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GENETIC DIVERSITY AND EVOLUTION

Deep Sequencing Reveals Compartmentalized HIV-1 in the Semen of Men with and without Sexually Transmitted Infection-Associated Urethritis

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ABSTRACT Concurrent sexually transmitted infections (STI) can increase the probability of HIV-1 transmission primarily by increasing the viral load present in semen. In this study, we explored the relationship of HIV-1 in blood and seminal plasma in the presence and absence of urethritis and after treatment of the concurrent STI. Primer ID deep sequencing of the V1/V3 region of the HIV-1 *env* gene was done for paired blood and semen samples from antiretroviral therapy (ART)-naive men living in Malawi with ($n = 19$) and without ($n = 5$) STI-associated urethritis; for a subset of samples, full-length *env* genes were generated for sequence analysis and to test entry phenotype. Cytokine concentrations in the blood and semen were also measured, and a reduction in the levels of proinflammatory cytokines was observed following STI treatment. We observed no difference in the prevalence of diverse compartmentalized semen-derived lineages in men with or without STI-associated urethritis, and these viral populations were largely stable during STI treatment. Clonal amplification of one or a few viral sequences accounted for nearly 50% of the viral population, indicating a recent bottleneck followed by limited viral replication. We conclude that the male genital tract is a site where virus can be brought in from the blood, where localized sustained replication can occur, and where specific genotypes can be amplified, perhaps initially by cellular proliferation but further by limited viral replication.

IMPORTANCE HIV-1 infection is a sexually transmitted infection that coexists with other STI. Here, we examined the impact of a concurrent STI resulting in urethritis on the HIV-1 population within the male genital tract. We found that viral populations remain largely stable even with treatment of the STI. These results show that viral populations within the male genital tract are defined by factors beyond transient inflammation associated with a concurrent STI.

KEYWORDS compartmentalization, HIV-1, genital tract, phylogeny, viral diversity

Nearly two million new HIV-1 infections occur worldwide every year, predominately through sexual transmission (1). Therefore, understanding the genotypic and phenotypic properties of HIV-1 present in the male genital tract is vital for treatment and prevention strategies. It is well-established that the probability of sexual transmis-

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sion of HIV-1 increases with an increasing viral load (2–5), and there are several factors that can influence the concentration of viral RNA present in semen. For example, stage of disease (6), $CD4^+$ T cell count (7), and the presence of inflammatory conditions (such as concurrent sexually transmitted infections [STI]) have all been demonstrated to increase the semen viral load (reviewed in reference 8).

For semen-mediated transmission events, the transmitted/founder virus is most proximal to the male genital tract at the time of transmission. Thus, the origin of virus in the male genital tract is relevant to a fuller understanding of HIV-1 transmission. Often the virus present in semen is similar to virus found in the blood (an equilibrated population), but there is also evidence that the male genital tract is able to support independent replication of HIV-1. This fact is inferred from observations of genetically distinct, or compartmentalized, HIV-1 populations in semen, compared to the virus found in the blood and other anatomical compartments (9–15). In addition, several studies (16, 17) have reported the presence of HIV-1 RNA in the semen of men who are on suppressive antiretroviral therapy and have undetectable blood plasma viral loads, implying that the male genital tract can influence viral replication independent of the periphery and harbor an independent viral reservoir. It is therefore important to elucidate the factors that promote the establishment and maintenance of compartmentalized viral lineages in the male genital tract.

In the current study, we examined the effects of STI-associated urethritis on the establishment and maintenance of compartmentalized lineages in the male genital tract by comparing viral sequences in the blood to those in seminal plasma using deep sequencing technology with Primer ID (18, 19). We hypothesized that STI-associated inflammation could act to recruit $CD4^+$ T cells into the genital tract, thereby promoting a mixing of viral populations in the blood and semen with a concomitant reduction in apparent compartmentalization, or, conversely, that the influx of cells could enhance the replication of locally produced virus and increase compartmentalization. We also examined the viral population dynamics between blood and semen over time to determine whether antibiotic treatment of the concurrent STI would impact HIV-1 compartmentalization. We detected no difference between the proportions of men who had compartmentalized, semen-derived lineages, grouped by the presence or absence of urethritis. Furthermore, antibiotic treatment of the STI did not observably impact the population dynamics between the blood and the semen, at least in the short term. We conclude that STI-associated inflammation is not a driving factor behind the establishment or maintenance of compartmentalized lineages in the semen and that compartmentalized viral replication can occur independently of inflammatory conditions.

RESULTS

Participant characteristics and sequence generation. Participants were part of a cohort of men based in Malawi that was established to examine the effect of STI-associated urethritis on seminal plasma HIV-1 viral load (20). In order to examine the relationship between urethritis associated with a concurrent sexually transmitted infection (STI) and the presence of compartmentalized virus in the genital tract, we selected a subset of men with ($n = 19$) and without ($n = 5$) STI-associated urethritis, with the sample size determined by availability of sufficient seminal plasma. All participants were chronically infected with subtype C HIV-1 and were antiretroviral therapy (ART) naive, as ART was not available in Malawi at the time of the study.

There was no difference in the blood viral load, semen viral load, or $CD4^+$ T cell count between the two groups at baseline (Table 1). HIV-1 RNA was extracted from paired blood plasma and seminal plasma, and Illumina MiSeq deep sequencing with Primer ID was used to generate HIV-1 *env* V1/V3 amplicons. The deep-sequencing output was collapsed into template consensus sequences (TCSs) for each Primer ID recovered to create a highly accurate sequence for each original RNA template sampled. An average of 62 TCSs were obtained from each compartment (blood and semen)

TABLE 1 Relevant clinical information for participants[a]

Patient	Viral load (log_{10} copies/ml)				CD4 count[b]	Diagnosed STI[c]
	Before STI treatment		After STI treatment			
	Plasma	Semen	Plasma	Semen		
Urethritis (n = 19)						
S003	5.23	5.48	5.51	4.8	ND	Gon, Ulc
S018	5.59	4.70	5.40	4.96	476	Gon, Tri, Ulc
S019	4.18	5.48	4.34	4.32	712	Gon
S031	4.95	4.62	4.63	4.92	333	Gon, Ulc
S053	6.11	6.08	5.88	5.38	318	Tri, Ulc
S070	4.73	3.92	4.96	4.46	670	Gon
S073	4.59	4.60	4.51	4.23	275	Gon
S075	5.59	5.20	5.90	4.08	ND	NPI
S101	5.83	5.89	5.79	5.20	235	Gon
S103	3.95	5.00	5.11	4.79	454	Gon
S146	4.79	4.96	5.58	5.53	258	Ulc
S172	4.80	5.18	4.28	4.62	344	Gon
S029	5.23	4.71	ND	ND	496	Gon
S017	5.79	4.40	ND	ND	178	NPI
S039	5.56	5.04	ND	ND	ND	Gon
S047	5.77	4.92	ND	ND	469	Gon
S099	5.57	5.82	ND	ND	ND	Gon, Tri
S148	5.08	5.98	ND	ND		Gon
S067	4.90	5.04	ND	ND		Gon
No urethritis (n = 5)						
C019	7.00	5.72	5.72	6.76	599	None
C073	4.82	3.76	ND	ND	ND	None
C082	5.49	4.20	ND	ND	305	None
C111	4.84	5.95	ND	ND	210	None
C061	5.56	6.51	ND	ND	262	None

[a]ND, not done.
[b]Number of cells per microliter of blood.
[c]Gon, gonorrhea; Tri, trichomoniasis; Ulc, genital ulcers; NPI, no pathogen identified.

for each participant (range, 12 to 200), giving us 95% power to detect minor populations present in most samples at a 1.5 to 5% frequency.

