, when they may be compartmentalized when exploring other genome regions. We cannot also exclude that these differences are due to differences in the study population (chronic versus acutely treated individuals), rebound dynamics, the type of vaccine administered, and/or the sequencing technology (454 versus Illumina MiSeq). Sequencing the near-full-length proviral genome (8, 37–39) would increase the sensitivity of these analyses, but these approaches require a high template input, which may limit the ability to explore reservoirs such as the genital tract.

Interestingly, we found higher genetic diversity in semen (as measured by Shannon entropy) in all coding regions compared to that in blood. It is possible that a considerable amount of divergence happened before viral suppression, but this is unlikely in our cohort of people who started ART very early during infection. As an alternative, there might be some degree of viral evolution in the genital tract during ART which requires further confirmation.

This study has several limitations. First, the study only included 12 individuals, and from these, we were only able to obtain semen sequencing data for 5, thereby limiting

TABLE 5 Wilcoxon pairwise comparisons of Shannon entropy between compartments[a]

Participant ID	Intervention	Time point	Region	Sample 1	Sample 2	Adjusted *P* value
ID3	Placebo	TP1	ENV	Semen	Blood	<0.01
			POL	Semen	Blood	<0.01
ID4	Placebo	TP1	ENV	Semen	Blood	<0.01
			GAG	Semen	Blood	0.016
			POL	Semen	Blood	0.067
		TP2	ENV	Semen	Blood	<0.01
			GAG	Semen	Blood	<0.01
			POL	Semen	Blood	0.02
ID5	Vaccine	TP1	ENV	Semen	Blood	0.044
			GAG	Semen	Blood	0.621
		TP2	ENV	Semen	Blood	<0.01
			GAG	Semen	Blood	<0.01
ID10	Vaccine	TP1	GAG	Semen	Blood	
			POL	Semen	Blood	<0.01
ID11	Vaccine	TP1	GAG	Semen	Blood	0.632

[a]Comparison between entropy measures across compartments was performed using a pairwise Wilcoxon adjusted test using the conservative Bonferroni correction method implemented in R package stats.

the power of subsequent statistical analysis. However, despite this small sample size, our study delivers a detailed characterization of semen virus in this unique prospective cohort with intensive clinical, laboratory, and informatics analyses. Second, all our participants started ART early in infection, allowing little baseline genetic diversity to compare rebounding virus populations. Furthermore, the sampling times for blood and semen were days to weeks after rebound, meaning virus replication could account for several nucleotides of difference between the initial rebounding virus and the sampled virus. On the same line, the short length of our amplicons also limited the total diversity among each rebounding virus population within a participant. Thus, theoretically, sequencing error and virus evolution or random change from rebound to sampling could account for all the diversity seen within each gene sequence comparison.

Also, low template input into the sequencing reaction and sequencing errors could cause biases or skewing in the sampling of the original viral population and negatively impact our ability to perform accurate analyses on these samples.

Template input was particularly low in some (but not all) seminal plasma samples, which likely explains the high rate of sequencing failures and negatively impacts our capacity to find unique clades within the seminal compartment. Other possible causes of sequencing failures might be primer mismatches and possibly PCR inhibition in semen.

Despite these limitations, our study suggests that the HIV RNA population sometimes rebounds independently within the genital tract and that the HIV DNA reservoirs in anatomic compartments might present additional obstacles to eradication and need to be actively targeted to achieve a complete cure. Our data suggest that failing to collect appropriate specimens (such as semen or cerebrospinal fluid) during structured ART interruption in the setting of cure trials might overlook some important events emanating from the anatomic compartments, which could be contributing to overall rebound.

MATERIALS AND METHODS

Ethics statement. The parent therapeutic vaccine clinical protocol (ClinicalTrials.gov identifier NCT01859325) was approved by the Institutional Review Board of the National Institutes of Allergy and Infectious Diseases (NIAID) and previously published (24). Semen samples for the genital tract substudy were collected under a separate protocol approved by the Human Research Protections Program at the University of Toronto. All adult subjects provided written informed consent.

Participants, samples, and clinical laboratory tests. Between September 2013 and February 2015, a total of 31 PWH enrolled in a randomized, double-blind, placebo-controlled clinical trial of HIV-MAG DNA vaccine prime and rVSVN4CT1gag booster vaccine (NCT01859325) (24). After the week 56 visit postintervention, all participants underwent treatment interruption to determine if vaccine administration resulted in an improved immune control of viral replication. Study participants were subsequently followed through week 96 postintervention for safety and efficacy parameters.

