

1 **Deep Sequencing Reveals Compartmentalized HIV-1 in the Semen of Men with and without**
2 **STI-associated Urethritis**

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30

31 **Abstract**

32 Concurrent sexually transmitted infections (STI) can increase the probability of HIV-1
33 transmission primarily by increasing the viral load present in semen. In this study, we explored the
34 relationship of HIV-1 in blood and seminal plasma in the presence and absence of urethritis and
35 after treatment of the concurrent STI. Primer ID deep sequencing of the V1/V3 region of the HIV-
36 1 *env* gene was done for paired blood and semen samples from ART-naïve men living in Malawi
37 with (n = 19) and without (n = 5) STI-associated urethritis; for a subset of samples full length *env*
38 genes were generated for sequence analysis and to test entry phenotype. Cytokine concentrations
39 in the blood and semen were also measured, and a reduction in the levels of pro-inflammatory
40 cytokines was observed following STI treatment. We observed no difference in the prevalence of
41 diverse compartmentalized semen-derived lineages in men with or without STI-associated
42 urethritis, and these viral populations were largely stable during STI treatment. Clonal
43 amplification of one or a few viral sequences accounted for nearly 50% of the viral population
44 indicating a recent bottleneck followed by limited viral replication. We documented a case of
45 superinfection where the new strain was restricted to the genital tract. We conclude that the male
46 genital tract is a site where virus can be brought in from the blood, where localized sustained
47 replication can occur, where a superinfecting strain can persist, and where specific genotypes can
48 be amplified perhaps initially by cellular proliferation but further by limited viral replication.

49

50 **Importance**

51 HIV-1 is a sexually transmitted infection that co-exists with other STIs. Here we examine
52 the impact of a concurrent STI resulting in urethritis on the HIV-1 population within the male
53 genital tract. We found that viral populations remain largely stable even with treatment of the STI.

54 These results show that viral populations within the male genital tract are defined by factors
55 beyond transient inflammation associated with a concurrent STI.

56

57

58 **Introduction**

59 Nearly two million new HIV-1 infections occur worldwide every year, predominately
60 through sexual transmission (1). Therefore, understanding the genotypic and phenotypic properties
61 of HIV-1 present in the male genital tract is vital for treatment and prevention strategies. It has
62 been well-established that the probability of sexual transmission of HIV-1 increases with an
63 increasing viral load (2-5), and there are several factors that can influence the concentration of
64 viral RNA present in semen. For example, stage of disease (6), CD4+ T cell count (7), and the
65 presence of inflammatory conditions (such as concurrent sexually transmitted infections [STI])
66 have all been demonstrated to increase the semen viral load (reviewed in (8)).

67 For semen-mediated transmission events, the transmitted/founder virus is most proximal
68 to the male genital tract at the time of transmission. Thus, the origin of virus in the male genital
69 tract is relevant to a fuller understanding of HIV-1 transmission. Often the virus present in semen
70 is similar to virus found in the blood (an equilibrated population), but there is also evidence that
71 the male genital tract is able to support independent replication of HIV-1. This fact is inferred from
72 observations of genetically distinct, or compartmentalized, HIV-1 populations in semen, as
73 compared to the virus found in the blood and other anatomical compartments (9-15). In addition,
74 several studies (16, 17) have reported the presence of HIV-1 RNA in the semen of men on
75 suppressive antiretroviral therapy, with undetectable blood plasma viral loads, implying that the
76 male genital tract can influence viral replication independent of the periphery and harbor an

77 independent viral reservoir. It is therefore important to elucidate the factors that promote the
78 establishment and maintenance of compartmentalized viral lineages in the male genital tract.

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

95

96 **Methods**

97

98 **Ethics Statement and Source of Clinical Samples.** Blood and semen samples were collected as
99 part of a study examining the effects of genital tract inflammation on HIV-1 semen viral load (20).

100 The study was approved by the Institutional Review Board at the University of North Carolina at
101 Chapel Hill. A subset of STI samples (12/19) were previously examined via a heteroduplex
102 tracking assay (9), and 2/5 control samples were previously examined via single genome
103 amplification (SGA) (10).

104

105 **Deep Sequencing with Primer ID.** Deep sequencing with Primer ID was performed as previously
106 described (19). Briefly, viral RNA was extracted from seminal and blood plasma using the
107 QIAamp Viral RNA Extraction Kit (Qiagen). Based on viral loads, up to 5,000 RNA copies (range:
108 196-5,000, mean: 3,161) were used for cDNA synthesis. cDNA was synthesized using the *env*
109 V1/V3 Primer ID primer (HXB2 positions 6585-7208): 5'-

110 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNCAGTCCATTTTGCT

111 CTACTAATGTTACAATGTGC-3' and SuperScript III Reverse Transcriptase (Invitrogen). The
112 final cDNA reaction contained the following: 0.5 mM dNTP mix (KAPA) 0.25 μ M V1-V3 reverse
113 primer, 5 mM DTT, 6 U RNaseOUT, and 30 U SuperScript III RT in a total volume of 60 μ l.