Compartmentalized, semen-derived lineages are observed in men with and without urethritis. As we were primarily interested in identifying diverse compartmentalized lineages, which represent independent replication over a period of time, rather than compartmentalized lineages that consist primarily of clonally amplified sequences, we initially collapsed sequences that were identical (within one nucleotide) into a single haplotype. After identical sequences were collapsed, an equal number of blood-derived and semen-derived sequences were used to construct maximum-likelihood phylogenetic trees for each participant, allowing us to compare the two populations at equivalent sampling depth. Compartmentalization was assessed using both the Slatkin-Maddison test (21) and the structured Slatkin-Maddison test (https://github.com/veg/hyphy-analyses/tree/master/SlatkinMaddison), which has been modified to reduce potentially spurious compartmentalization detection in trees with large numbers of sequences. When both tests resulted in a P value of <0.05, the tree was deemed compartmentalized. When one test indicated compartmentalization while the other did not, trees were inspected visually for the presence of diverse, semen-dominated lineages.

Among the 24 men, we observed various degrees of compartmentalization, ranging from nearly complete separation of blood- and semen-derived sequences to minor compartmentalization in 6/24 (25%) participants. In men with urethritis, compartmentalization was detected in 6/19 (32%) men, while viral populations were equilibrated between the blood and semen in 13/19 (68%) men. In men without urethritis, we observed minor compartmentalization in 1/5 (20%) individuals and equilibrated viral

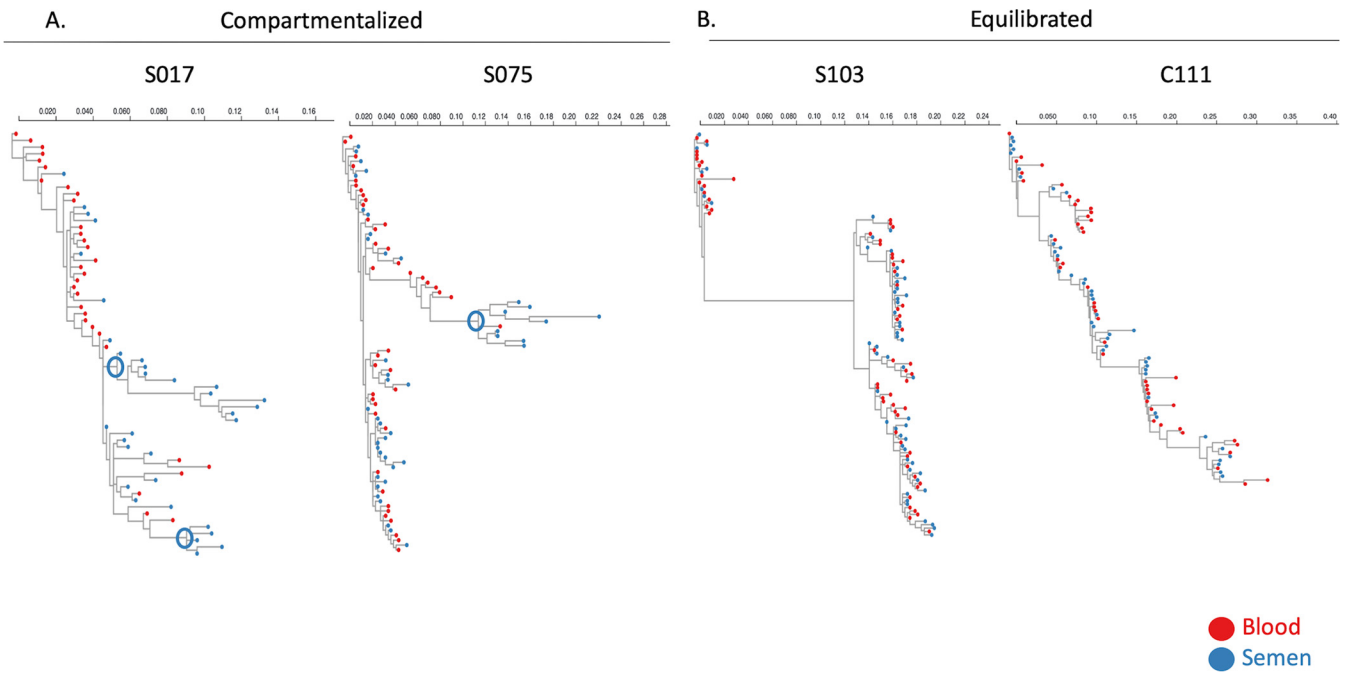

FIG 1 Semen-derived HIV-1 *env* V1/V3 sequences can be equilibrated or compartmentalized. Representative maximum-likelihood *env* V1/V3 phylogenetic trees depicting compartmentalization between the blood- and semen-derived lineages (A) and equilibration between blood- and semen-derived lineages (B) are shown. Blood-derived sequences are in red; semen-derived sequences are in blue. Circles indicate compartmentalized nodes.

populations in 4/5 (80%) individuals. Thus, both compartmentalized and equilibrated HIV-1 populations were found in men with and without urethritis (Fig. 1 and Table 2) at statistically indistinguishable frequencies. This suggests that urethritis does not alter the establishment of compartmentalized lineages, although the generalizability of this conclusion is limited by the number of samples studied.

HIV-1 population dynamics between blood and semen remain largely stable after STI treatment. To examine the effects of antibiotic treatment of the STI on HIV-1 population dynamics, we compared pretreatment and posttreatment time points in 13 men (12 with urethritis, 1 without urethritis). Samples were obtained an average of 12 days after antibiotic treatment had been initiated (range, 7 to 14 days). Maximum-likelihood phylogenetic trees were built as described above, and the relationship between blood- and semen-derived sequences (i.e., equilibrated or compartmentalized) was determined at each time point. In 12/13 men, the relationship did not change following STI treatment (Fig. 2). In one individual, S101, semen- and blood-derived lineages were equilibrated at the pre-STI treatment time point but compartmentalized posttreatment, as determined by both the standard Slatkin-Maddison test and the structured Slatkin-Maddison test ($P < 0.0001$) (Table 2).