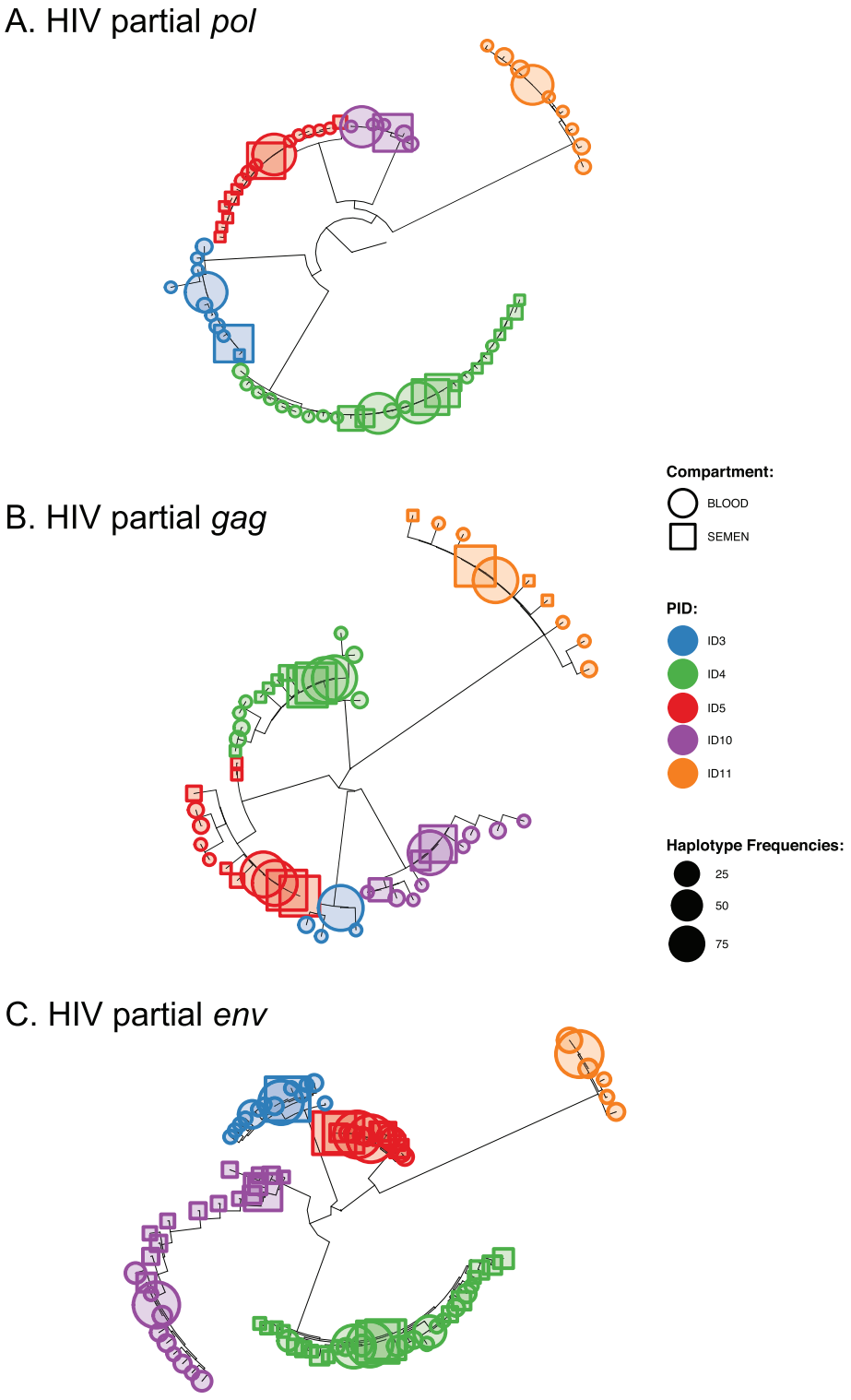

FIG 2 (A to C) Approximate maximum likelihood phylogeny, including all data, from 5 study participants with available sequencing data. All sequences clustered by study participant, which confirm the lack of interparticipant contamination. Participants ID3 and ID4 were assigned to the placebo group, while ID5, ID10, and ID11 were assigned to the vaccine active group.