114 Initially a mixture containing dNTPs, cDNA primer and RNA template was incubated at 65°C for
115 5 minutes, followed by 4°C for 2 minutes. Then DTT, RNaseOUT and SuperScript III were added

116 and the reactions were incubated for one hour at 50°C, followed by one hour at 55°C. Samples
117 were then heated to 70°C for 15 minutes to inactivate the SuperScript III prior to addition of RNase

118 H (2 units) and a final incubation at 37°C for 20 minutes. cDNA was purified using Agencourt
119 RNAClean XP beads (Beckman Coulter) at a volume ratio of 0.6:1 beads: cDNA. The beads were

120 washed four times with 70% ethanol. Purified cDNA was eluted in 24 μ l molecular grade water
121 (Corning), and the purification was repeated with a bead:cDNA ratio of 0.6:1. The purified cDNA

122 was again eluted in 24 μ l molecular grade water and stored at -20°C. All of the cDNA (24 μ l) was

123 used for PCR amplification. KAPA 2G Robust HotStart Polymerase was used as the first-round
124 PCR enzyme along with the following forward primer: 5'-
125 GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTTATGGGATCAAAG
126 CCTAAAGCCATGTGTA-3' corresponding to the HIV-1 *env* V1/V3 region. Following
127 amplification, PCR products were purified using AmpureXP beads (Beckman Coulter) at a ratio
128 of 0.7:1 beads: DNA. Beads were washed four times using 70% ethanol, and the purified DNA
129 was eluted in 50 µl of DNase-free water (Corning). The second round of PCR consisted of 2 µl of
130 purified first-round PCR product along with the KAPA HiFi Robust Polymerase enzyme and
131 served to incorporate MiSeq adaptors and index oligonucleotides that allowed for multiplexing of
132 samples.

133
134 **MiSeq Library Preparation and Quality Control.** Amplicons were visualized on a 1.2% agarose
135 gel. Gel extraction was performed using the MinElute Gel Extraction Kit (Qiagen) according to
136 manufacturer's instructions. Purified DNA was eluted in 10 µl of EB Buffer (Qiagen) and
137 quantified using the Qubit dsDNA Broad Range Assay (Thermo Fisher). Samples were pooled in
138 equimolar concentrations and the final library was purified using AmpureXP beads at a ratio of
139 0.7:1 beads: DNA. Libraries were submitted to the UNC High Throughput Sequencing Facility for
140 generation of 2x300 base paired-end reads using the Illumina MiSeq platform.

141
142 **Phylogenetic and Compartmentalization Analyses.** Compartmentalization of viral populations
143 was assessed using two tree-based methods: the Slatkin-Maddison (S-M) test (21) and the presence
144 of a genetically diverse, semen-derived lineage. The S-M test was performed on phylogenetic trees
145 that had equal numbers of semen and blood-derived V1/V3 sequences, after collapsing identical

146 sequences in each compartment to focus on diverse populations rather than clonally amplified
147 populations. The standard Slatkin-Maddison test was modified to account for the structure of the
148 tree, with the leaves of each node being permuted sequentially before inferring migrations (Pond
149 et al, manuscript in preparation, [https://github.com/veg/hyphy-](https://github.com/veg/hyphy-analyses/tree/master/SlatkinMaddison)
150 [analyses/tree/master/SlatkinMaddison](https://github.com/veg/hyphy-analyses/tree/master/SlatkinMaddison)). Trees were considered compartmentalized if 10,000
151 permutations of the Standard Slatkin-Maddison test or 50,000 permutations the Structured Slatkin-
152 Maddison test yielded a p-value <0.05 and there was a semen-derived, genetically diverse lineage.
153 Both S-M tests are implemented in the standard analysis “sm” in HyPhy v2.5.