Next, we compared within-compartment viral diversity before and after STI treatment. To this end, we inferred maximum-likelihood phylogenetic trees containing equal numbers of semen-derived sequences from the pre- and post-STI-treatment time points for the 13 men described above. Both the standard Slatkin-Maddison and the structured Slatkin-Maddison tests were performed on the trees in order to determine whether the pre- and post-STI-treatment semen sequences constituted distinct clades. In 12 of 13 men, the semen populations before and after STI treatment were not significantly different from one another; i.e., populations that existed before STI treatment were still readily observable after STI treatment. Of particular interest were the individuals with compartmentalized, semen-derived lineages. In 2 of the 3 men with compartmentalized lineages at both time points, the lineage that was responsible for the compartmentalization was the same before and after STI treatment (Fig. 2A and B). Thus, not only was the relationship between compartments unchanged, but the

TABLE 2 Summary of the methods used to determine the relationship between blood-derived and semen-derived HIV-1 *env* V1/V3 sequences[a]

Patient	Before STI treatment				After STI treatment			
	No. of HIV *env* V1/V3 sequences	Structured Slatkin-Maddison value	Standard Slatkin-Maddison value	Visual inspection	No. of HIV *env* V1/V3 sequences	Structured Slatkin-Maddison value	Standard Slatkin-Maddison value	Visual inspection
Urethritis (n = 19)								
S003	187	0.15	**0.0009**	Equilibrated	25	0.788	0.392	Equilibrated
S018	120	0.327	0.186	Equilibrated	24	0.926	0.819	Equilibrated
S019	48	0.076	**0.004**	Equilibrated	22	0.335	**0.03**	Equilibrated
S031	200	**0**	**0**	Compartmentalized	306	**0**	**0**	Compartmentalized
S053	29	0.461	**0.027**	Equilibrated	23	0.396	0.111	Equilibrated
S070	15	0.946	0.539	Minor compartment	12	0.262	**0.017**	Minor compartment
S073	24	0.882	0.734	Equilibrated	8	0.428	0.428	Equilibrated
S075	43	0.263	**0.003**	Minor compartment	42	0.515	**0.06**	Minor compartment
S101	13	0.653	0.15	Equilibrated	103	**0**	**0**	Compartmentalized
S103	62	0.904	0.902	Equilibrated	10	0.322	0.098	Equilibrated
S146	71	0.251	**0.004**	Minor compartment	45	0.36	**0.033**	Minor compartment
S172	32	0.805	0.277	Equilibrated	24	0.96	0.89	Equilibrated
S029	47	0.713	0.17	Equilibrated	ND	ND	ND	ND
S017	32	**0.003**	**0**	Minor compartment	ND	ND	ND	ND
S039	19	0.825	0.61	Equilibrated	ND	ND	ND	ND
S047	25	0.916	0.383	Equilibrated	ND	ND	ND	ND
S099	200	**0.002**	**0.0009**	Compartmentalized	ND	ND	ND	ND
S148	82	0.294	**0.014**	Equilibrated	ND	ND	ND	ND
S067	98	0.203	**0.01**	Equilibrated	ND	ND	ND	ND
No urethritis (n = 5)								
C019	25	0.787	0.369	Equilibrated	32	0.649	**0.029**	Equilibrated
C073	15	0.795	0.111	Equilibrated	ND	ND	ND	ND
C082	12	0.537	0.248	Equilibrated	ND	ND	ND	ND
C111	46	0.572	**0.025**	Equilibrated	ND	ND	ND	ND
C061	34	0.404	**0.006**	Minor compartment	ND	ND	ND	ND

[a]Bold values indicate statistically significant compartmentalization as determined by the indicated method. ND, not done.

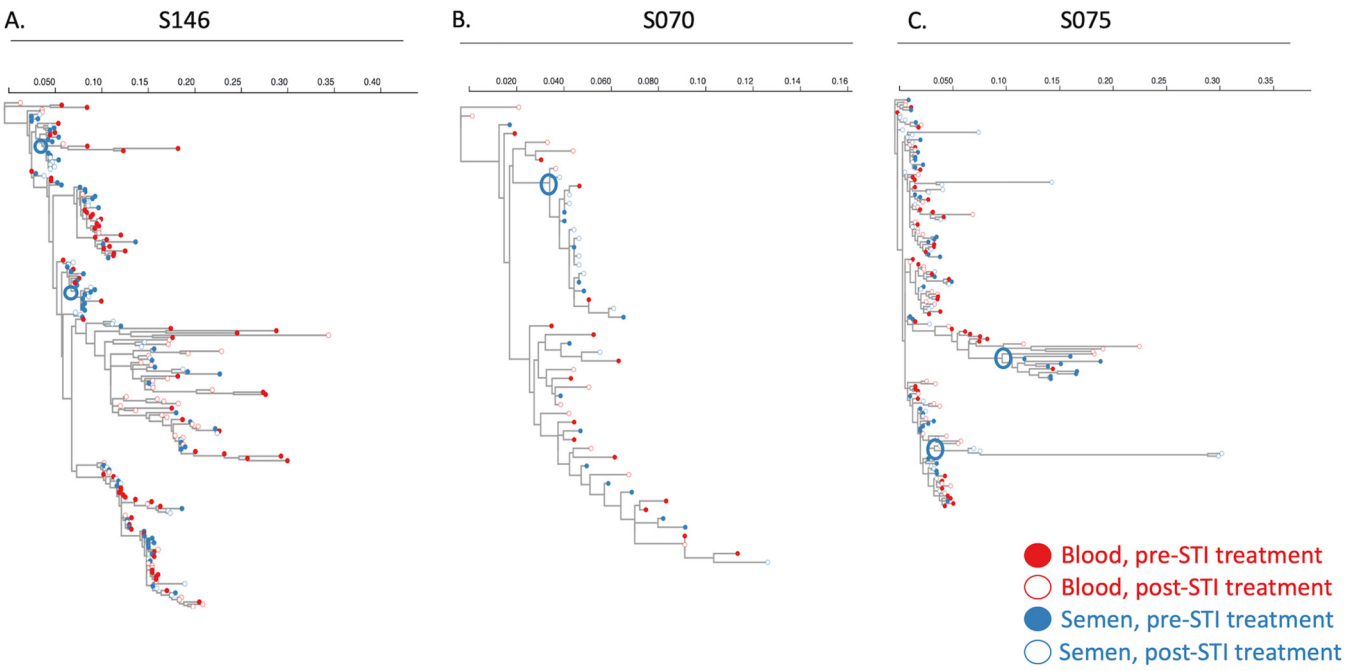

FIG 2 HIV-1 population dynamics between blood and semen remain unchanged after STI treatment. Maximum-likelihood phylogenetic trees depicting blood-derived *env* V1/V3 sequences (red) and semen-derived *env* V1V3 sequences (blue) are shown. Sequences from before STI treatment are shown in filled circles, and sequences from after STI treatment are shown in open circles. In panels A and B, the same compartmentalized lineage appearing in the semen before and after STI treatment (circled nodes) is seen. In panel C, a different semen-derived, compartmentalized lineage at the pre- and post-STI-treatment time points is seen.

specific lineages themselves persisted. However, in one individual, the compartmentalized, semen-derived lineage that was detected before STI treatment was not detected at the second time point, but a new compartmentalized lineage was observed (Fig. 2C).