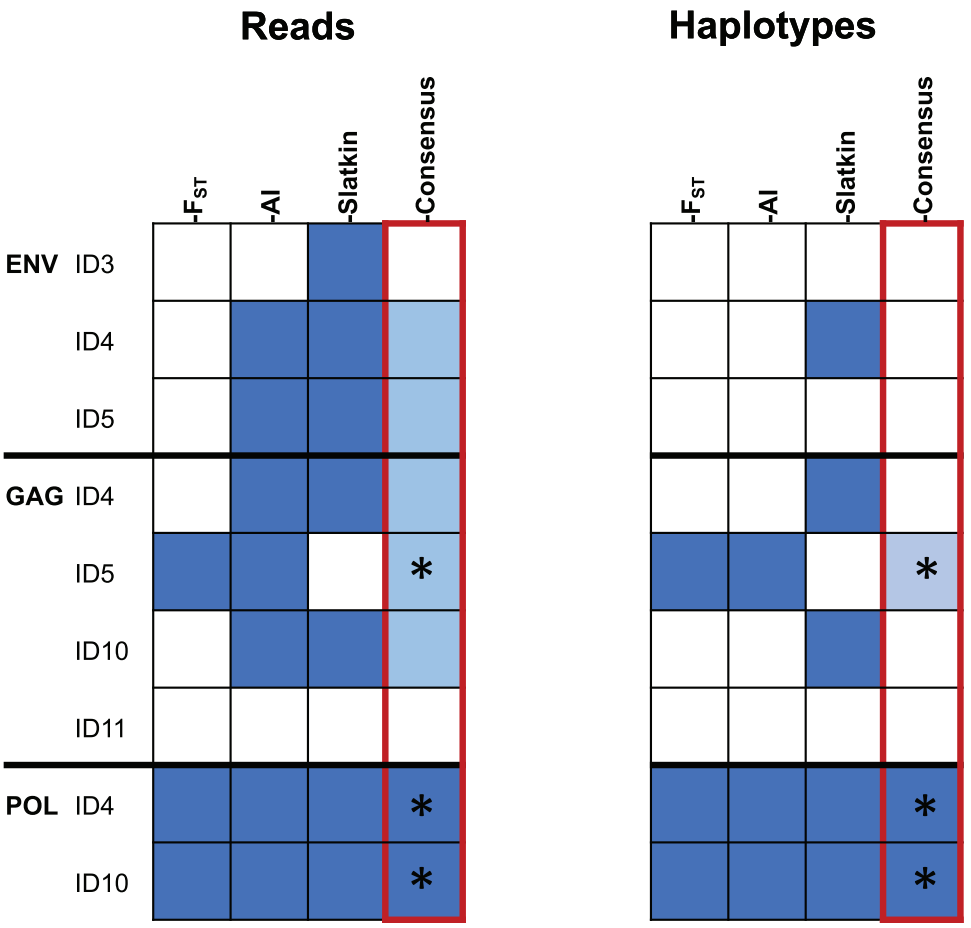

FIG 3 Genetic compartmentalization results for each participant and each coding region. Compartmentalization was assessed using one distance-based and two tree-based approaches: (i) the fixation index F_{ST} test (55), (ii) Simmonds association index (AI) (52), and (iii) the Slatkin-Maddison (SM) test (54). Blue denotes compartmentalization. "Consensus" denotes the final result: light blue indicates significant results in at least 2 of 3 tests, dark blue indicates significant results in all tests. *, compartmentalization was declared only if the results of at least 2/3 tests using both reads and haplotypes agreed. (Left) Results when comparing all reads from blood and semen. (Right) Results with reads collapsed to haplotypes. See Materials and Methods for details. Of note, no compartmentalization analysis was performed for ID3 in the *pol* region because only one haplotype was obtained.

A subset of 12 participants agreed to enroll in a genital tract substudy. Semen was collected at baseline (before ART interruption) and approximatively every 2 weeks for a median of 18 weeks (range, 12 to 27 weeks) post-ART interruption. Semen was processed as previously described (40–43) by masturbation into a sterile container containing 10 ml of RPMI with penicillin-streptomycin following 48 h of abstinence, and was processed within 2 h. Semen was centrifuged at 850 × *g* for 10 min, and semen plasma HIV RNA viral load assayed using the Abbott RealTime HIV-1 assay (Abbott Molecular Diagnostics; limit of detection, 40 copies/ml). Due to occasional spillage of RPMI during semen collection and/or sample transport, correction for semen dilution assumed a semen volume of 2 ml, as previously validated.

All study participants were asked to practice condom-protected sex for the duration of the study, and sexually transmitted infection (STI) screening (syphilis serology and urine molecular diagnostics for *Neisseria gonorrhoeae* and *Chlamydia trachomatis*) was performed at study screening, at baseline, and at each semen donation visit. No STIs were diagnosed in any participant for the duration of the study.