154

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

168

169 **Construction of HIV-1 *env* clones.** Amplicons of the full-length HIV-1 *env* gene from the first
170 round PCR with confirmed sequences were subjected to an additional round of PCR using the
171 Phusion hot-start high fidelity DNA polymerase (Invitrogen) and the primers cEnvA (5'-
172 CACCGGCTTAGGCATCTCCTATAACCAGGAAGAA-3') and EnvN (5'-
173 CTGCCAATCAGGGAAGTAGCCTTGTGT-3') following the manufacturer's instructions.
174 HIV-1 *env* amplicons were then gel purified using the Qiagen QIAQuick Gel Extraction Kit. An
175 aliquot of 50 ng of purified HIV-1 *env* DNA was used to clone into the pcDNA 3.1D/V5-His-
176 TOPO vector (Invitrogen) and MAX Efficiency Stlb2 competent cells (Life Technology) per the
177 manufacturer's instructions.

178

179 **Env-pseudotyped viruses.** Env-pseudotyped luciferase reporter viruses were generated by co-
180 transfection of 810 ng of an *env* expression vector and 810 ng of pZM247Fv2 Δ env backbone (22)
181 using 293T cells and the Fugene 6 reagent and protocol (Promega). Five hours after transfection,
182 the medium was changed. Forty-eight hours after transfection, the medium was harvested, filtered
183 through a 0.45 μ m filter, and aliquoted into 0.6 ml tubes. Aliquots were stored at -80°C until use.

184

185 **Single-cycle infection of 293-Affinofile cells.** The ability of HIV-1 Env proteins to mediate
186 infection of cells expressing low densities of CD4 was assessed as previously described (23-25).
187 Briefly, experiments were carried out in black, flat-bottomed, 96-well plates. A solution of 100 μ l
188 of 293-Affinofile cells at a density of 1.8×10^5 cells/ml was added to the inner 60 wells of each 96-
189 well plate. All 293-Affinofile cells were induced to express high levels of CCR5 expression using
190 Ponesterone A. CD4 expression was induced in half of the cells using Doxycycline. Twenty-four
191 hours after CCR5 and/or CD4 induction, cells were spinoculated (26) with previously titered Env-

192 pseudotyped viruses (849 x g for 2 hours at 37°C). Following spinoculation, cells were incubated
193 at 37°C for forty-eight hours. Cells were then washed twice with PBS and lysed with 1x Renilla
194 luciferase assay lysis buffer diluted in distilled water. Following lysis, plates were kept at -80°C
195 overnight. The following day, plates were thawed at room temperature and read using a
196 luminometer. A 50 µl aliquot of Renilla assay reagent was injected into the luminometer per well,
197 and relative light units (RLUs) were recorded over 5 seconds with a 2-second delay.

198
199 **Cytokine Evaluation:** Cytokine concentrations in blood plasma and seminal plasma were
200 quantified using a Luminex® bead-based multiplex assay (R&D Systems). Specifically, TNF- α ,
201 IL-6, CXCL10, IL-10, CCL2, IL-1 β , and IFN- γ concentrations were determined. All assays were
202 performed following the manufacturer's instructions.

203
204 **Data availability:** The full length *env* gene sequences will be deposited in GenBank on
205 acceptance and the accession numbers included in proof. The MiSeq sequences will be
206 deposited in the Sequencing Read Archive and the accession numbers included in proof.

207

208

209 **Results**

210

211 **Participant characteristics and sequence generation.** Participants were part of a cohort of men
212 based in Malawi that was established to examine the effect of STI-associated urethritis on seminal
213 plasma HIV-1 viral load (20). In order to examine the relationship between urethritis associated
214 with a concurrent sexually transmitted infection (STI) and the presence of compartmentalized virus

215 in the genital tract, we selected a subset of men with ($n = 19$) and without ($n = 5$) STI-associated
216 urethritis, with the sample size determined by availability of sufficient seminal plasma. All
217 participants were chronically infected with HIV-1, and antiretroviral therapy (ART) naive, as ART
218 was not available in Malawi at the time of the study.

219 Participant characteristics are shown in Table 1. There was no difference in the blood viral
220 load, semen viral load, or CD4+ T cell count between the two groups at baseline. HIV-1 RNA was
221 extracted from paired blood plasma and seminal plasma, and Illumina MiSeq deep sequencing
222 with Primer ID was used to generate HIV-1 *env* V1/V3 amplicons. The deep sequencing output
223 was collapsed into Template Consensus Sequences (TCS) for each Primer ID recovered to create
224 a highly accurate sequence for each original RNA template sampled. An average of 62 TCSs were
225 obtained from each compartment (blood and semen) for each participant (range: 12-200), giving
226 us 95% power to detect minor populations present in most samples at a 1.5-5% frequency.