Clonal amplification of blood- and semen-derived sequences was observed in men with and without urethritis. As we were primarily interested in the presence of diverse, compartmentalized lineages, rather than compartmentalized lineages composed of a clonally expanded population, we collapsed sequences that were identical to within one nucleotide into a single haplotype. In doing so, we observed that a large proportion of both blood- and semen-derived V1/V3 sequences were identical or nearly identical. Such an observation could be made because of the PCR amplification step prior to sequencing, where the original templates are repetitively sequenced, a phenomenon called PCR resampling; however, the use of Primer ID to tag each original template before PCR avoids this problem, allowing us to infer the presence of identical or nearly identical sequences within the viral population *in vivo*. For blood-derived sequences, means of only 41% and 48% of sequences were unique in men with and without urethritis, respectively ($P = 0.4237$) (Fig. 3A). For semen-derived sequences, means of only 44% and 62% of sequences were unique in men with and without urethritis, respectively ($P = 0.086$) (Fig. 3C). The proportion of unique sequences observed in blood and semen remained stable before and after STI treatment in men with urethritis (Fig. 3B and D). This result indicates that a significant fraction of the population in each compartment was in a genetic bottleneck or had recently gone through a bottleneck.

We considered the possibility that the short *env* V1/V3 amplicon (527 bases) would overestimate the percentage of sequences that were identical across the entirety of *env*. To evaluate this possibility, we performed single-genome amplification (SGA) of full-length HIV-1 *env* genes (~2,500 bases) from the blood and semen of four men (three with urethritis and one without). We obtained an average of 30 full-length *env* sequences from each participant. In two of the four cases, we observed identical

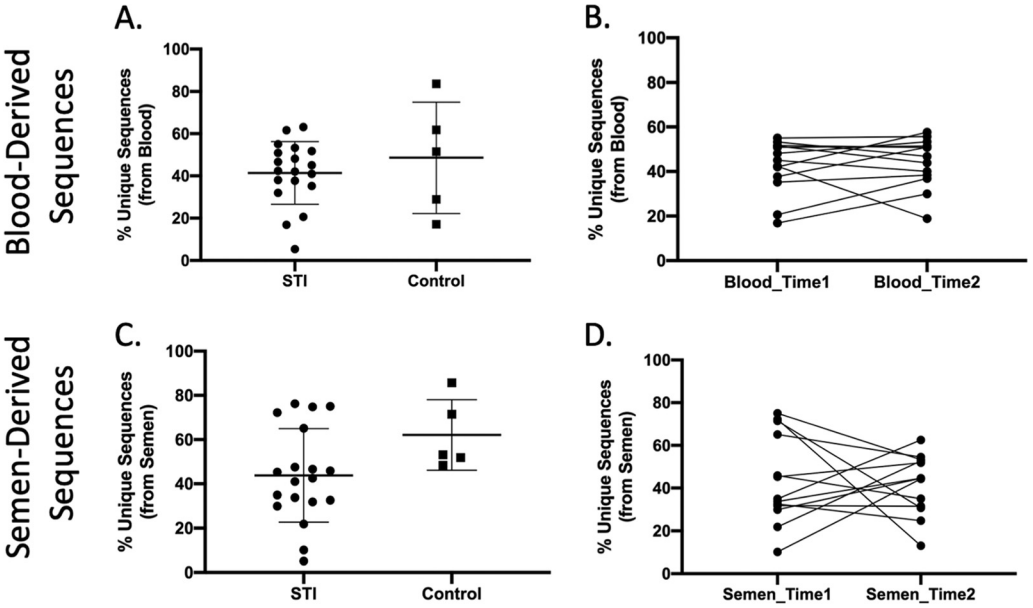

FIG 3 Clonal amplification of identical sequences is observed in both blood- and semen-derived viruses in men with and without urethritis. (A and C) Analysis of the percentage of V1/V3 sequences that are not identical, derived from blood (A) or semen (C). (B and D) The percent unique sequences remains stable over time in both the blood (B) and semen (D). An unpaired *t* test was performed on all comparisons, and none were found to be statistically significant.

sequences across the entirety of *env*. When we trimmed the full-length sequences and analyzed only the V1/V3 region used in our deep sequencing, we observed identical or nearly identical sequences in all four participants. Sequences that were identical in the V1/V3 region but not identical across the entire envelope differed by only a few nucleotides. Such differences are consistent with the low-level diversity generated from recent viral replication from a unique ancestor/bottleneck (Fig. 4; also, see Fig. S1 to S3 in the supplemental material). Thus, while examining only the V1/V3 region does increase the number of sequences that appear identical, the overall viral diversity of those variants is low and consistent with recent clonal expansion involving a bottleneck with subsequent viral replication to introduce modest diversity. In a control experiment, we generated 8 *env* amplicons from virus produced from the cell line 8E5, which contains a single defective viral genome. When the 8 amplicons were sequenced, we observed a single substitution mutation and a single frameshift mutation (data not shown). The low level of diversity observed in the viral populations *in vivo* was in most cases greater than the level observed in the control amplification, consistent with ongoing viral replication after a recent bottleneck rather than just virus production from a clonally expanded cell.

Semen-derived HIV-1 Env proteins are T-cell tropic. HIV-1 primarily infects $CD4^+$ T cells, which have a high density of the CD4 protein on their cell surface, which is typically required by the virus for efficient entry. However, viruses that have been replicating independently in anatomically distinct regions, such as the central nervous system (CNS), where $CD4^+$ T cells are less abundant, can evolve the ability to enter cells expressing CD4 at lower densities, such as macrophages. This has been observed for compartmentalized lineages derived from both the CNS (22) and, in one case, the male genital tract (23). In order to determine whether compartmentalized, semen-derived lineages from our cohort have the ability to enter cells expressing a low density of CD4, we produced pseudotyped virus that expressed participant-derived Env surface proteins, obtained through SGA. These viruses were used to infect Affinofile cells that had been induced to express either high or low densities of CD4. The amount of luciferase produced by the cells was quantified and used as a surrogate measure of infectivity. As shown in Fig. 5, semen-derived HIV-1 *env* genes, from both compartmentalized and