HIV RNA extraction and next-generation sequencing from blood plasma and seminal supernatant. Nucleic acid was concentrated from 1 ml of blood plasma by high-speed centrifugation. Subsequently, RNA was isolated according to the manufacturer's instructions (QIAamp viral RNA minikit; Qiagen, Hilden, Germany), and cDNA was produced (RETROscript kit; Applied Biosystems/Ambion, Austin, TX). For semen, viral population was concentrated from 2 to 6 ml of supernatant in order to maximize template input in each MiSeq reaction mixture (depending on viral load and sample availability). Three coding regions—*gag* p24 (HXB2 coordinates 1366 to 1619), *pol* RT (2708 to 3242), and *env* C2-V3 (6928 to 7344)—were amplified by PCR with region-specific primers, as previously described (44).

Sequence processing and bioinformatics analysis. (i) Sequencing. The Illumina MiSeq instrument and MiSeq reagent kit V3 600-cycle paired-end sequencing kits (MS-102-2003/MS-102-3003) were used to sequence the DNA libraries. The median number of reads per amplicon was 320,452 (IQR, 198,053 to 1,494,661).

FIG 4 Phylogenetic trees illustrating different HIV RNA populations in blood and semen. Topologies showed intermingled sequences from blood and seminal plasma, confirming the lack of viral compartmentalization for most participants and regions. Phylogenetic trees for *pol* D4 and *pol* D10 confirm the presence of monophyletic clade originating from semen, but a definitive evaluation is limited due to the short length of the sequences and the overall limited diversity in our data set. Participants ID3 and ID4 were assigned to the placebo group, while ID5, ID10, and ID11 were assigned to the vaccine active group.

(ii) Read mapping and filtering. The reads were analyzed using a custom pipeline adapted from Zanini et al. (45). Briefly, (i) reads were first mapped onto the HIV-1 reference HxB2, (ii) mapped reads were classified into partial *gag*, *pol*, and *env* regions (ambiguous reads were discarded) and trimmed for PHRED quality above or equal to 30, (iii) a consensus sequence was computed for each 3 regions in each sample from a subset of the reads, using a chain of overlapping local multiple sequence alignments, (iv)

reads were remapped against their own consensus, (v) reads were trimmed for mapping errors at the edges (small indels), (vi) filtered reads were mapped a third time against a patient-specific consensus sequence from the initial time point (day 0), and (vii) reads were refiltered and checked again for cross-contamination.

(iii) Recombination screening. We screened all sets of representative reads for evidence of recombination using GARD (46).

(iv) Diversity. Viral diversity was assessed by measuring the Shannon entropy index from all cleaned reads mapped to HIV partial *gag*, *pol*, and *env* regions (47). Comparison between entropy measures across participants was performed using a pairwise Wilcoxon adjusted test using the conservative Bonferroni correction method implemented in R package stats (48, 49).

(v) Phylogenetic analysis. HIV haplotypes above a minimal frequency threshold of 0.01 were extracted from reads covering the *gag*, *pol*, and *env* regions and were used to construct maximum likelihood (ML) phylogenies using IQtree (50).

(vi) Population structure. Compartmentalization was assessed using two tree-based and one distance-based approach:

(1) To quantify the overall spatial structure of the phylogenies, we first measured the phylogenetic association in the location trait data (i.e., semen or blood) (51, 52). Using the posterior set of trees, we calculated the Simmonds association index (AI) (52) for each discrete "location trait" (i.e., compartment) using BaTS v1.0 (53). To provide statistical evidence for genetic segregation of the HIV populations, the reported *P* value was inferred as the proportion of trees from the null distribution equal to, or more extreme than, the median posterior estimate of the statistic from the posterior set of trees.

(2) The tree-based Slatkin-Maddison (SM) test (54): trees were considered compartmentalized with the SM test if 10,000 permutations of the SM test yielded a *P* value of <0.05.

(3) The fixation index (55) defined as $F_{ST} = 1 - \frac{\pi_I}{\pi_D}$, where π_I is the estimate of mean pairwise intracompartment genetic distance (TN93) (56) and π_D is its intercompartment counterpart (22).

These methods were repeated after collapsing identical reads into haplotypes to consider the haplotype relative abundance. HIV populations from each of the 3 HIV coding regions were considered compartmentalized if at least 2 of 3 methods showed evidence of compartmentalization consistently before and after collapsing reads into haplotypes.

Data availability. All read files are available in the NCBI Sequence Read Archive under accession numbers SAMN14848556 to SAMN14848588.

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We have no conflicts of interest.

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