227
228 **Compartmentalized, semen-derived lineages are observed in men with and without**
229 **urethritis.** As we were primarily interested in identifying diverse compartmentalized lineages,
230 which represent independent replication over a period of time, rather than compartmentalized
231 lineages that consist primarily of clonal sequences, we initially collapsed sequences that were
232 identical to within one nucleotide into a single haplotype. After identical sequences were collapsed,
233 an equal number of blood-derived and semen-derived sequences were used to construct neighbor-
234 joining phylogenetic trees for each participant, allowing us to compare the two populations at
235 equivalent sampling depth. Compartmentalization was assessed using both the Slatkin-Maddison
236 test (21), and the Structured Slatkin-Maddison test (Pond et al, in preparation,
237 <https://github.com/veg/hyphy-analyses/tree/master/SlatkinMaddison>), which has been modified to

238 reduce potentially spurious compartmentalization detection in trees with large numbers of
239 sequences. When both tests resulted in a P value < 0.05 , the tree was deemed compartmentalized.
240 When one test indicated compartmentalization while the other did not, trees were inspected
241 visually for the presence of diverse, semen-dominated lineages.

242 Among the 24 men, we observed varying degrees of compartmentalization, ranging from
243 near-complete separation of blood and semen-derived sequences, to minor compartmentalization
244 in 6/24 (25%) participants. In men with urethritis, compartmentalization was detected in 5/19
245 (26%) men, while viral populations were equilibrated between the blood and semen in 13/19 (68%)
246 men. One individual with urethritis was superinfected, with the superinfecting population
247 constituting a distinct, semen-only lineage. In men without urethritis, we observed minor
248 compartmentalization in 1/5 (20%) individuals, and equilibrated viral populations in 4/5 (80%)
249 individuals. Thus, both compartmentalized and equilibrated HIV-1 populations were found in men
250 with and without urethritis (Figure 1 and Table 2) at statistically indistinguishable frequencies,
251 although the generalizability of this conclusion is limited by the number of samples studied.

252

253 **HIV-1 population dynamics between blood and semen remain largely stable after STI**
254 **treatment.** To examine the effects of antibiotic treatment of the STI on HIV-1 population
255 dynamics, we compared pre-treatment and post-treatment time points in 13 men (12 with urethritis,
256 1 without urethritis). Samples were obtained an average of 12 days after antibiotic treatment had
257 been initiated (range: 7-14 days). Neighbor-joining phylogenetic trees were built as described
258 above, and the relationship between blood and semen-derived sequences (i.e., equilibrated or
259 compartmentalized) was determined at each time point. In 12/13 men, the relationship did not
260 change following STI treatment (Figure 2). In one individual, S101, semen and blood-derived

261 lineages were equilibrated in the pre-STI treatment time point, but compartmentalized post-
262 treatment, as determined by both the Standard Slatkin-Maddison test and the Structured Slatkin-
263 Maddison test ($p < 0.0001$, Table 2).

264 Next, we compared within-compartment viral diversity before and after STI treatment. To
265 this end, we inferred neighbor-joining phylogenetic trees containing equal numbers of semen-
266 derived sequences from the pre and post STI treatment time points for the 13 men described above.
267 Both the Standard Slatkin-Maddison and the Structured Slatkin-Maddison tests were performed
268 on the trees in order to determine whether the pre- and post-STI treatment semen sequences
269 constituted distinct clades. In 12 of 13 men, the semen populations before and after STI treatment
270 were not significantly different from one another – i.e., populations that existed before STI
271 treatment were still readily observable after STI treatment. Of particular interest were the
272 individuals with compartmentalized, semen-derived lineages. In 2 of the 3 men with
273 compartmentalized lineages at both time points, the lineage that was responsible for the
274 compartmentalization was the same before and after STI treatment (Figure 2A and 2B). Thus, not
275 only was the relationship between compartments unchanged, but the specific lineages themselves
276 persisted. However, in one individual the compartmentalized, semen-derived lineage that was
277 detected before STI treatment was not detected at the second time point but a new
278 compartmentalized lineage was observed (Figure 2C).

279

280 **Clonal amplification of blood and semen-derived sequences is observed in men with and**
281 **without urethritis.** As we were primarily interested in the presence of diverse, compartmentalized
282 lineages, rather than compartmentalized lineages comprised of a clonally expanded population, we
283 collapsed sequences that were identical to within one nucleotide into a single haplotype. In doing

284 so, we observed that a large proportion of both blood and semen-derived V1/V3 sequences were
285 identical or nearly identical. Such an observation could be made because of the PCR amplification
286 step prior to sequencing where the original templates are repetitively sequenced, a phenomenon
287 called PCR resampling; however, the use of Primer ID to tag each original templates before PCR
288 avoids this problem allowing us to infer the presence of identical or near identical sequences within
289 the viral population in vivo. For blood-derived sequences, a mean of only 41% and 48% of
290 sequences were unique in men with and without urethritis, respectively ($p = 0.4237$, Figure 3A).
291 For semen-derived sequences, a mean of only 44% and 62% of sequences were unique in men
292 with and without urethritis, respectively ($p = 0.086$, Figure 3C). The proportion of unique
293 sequences observed in blood and semen remained stable before and after STI treatment in men
294 with urethritis (Figure 3B and 3D). This result indicates that a significant fraction of the population
295 in each compartment was in a genetic bottleneck or had recently gone through a bottleneck.