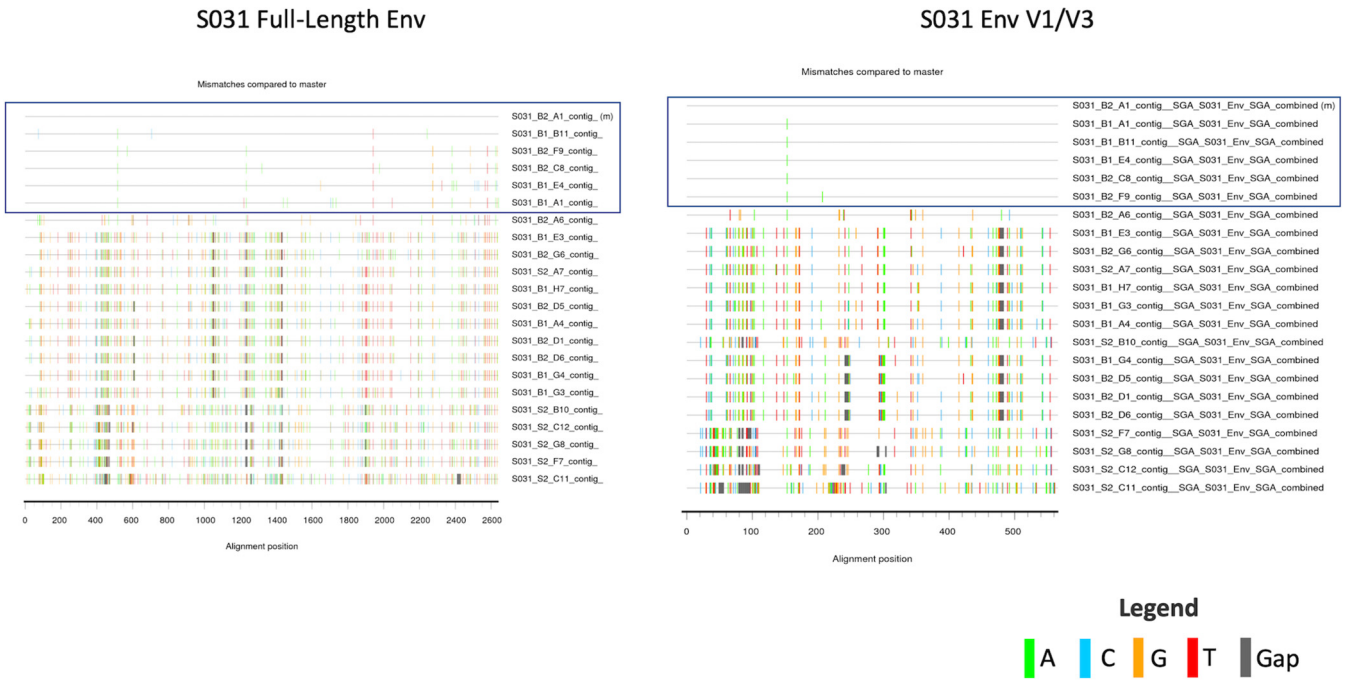

FIG 4 Highlighter plot of paired full-length *env* and *env* V1/V3 sequences. SGA-derived full-length envelope sequences and the corresponding V1/V3 region only are shown on the left and right, respectively. Nucleotide changes relative to the master (top) sequence are indicated by colored vertical tick marks. Boxed sequences represent those that are identical in the V1/V3 amplicon and nearly identical over the full envelope amplicon.

equilibrated lineages, encoded Env proteins that require a high density of CD4 for efficient cell entry, indicating that they were being selected for replication in T cells.

Cytokine/chemokine dynamics during treatment of the STI. To better understand the magnitude of the inflammation present within the genital tract during a concurrent sexually transmitted infection, we measured the concentrations of seven inflammatory cytokines and chemokines present in the blood and semen before and after treatment of the STI. To differentiate between STI-induced inflammation and HIV-induced inflammation, we included samples from HIV-positive individuals not experiencing urethritis. As shown in Fig. 6A, there was a group of cytokines (tumor necrosis factor alpha [TNF-α], interleukin 6 [IL-6], and IL-1β) whose concentrations were increased in the semen of men with urethritis at the pretreatment time point and subsequently decreased after STI treatment; although the pattern was consistent across these analytes, the small sample size limited statistical power. A second group of cytokines/chemokines, including CXCL10, IL-10, gamma interferon (IFN-γ), and CCL2, were at similar concentrations in men with and without urethritis, as well as before and after STI treatment. A subset of four cytokines/chemokines (TNF-α, IL-10, CCL2, and CXCL10) were measured in blood as well (Fig. 6B). There was no difference in the concentration of any of these analytes at any time point in men with or without urethritis, suggesting that STI-associated inflammation is limited to the genital tract and largely resolves with antibiotic treatment.

DISCUSSION

In this study, we used Primer ID deep sequencing to examine the prevalence of compartmentalized HIV-1 populations in the male genital tract in men with and without STI-associated urethritis. Urethritis did not impact the prevalence of genital tract compartmentalization. In further support of this conclusion, we observed similar viral population dynamics between the blood and genital tract before and after STI treatment, while simultaneously observing a decrease in genital tract inflammation.

Numerous studies (12–14, 24–28) have examined the prevalence of male genital tract compartmentalization of HIV-1, sometimes with discordant results. Some of these

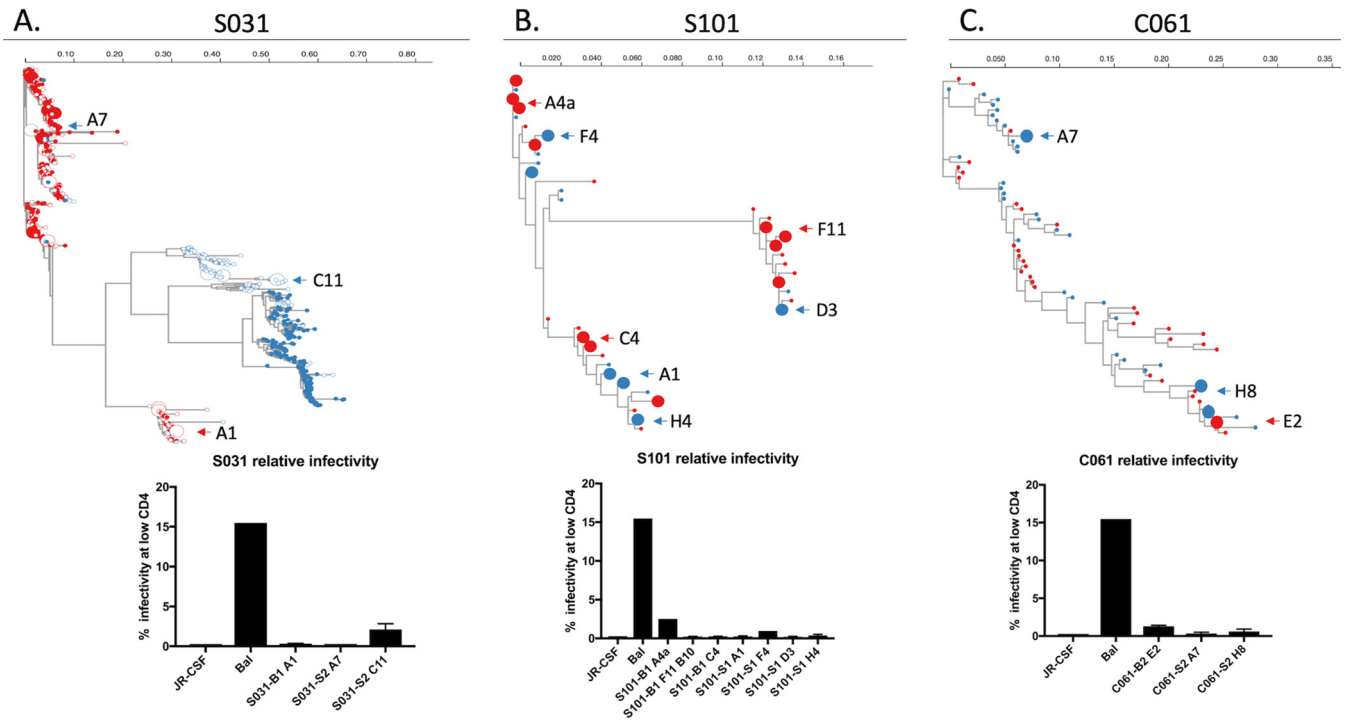

FIG 5 SGA-derived HIV-1 envelopes from the semen are T-cell tropic. Maximum-likelihood trees of *env* V1/V3 blood (red)- and semen (blue)-derived sequences are shown. The graphs below depict the ability of SGA-derived envelopes from blood and semen to enter cells expressing low densities of CD4. Colored arrows on the trees depict the locations of the envelopes used in the graphs below. JR-CSF and Bal are T-tropic and M-tropic controls, respectively. Data are the averages from three biological replicates.