296 We considered the possibility that the short *env* V1/V3 amplicon (527 bases) would over-
297 estimate the percentage of sequences that were identical across the entirety of *env*. To evaluate this
298 possibility, we performed single genome amplification (SGA) of full-length HIV-1 *env* genes
299 (~2500 bases) from the blood and semen of four men (3 with urethritis and 1 without). We obtained
300 an average of 30 full-length *env* sequences from each participant. In two of the four cases, we
301 observed identical sequences. When we trimmed the full-length sequences and analyzed only the
302 V1/V3 region used in our deep sequencing, we observed identical or nearly identical sequences in
303 all four participants. Sequences that were identical in the V1/V3 region but different across the
304 entire envelope had only a few nucleotide changes between them, consistent with the low-level
305 diversity generated from recent viral replication from a unique ancestor/bottleneck (Figure 4, and
306 Supplemental Figures 1-3). Thus, while examining only the V1/V3 region does increase the

307 number of sequences that appear identical, the overall viral diversity of those variants is low and
308 consistent with recent clonal expansion involving a bottleneck with subsequent viral replication to
309 introduce modest diversity. In a control experiment we generated 8 *env* amplicons from virus
310 produced from the cell line 8E5, which contains a single defective viral genome. When the 8
311 amplicons were sequenced we observed a single substitution mutation and a single frameshift
312 mutation (data not shown). The low-level diversity observed in the viral populations in vivo were
313 in most cases greater than the level observed in the control amplification, consistent with ongoing
314 viral replication after a recent bottleneck rather than just virus production from a clonally expanded
315 cell.

316

317 **Semen-derived HIV-1 envelopes are T-cell tropic.** HIV-1 primarily infects CD4⁺ T cells, which
318 have a high density of the CD4 protein on their cell surface that is typically required by the virus
319 for efficient entry. However, viruses that have been replicating independently in anatomically
320 distinct regions such as the central nervous system where CD4⁺ T cells are less abundant, can
321 evolve the ability to enter cells expressing lower densities of CD4, such as macrophages. This has
322 been observed for compartmentalized lineages derived from both the CNS (27) and, in one case,
323 the male genital tract (28). In order to determine whether compartmentalized, semen-derived
324 lineages from our cohort have the ability to enter cells expressing a low density of CD4, we
325 performed SGA of full-length HIV-1 *env* genes using viral RNA as the template for cDNA
326 synthesis followed by PCR done at template end-point dilution. Amplicons were sequenced to
327 ensure that a single cDNA template initiated each amplification. A subset of the semen-derived
328 HIV-1 *env* gene amplicons were cloned into an expression vector then used to pseudotype a virus
329 made by cotransfecting with a Δenv HIV-1 backbone plasmid with a *renilla* luciferase reporter in

330 order to produce pseudotyped virus that expressed participant-derived Env surface proteins.
331 Pseudotyped virus was used to infect Affinofile cells that had been induced to express either high
332 or low densities of CD4. The amount of luciferase produced by the cells was quantified and used
333 as a surrogate measure of infectivity. As shown in Figure 5, semen-derived HIV-1 *env* genes, from
334 both compartmentalized and equilibrated lineages, encoded Env proteins that require a high
335 density of CD4 for efficient cell entry, indicating that they were being selected for replication in T
336 cells.

337
338 **Cytokine/chemokine dynamics during treatment of the STI.** To better understand the
339 magnitude of the inflammation present within the genital tract during a concurrent sexually
340 transmitted infection, we measured the concentrations of seven inflammatory cytokines and
341 chemokines present in the blood and semen before and after treatment of the STI. To differentiate
342 between STI-induced inflammation and HIV-induced inflammation, we included samples from
343 HIV+ individuals not experiencing urethritis. As shown in Figure 6A, there was a group of
344 cytokines (TNF- α , IL-6, and IL-1 β) whose concentrations were increased in the semen of men
345 with urethritis at the pre-treatment time point, and subsequently decreased after STI treatment. A
346 second group of cytokines/chemokines, including CXCL10, IL-10, IFN- γ , and CCL2, were at
347 similar concentrations in men with and without urethritis, as well as before and after STI treatment.
348 A subset of four cytokines/chemokines (TNF- α , IL-10, CCL2 and CXCL10) were measured in
349 blood as well (Figure 6B). There was no difference in the concentration of any of these analytes at
350 any time point in men with or without urethritis, suggesting that STI-associated inflammation is
351 limited to the genital tract and largely resolves with antibiotic treatment.

352

353 **Identification and characterization of a super-infection initiated in the male genital tract.** In

354 one participant with urethritis, S031, we observed a distinct, semen-only lineage in both the pre-
355 and post-STI treatment time points (Figure 7A). Though the separate, semen-only lineage persisted
356 across two time points, we did note that fewer semen-derived sequences were in semen-only clades
357 in the second time point (post-STI treatment) as compared to the first time point (pre-STI
358 treatment), suggesting that viral populations in the blood had been mixing with viral populations
359 in the genital tract. Importantly, we observed semen-derived sequences that clustered with the
360 blood-derived sequences at both time points, thus making contamination or sample mis-labeling
361 an unlikely explanation for our observation (Figure 7A). When we constructed a neighbor-joining
362 phylogenetic tree using blood- and semen-derived sequences from this participant and four others,
363 we noted that the semen-derived sequences from S031 were as distinct from the blood-derived
364 sequences as all participants were from one another, suggesting the presence of a superinfection
365 (Figure 7B). A highlighter plot was used to identify the presence of recombinant lineages within
366 the blood- and semen-derived sequences (Figure 7C), further supporting the notion of
367 superinfection.

368

369

370 **Discussion**

371

372 There have been numerous studies (12-14, 29-33) that have examined the prevalence of
373 male genital tract compartmentalization of HIV-1, sometimes with discordant results. Some of
374 these studies (11, 29, 30) examined the phenomenon of compartmentalization through the use of
375 bulk amplification and/or cloning prior to sequencing; however these approaches have been shown

376 to introduce sequencing artifacts, such as PCR-mediated recombination and sequence resampling
377 (34-36). The use of deep sequencing with Primer ID in the current study corrects for PCR and
378 sequencing errors through the creation of a template consensus sequence for each Primer ID-
379 tagged cDNA (the template for PCR), while simultaneously allowing for the precise quantification
380 of the total number of templates sequenced, i.e. the sample size of sampling of the viral sequence
381 population (19). Thus, we can be confident that the viral variants we analyze are an accurate
382 representation of the diversity found *in vivo*.

383 The observation that STI-associated urethritis does not significantly impact the degree of
384 HIV-1 compartmentalization within the male genital tract raises several points. First, the
385 mechanism underlying the establishment of compartmentalized lineages within the male genital
386 tract remains unknown. This study sought to compare two possibilities: that STI-associated
387 inflammation would lead to compartmentalized replication, or alternatively serve to recruit HIV-
388 infected CD4+ T cells into the genital tract, thereby equilibrating the viral populations found in
389 the blood and semen. We observed compartmentalization in 26% of men with urethritis, and 20%
390 of men without urethritis; thus, given this number of participants we did not detect a difference in
391 the extent of compartmentalization with and without and STI-associated urethritis. In the overall
392 cohort, we observed compartmentalization in the genital tract of 25% of men. This prevalence of
393 compartmentalized lineages in the genital tract is similar to what was observed in a previous study
394 (9) that used a heteroduplex tracking assay to examine the relationship between blood and semen-
395 derived *env* V3 populations in men with and without urethritis. In this earlier study, they observed
396 discordant V3 populations between the blood and semen of 40% of men. Importantly, there was
397 no difference in the V3 population dynamics between the blood and semen of men with urethritis,
398 compared to men without urethritis. Later, Anderson and colleagues (10) utilized single genome

399 amplification to examine the relationship between blood and semen-derived HIV-1 envelopes in
400 men without urethritis. Here, they reported a 31% prevalence of compartmentalization in the
401 genital tract. They also observed clonal amplification in the semen of men without urethritis.
402 Compartmentalized populations in the genital tract have also been observed in the context of acute
403 HIV-1 infection. In a recent study by Chaillon *et al.* (31), deep sequencing was used to examine
404 HIV-1 populations in blood and semen in early infection. They observed compartmentalization in
405 2 of 6 participants at baseline (a median of 81 days after the estimated date of infection).