studies (11, 24, 25) examined the phenomenon of compartmentalization through the use of bulk amplification and/or cloning prior to sequencing; however, these approaches have been shown to introduce sequencing artifacts, such as PCR-mediated recombination and sequence resampling (29–31). The use of deep sequencing with Primer ID in the current study corrects for PCR and sequencing errors through the creation of a template consensus sequence for each Primer ID-tagged cDNA (the template for PCR), while simultaneously allowing the precise quantification of the total number of templates sequenced, i.e., the sample size of sampling of the viral sequence population (19). Thus, we can be confident that the viral variants we analyze are an accurate representation of the diversity found *in vivo*.

We observed compartmentalization in 32% of men with urethritis and 20% of men without urethritis; thus, given this number of participants, we did not detect a difference in the extent of compartmentalization with and without and STI-associated urethritis. In the overall cohort, we observed compartmentalization in the genital tracts of 29% of men. This prevalence of compartmentalized lineages in the genital tract is similar to what was observed in a previous study (9) that used a heteroduplex tracking assay to examine the relationship between blood- and semen-derived *env* V3 populations in men with and without urethritis. In this earlier study, they observed discordant V3 populations between the blood and semen of 40% of men. Importantly, there was no difference in the V3 population dynamics between the blood and semen of men with urethritis and those of men without urethritis. Later, Anderson and colleagues (10) utilized single-genome amplification to examine the relationship between blood- and semen-derived HIV-1 envelopes in men without urethritis. They reported a 31% prevalence of compartmentalization in the genital tract. They also observed clonal amplification in the semen of men without urethritis. Compartmentalized populations in the genital tract have also been observed in the context of acute HIV-1 infection. In a study by Chaillon et al. (26), deep sequencing was used to examine HIV-1 populations in

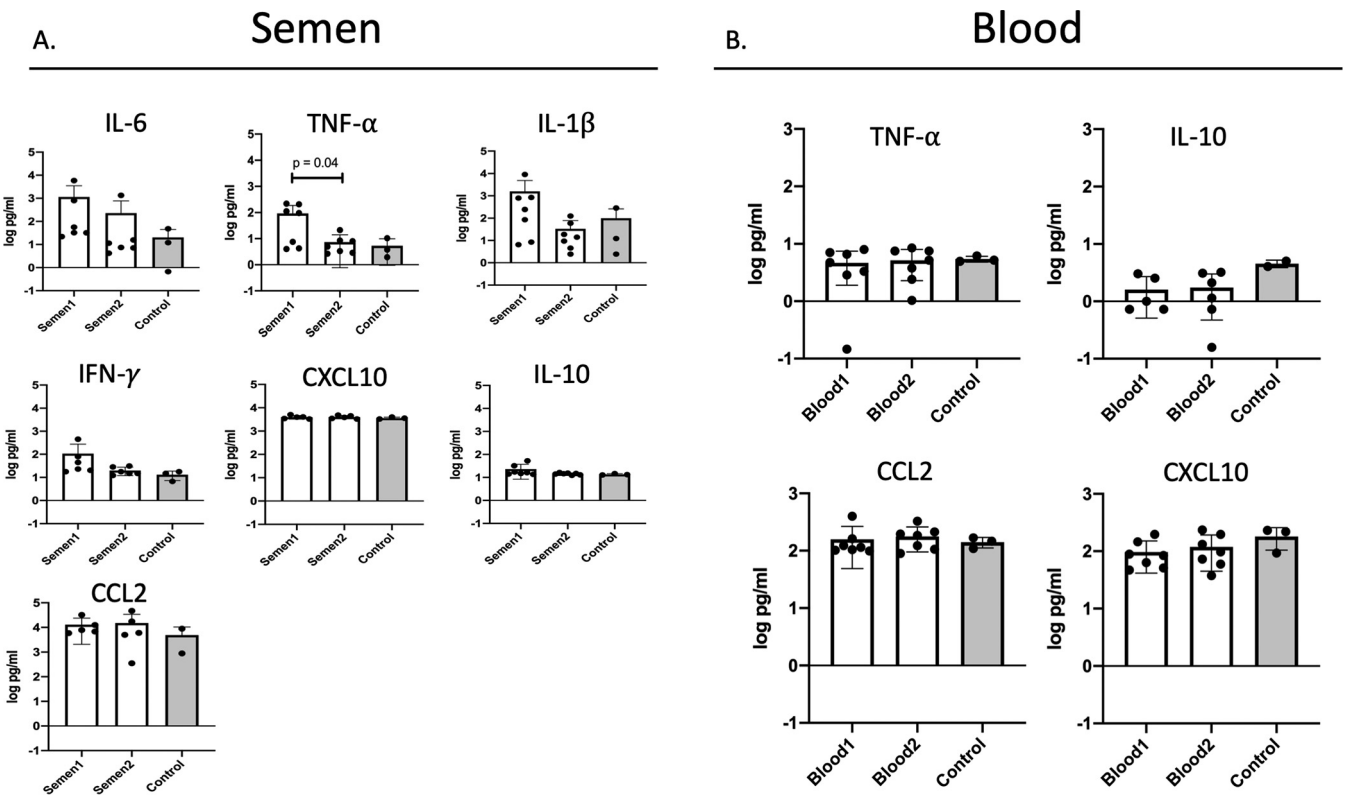

FIG 6 Cytokine/chemokine analysis in semen and blood before and after antibiotic treatment of the STI. Cytokine/chemokine concentrations in semen (A) and blood (B) were measured before (Blood1 and Semen1) and after (Blood2 and Semen2) STI treatment. The values were compared to those from a group of HIV-positive men without a concurrent STI (controls [gray bars]). A paired *t* test was used to generate the *P* value.

blood and semen in early infection. They observed compartmentalization in 2 of 6 participants at baseline (a median of 81 days after the estimated date of infection).

The observation that inflammation does not alter the frequency with which we detect semen-specific HIV-1 lineages suggests that when compartmentalized lineages are present, they are most likely produced by cells in anatomical areas that are not in direct contact with the periphery. In one extreme case of this type of isolation, we previously observed the presence of a macrophage-tropic variant in semen (23). In the current study, all of the viruses tested were T-cell-tropic, requiring a high density of CD4 for efficient entry into cells. In addition, all were predicted to use CCR5 as a coreceptor based on genotypic predictions of the V3 loop sequence (data not shown). This result is important, as a recent article by Ganor and colleagues (32) reported the presence of macrophage-tropic viral variants in urethral tissues, suggesting the possibility of a urethral reservoir. However, it appears that such variants are not shed in the semen at a detectable level.

Compartmentalization in the male genital tract has largely been defined as a transient phenomenon (25, 26). Here, we examined how antibiotic treatment of a concurrent sexually transmitted infection (primarily gonorrhea or trichomoniasis) impacted the relationship between blood- and semen-derived HIV-1 *env* V1/V3 sequences. We found that viral variants present before STI treatment remained detectable after STI treatment and, furthermore, that the relationship between blood- and semen-derived sequences remained consistent throughout the course of STI coinfection. In only one participant out of 13 did we detect a change in the relationship between blood- and semen-derived sequences over time. In this instance, the depth of sampling pre-STI treatment was relatively poor, while the sampling post-STI treatment was much greater. Thus, it is quite possible that the relatively few sequences obtained pretreatment obscured the presence of the compartmentalized lineage that we observed

posttreatment. It is also important to note that while gonococcal infections are cleared rapidly from the urogenital tract after a single antibiotic treatment (33), the underlying immune activation can persist, as demonstrated by the fact that in men with an STI, HIV-1 viral loads in semen were still higher than in men without an STI, even after effective antibiotic treatment (20), although we were able to measure some diminution of inflammation with a change in some inflammatory markers. Therefore, while we do observe stable relationships between blood- and semen-derived sequences before and after STI treatment, our conclusions are limited by the relatively short period of follow-up. It is worth noting that in one participant, virus in the semen was compartmentalized relative to the blood both before and after STI treatment but the compartmentalized lineage in the semen changed between the two time points. Both lineages, while minor, were complex in sequence composition, and thus the latter one did not evolve over the short period of time between the two samplings. Thus, there must have been reduced production of one lineage and the appearance of a pre-existing lineage over a relatively short period of time.

The identification of identical or nearly identical sequences in the blood in chronic untreated infection is relatively infrequent, consistent with a large population size and replication in the context of the error-prone nature of HIV-1 reverse transcription (34–37). However, we observed a striking number of identical sequences in the blood and semen of men with and without urethritis. In the absence of therapy, the viral load of viruses with similar sequences is much higher than that seen in the presence of therapy, suggesting either that the corresponding cellular expansion is much greater or that the virus comes from another source, i.e., replication, after passing through a recent genetic bottleneck. This question becomes even more relevant when the shorter amplicon associated with deep sequencing is used, as a significant fraction of the viral sequences cluster into lineages of identical sequences. In order to determine if the identical sequences from deep sequencing observed in the absence of therapy in these men were truly clonal, we compared sequences obtained from deep sequencing to those obtained as full-length *env* genes using template endpoint dilution PCR (SGA). We found that the sequences that were identical in the deep sequencing data set were in a population of similar but not identical sequences when the larger region of the genome was analyzed (Fig. 4; also, see Fig. S1 to S3). We conclude that these populations are present at their detected level due to ongoing viral replication. However, the high level of similarity in these sequences implies a recent genetic bottleneck prior to expansion by viral replication, although the nature of that bottleneck remains unknown, and it could still be due to clonal expansion of an infected cell amplified by a burst of local replication. It is possible that this phenomenon is mediated by an infected antigen-specific cell that undergoes amplification due to the presence of the STI.

HIV-1 infection is associated with dysregulation of seminal cytokines (38, 39) as well as an increased semen/blood cytokine ratio (10, 38, 39). This proinflammatory environment has been suggested to increase viral replication, as semen viral load often correlates with cytokine levels (40), as well as the fact that several cytokines, including TNF-α, directly act on the virus to increase replication (41) (reviewed in reference 42). A similar phenomenon is observed in men with classical STI such as gonorrhea or trichomoniasis (20). We analyzed cytokine/chemokine levels in the blood and semen of men with and without STI-associated urethritis to determine whether inflammation increased with the presence of a concurrent STI infection and whether such inflammation had resolved during the 2-week period of follow-up. Among the seven cytokines/chemokines analyzed (IL-6, TNF-α, IL-1β, IFN-γ, CXCL10, IL-10, and CCL2), only TNF-α was significantly increased in the semen of men with STI-associated urethritis, compared to HIV-positive men without urethritis. However, levels of IL-6 and IL-1β were also increased in men with urethritis, though the difference was not statistically significant. Importantly, the levels of TNF-α, IL-6, and IL-1β all decreased to levels similar to those in men without urethritis after STI treatment. Thus, we observed that men with urethritis have an enhanced proinflammatory environment compared to HIV-positive men without urethritis and that this difference is reduced following antibiotic treat-

ment of the STI. As expected, cytokine levels in the blood were similar in men with and without STI-associated urethritis and remained unchanged following STI treatment. This result further supports our finding that inflammation due to STI-associated urethritis does not impact the formation of compartmentalized lineages in the male genital tract.

MATERIALS AND METHODS

Ethics statement and source of clinical samples. Blood and semen samples were collected as part of a study examining the effects of genital tract inflammation on HIV-1 semen viral load (20). The study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. A subset of STI samples (12/19) had previously been examined via a heteroduplex tracking assay (9), and 2/5 control samples had previously been examined via single-genome amplification (SGA) (10).

Deep sequencing with Primer ID. Deep sequencing with Primer ID was performed as previously described (19). Briefly, viral RNA was extracted from seminal and blood plasma using the QIAamp viral RNA extraction kit (Qiagen). Based on viral loads, up to 5,000 RNA copies (range, 196 to 5,000; mean, 3,161) were used for cDNA synthesis. cDNA was synthesized using the *env* V1/V3 Primer ID primer (HXB2 positions 6585 to 7208; 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNCAGTCCATTTTG CTCTACTAATGTTACAATGTGC-3′) and SuperScript III reverse transcriptase (Invitrogen). The final cDNA reaction mixture contained 0.5 mM deoxynucleoside triphosphate (dNTP) mix (KAPA), 0.25 μM V1/V3 reverse primer, 5 mM dithiothreitol (DTT), 6 U RNaseOUT, and 30 U SuperScript III RT in a total volume of 60 μl. Initially, a mixture containing dNTPs, cDNA primer, and RNA template was incubated at 65°C for 5 min, followed by 4°C for 2 min. Then DTT, RNaseOUT, and SuperScript III were added, and the reaction mixtures were incubated for 1 h at 50°C, followed by 1 h at 55°C. Samples were then heated to 70°C for 15 min to inactivate the SuperScript III prior to addition of RNase H (2 units) and subjected to a final incubation at 37°C for 20 min. cDNA was purified using Agencourt RNAclean XP beads (Beckman Coulter) at a bead-to-cDNA volume ratio of 0.6:1. The beads were washed four times with 70% ethanol. Purified cDNA was eluted in 24 μl molecular-grade water (Corning), and the purification was repeated with a bead-to-cDNA ratio of 0.6:1. The purified cDNA was again eluted in 24 μl molecular-grade water and stored at −20°C. All (24 μl) of the cDNA was used for PCR amplification. KAPA 2G Robust HotStart polymerase was used as the first-round PCR enzyme along with the forward primer 5′-GCCTCCCTCGC GCCATCAGAGATGTGTATAAGAGACAGNNNNTTATGGGATCAAAGCCTAAAGCCATGTGTA-3′, corresponding to the HIV-1 *env* V1/V3 region. Following amplification, PCR products were purified using AmpureXP beads (Beckman Coulter) at a bead-to-DNA ratio of 0.7:1. Beads were washed four times using 70% ethanol, and the purified DNA was eluted in 50 μl of DNase-free water (Corning). The second round of PCR consisted of 2 μl of purified first-round PCR product along with the KAPA HiFi Robust polymerase and served to incorporate MiSeq adaptors and index oligonucleotides that allowed for multiplexing of samples.