406 The second noteworthy point pertains to the source of HIV-1 shed in the semen. HIV-1
407 (12, 13, 37) and/or SIV (38) RNA has been recovered from a variety of male genital tract tissues
408 including the urethra, prostate, testis, seminal vesicles, vas deferens and epididymis. Our
409 observation that inflammation does not alter the frequency with which we detect semen-specific
410 HIV-1 lineages suggests that, when compartmentalized lineages are present, they are most likely
411 produced by cells in anatomical areas that are not in direct contact with the periphery. In one
412 extreme case of this type of isolation we previously observed the presence of a macrophage-tropic
413 variant in semen (28) which suggests that in this case there was sufficient depletion of CD4⁺ T
414 cells, without replenishment, that the virus evolved to expand its target cell specificity. In the
415 current study all of the viruses tested were T cell-tropic, requiring a high density of CD4 for
416 efficient entry into cells. In addition, all were predicted to use CCR5 as a coreceptor based on
417 genotypic predictions of the V3 loop sequence (data not shown). This result is important as a recent
418 report by Ganor and colleagues (39) reported the presence of macrophage-tropic viral variants in
419 urethral tissues, suggesting the possibility of a urethral reservoir. However, it appears such variants
420 are not shed in the semen.

421 Compartmentalization in the male (30, 31) genital tract has largely been defined as a
422 transient phenomenon. Here, we examined how antibiotic treatment of a concurrent sexually
423 transmitted infection (primarily gonorrhea or trichomonas) impacted the relationship between
424 blood- and semen-derived HIV-1 *env* V1/V3 sequences. We found that viral variants present
425 before STI treatment remained detectable after STI treatment, and furthermore, that the
426 relationship between blood and semen-derived sequences remained consistent throughout the
427 course of STI co-infection. In only one participant out of 13 did we detect a change in the
428 relationship between blood and semen-derived sequences over time. In this instance, the depth of
429 sampling pre-STI treatment was relatively poor, with only 13 V1/V3 sequences recovered per
430 compartment, while the sampling post-STI treatment was much greater (103 sequences per
431 compartment). Thus, it is quite possible that the relatively few sequences obtained pre-treatment
432 obscured the presence of the compartmentalized lineage that we observed post-treatment. It is also
433 important to note that while gonococcal infections are cleared rapidly from the urogenital tract
434 after a single antibiotic treatment (40), the underlying immune activation can persist, as
435 demonstrated by the fact that in men with an STI, HIV-1 viral loads in semen were still higher
436 than in men without an STI, even after effective antibiotic treatment (20), although we were able
437 to measure some diminution of inflammation with a change in some inflammatory markers.
438 Therefore, while we do observe stable relationships between blood and semen-derived sequences
439 both before and after STI treatment, our conclusions are limited by the relatively short period of
440 follow-up. It is worth noting that in one participant, virus in the semen was compartmentalized
441 relative to the blood both before and after STI treatment but the compartmentalized lineage in the
442 semen changed between the two time points. Both lineages, while minor, were complex in
443 sequence composition and thus the latter one did not evolve over the short period of time between

444 the two samplings. Thus, there must have been reduced production of one lineage and the
445 appearance of a pre-existing lineage over a relatively short period of time.

446 Given the error-prone nature of HIV-1 reverse transcription (41), a single
447 transmitted/founder viral variant rapidly evolves into a diverse population within an infected
448 individual (42-44). As such, the identification of identical or nearly identical sequences in the
449 blood in chronic untreated infection is relatively infrequent. There are two mechanisms to consider
450 that can explain the presence of such sequences. In people on therapy there can be low level
451 production of virus particles with identical sequences and this is thought to be due to clonal
452 expansion of an infected cell (45-47) some of which can produce a low level of infectious virus
453 (48, 49). In the absence of therapy, the viral load of viruses with similar sequences is much higher,
454 suggesting either that the corresponding cellular expansion is much greater or that the virus comes
455 from another source, i.e. replication, after passing through a recent genetic bottleneck. This
456 question becomes even more relevant using the shorter amplicon associated with deep sequencing
457 as a significant fraction of the viral sequences cluster into lineages of identical sequences. In order
458 to determine if the identical sequences from deep sequencing observed off therapy in these men
459 were truly clonal, we compared sequences obtained from deep sequencing to those obtained as full
460 length *env* genes using template end-point dilution PCR (SGA). We found that the sequences that
461 were identical in the deep sequencing data set were in a population of similar but not identical
462 sequences when the larger region of the genome was analyzed (Figure 4, Supplemental Figure 1-
463 3). We conclude that these populations are present at their detected level due to ongoing viral
464 replication. However, the high level of similarity in these sequences implies a recent genetic
465 bottleneck prior to expansion by viral replication, although the nature of that bottleneck remains
466 unknown and could still be due to clonal expansion of an infected cell amplified by a burst of local

467 replication. It is possible that this phenomenon is mediated by an infected antigen-specific cell that
468 undergoes amplification due to the presence of the STI.