MiSeq library preparation and quality control. Amplicons were visualized on a 1.2% agarose gel. Gel extraction was performed using the MinElute gel extraction kit (Qiagen) according to the manufacturer's instructions. Purified DNA was eluted in 10 μl of buffer EB (Qiagen) and quantified using the Qubit double-stranded-DNA broad-range assay (Thermo Fisher). Samples were pooled in equimolar concentrations, and the final library was purified using AmpureXP beads at a bead-to-DNA ratio of 0.7:1. Libraries were submitted to the UNC High Throughput Sequencing Facility for Illumina MiSeq 2 × 300-base paired-end sequencing.

Phylogenetic and compartmentalization analyses. Compartmentalization of viral populations was assessed using two tree-based methods: the Slatkin-Maddison (S-M) test (21) and the presence of a genetically diverse, semen-derived lineage. The S-M test was performed on phylogenetic trees that had equal numbers of semen- and blood-derived V1/V3 sequences, after identical sequences in each compartment had been collapsed to focus on diverse populations rather than clonally amplified populations. The standard Slatkin-Maddison test was modified to account for the structure of the tree, with the leaves of each node being permutated sequentially before migrations were inferred (Pond et al., unpublished; https://github.com/veg/hyphy-analyses/tree/master/SlatkinMaddison). Trees were considered compartmentalized if 10,000 permutations of the standard Slatkin-Maddison test or 50,000 permutations of the structured Slatkin-Maddison test yielded a P value of <0.05 and there was a semen-derived, genetically diverse lineage. Both S-M tests are implemented in the standard analysis "sm" in HyPhy v2.5 (43).

Single-genome amplification. Single-genome amplification (SGA), or template endpoint dilution PCR, was performed as previously described (10). Briefly, viral RNA was extracted using a QIAamp viral RNA extraction kit (Qiagen). cDNA was synthesized using an oligo(dT) primer and SuperScript III RT (Invitrogen). Template cDNA was diluted such that <30% of reactions were positive in the subsequent PCR. Nested PCR was performed using Platinum *Taq* High Fidelity polymerase (Invitrogen) and the following primers: for PCR-1, 5′-GGGTTTATTACAGGGACAGCAGAG-3′ (Vif1) and 5′-TAAGCCTCAATAAAG CTTGCCTTGAGTGC-3′ (OFM19); for PCR-2, 5′-GGCTTAGGCATCTCCTATGGCAGGAAGAA-3′ (EnvA) and 5′-ACACAAGGCTACTTCCCTGGATTGGCAG-3′ (EnvN). SGA products were fully sequenced from both directions to confirm the presence of a single template. Amplicons with evidence of multiple templates (i.e., double peaks on the chromatogram) were not used in downstream applications.

Construction of HIV-1 *env* clones. Amplicons of the full-length HIV-1 *env* gene from the first-round PCR with confirmed sequences were subjected to an additional round of PCR using the Phusion Hot-Start

High Fidelity DNA polymerase (Invitrogen) and the primers cEnvA (5′-CACCGGCTTAGGCATCTCCTATAC CAGGAAGAA-3′) and EnvN (5′-CTGCCAATCAGGGAAGTAGCCTTGTGT-3′) following the manufacturer's instructions. HIV-1 *env* amplicons were subsequently gel purified using the Qiagen QIAquick gel extraction kit. An aliquot of 50 ng of purified HIV-1 *env* DNA was used to clone into the pcDNA 3.1D/V5-His-TOPO vector (Invitrogen), and MAX Efficiency Stlb2 competent cells (Life Technology) were transformed per the manufacturer's instructions.

Env-pseudotyped viruses. Env-pseudotyped luciferase reporter viruses were generated by cotransfection of 810 ng of an *env* expression vector and 810 ng of pZM247Fv2Δenv backbone (44) with the Fugene 6 reagent (Promega). The mixture of DNA and Fugene 6 reagent was added to 4.8×10^5 293T cells plated at a density of 2.4×10^5 cells/ml in 6-well plates, following the Fugene 6 protocol. Five hours after transfection, the medium was changed. Forty-eight hours after transfection, the medium was harvested, filtered through a 0.45-μm filter, and aliquoted into 0.6-ml tubes. Aliquots were stored at −80°C until use.

Single-cycle infection of 293-Affinofile cells. The ability of HIV-1 Env proteins to mediate infection of cells expressing low densities of CD4 was assessed as previously described (45–47). Briefly, experiments were carried out in black, flat-bottomed, 96-well plates. A solution of 100 μl of 293-Affinofile cells at a density of 1.8×10^5 cells/ml was added to the inner 60 wells of each 96-well plate. All 293-Affinofile cells were induced to express high levels of CCR5 expression using ponesterone A. CD4 expression was induced in half of the cells using doxycycline. Twenty-four hours after CCR5 and/or CD4 induction, cells were spinoculated (48) with Env-pseudotyped viruses whose titers had been previously determined ($849 \times g$ for 2 h at 37°C). Following spinoculation, cells were incubated at 37°C for 48 h. Cells were then washed twice with phosphate-buffered saline (PBS) and lysed with 1× *Renilla* luciferase assay lysis buffer diluted in distilled water. Following lysis, plates were kept at −80°C overnight. The following day, plates were thawed at room temperature and read using a luminometer. A 50-μl aliquot of *Renilla* assay reagent was injected into the luminometer per well, and relative light units (RLUs) were recorded over 5 s with a 2-s delay.

Cytokine evaluation. Cytokine concentrations in blood plasma and seminal plasma were quantified using a Luminex bead-based multiplex assay (R&D Systems). Specifically, TNF-α, IL-6, CXCL10, IL-10, CCL2, IL-1β, and IFN-γ concentrations were determined. All assays were performed following the manufacturer's instructions.

Data availability. The full-length *env* gene sequences have been deposited in GenBank under accession numbers MT227374 to MT227495. The MiSeq sequences are available under BioProject accession number PRJNA613442.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4.4 MB.

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UNC is pursuing intellectual property protection for Primer ID, and R.S. is listed as a coinventor and has received nominal royalties.

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