469 HIV-1 infection is associated with dysregulation of seminal cytokines (50, 51) as well as
470 an increased semen: blood cytokine ratio (10, 50, 51). This pro-inflammatory environment has
471 been suggested to increase viral replication as semen viral load often correlates with cytokine
472 levels (52), as well as the fact that several cytokines, including TNF- α , directly act on the virus to
473 increase replication (53) (reviewed in (54)). A similar phenomenon is observed in men with
474 classical STIs such as gonorrhea or trichomonas (20). We analyzed cytokine/chemokine levels in
475 the blood and semen of men with and without STI-associated urethritis to determine if
476 inflammation increased with the presence of a concurrent STI infection and whether such
477 inflammation had resolved during the two-week period of follow-up. Among the seven
478 cytokines/chemokines analyzed (IL-6, TNF- α , IL-1 β , IFN- γ , CXCL10, IL-10 and CCL2) only
479 TNF- α was significantly increased in the semen of men with STI-associated urethritis, compared
480 to HIV-positive men without urethritis. However, levels of IL-6 and IL-1 β were also increased in
481 men with urethritis, though the difference was not statistically significant. Importantly, the levels
482 of TNF- α , IL-6 and IL-1 β all decreased to levels similar to that of men without urethritis after STI
483 treatment. Thus, we observed that men with urethritis have an enhanced pro-inflammatory
484 environment compared to HIV-positive men without urethritis, and that this difference is reduced
485 following antibiotic treatment of the STI. As expected, cytokine levels in the blood were similar
486 in men with and without STI-associated urethritis and remained unchanged following STI
487 treatment. This result further supports our finding that inflammation due to STI-associated
488 urethritis does not impact the formation of compartmentalized lineages in the male genital tract.

489

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513 References

- 514 1. UNAIDS. 2019. UNAIDS Global AIDS Update 2019.
- 515 2. Quinn TC, Wawer MJ, Sewankambo N, Serwadda D, Li C, Wabwire-Mangen F, Meehan
516 MO, Lutalo T, Gray RH. 2000. Viral load and heterosexual transmission of human
517 immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med* 342:921-929.
- 518 3. Gray RH, Wawer MJ, Brookmeyer R, Sewankambo NK, Serwadda D, Wabwire-Mangen
519 F, Lutalo T, Li X, vanCott T, Quinn TC, Rakai Project T. 2001. Probability of HIV-1
520 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in
521 Rakai, Uganda. *Lancet* 357:1149-1153.
- 522 4. Wawer MJ, Gray RH, Sewankambo NK, Serwadda D, Li X, Laeyendecker O, Kiwanuka
523 N, Kigozi G, Kiddugavu M, Lutalo T, Nalugoda F, Wabwire-Mangen F, Meehan MP,
524 Quinn TC. 2005. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection,
525 in Rakai, Uganda. *J Infect Dis* 191:1403-1409.
- 526 5. Baeten JM, Kahle E, Lingappa JR, Coombs RW, Delany-Moretlwe S, Nakku-Joloba E,
527 Mugo NR, Wald A, Corey L, Donnell D, Campbell MS, Mullins JI, Celum C, Partners in
528 Prevention HSVHIVTST. 2011. Genital HIV-1 RNA predicts risk of heterosexual HIV-1
529 transmission. *Sci Transl Med* 3:77ra29.
- 530 6. de Vincenzi I. 1994. A longitudinal study of human immunodeficiency virus transmission
531 by heterosexual partners. European Study Group on Heterosexual Transmission of HIV.
532 *N Engl J Med* 331:341-346.
- 533 7. Dyer JR, Eron JJ, Hoffman IF, Kazembe P, Vernazza PL, Nkata E, Costello Daly C,
534 Fiscus SA, Cohen MS. 1998. Association of CD4 cell depletion and elevated blood and

- 535 seminal plasma human immunodeficiency virus type 1 (HIV-1) RNA concentrations with
536 genital ulcer disease in HIV-1-infected men in Malawi. *J Infect Dis* 177:224-227.
- 537 8. Galvin SR, Cohen MS. 2004. The role of sexually transmitted diseases in HIV
538 transmission. *Nat Rev Microbiol* 2:33-42.
- 539 9. Ping LH, Cohen MS, Hoffman I, Vernazza P, Seillier-Moiseiwitsch F, Chakraborty H,
540 Kazembe P, Zimba D, Maida M, Fiscus SA, Eron JJ, Swanstrom R, Nelson JA. 2000.
541 Effects of genital tract inflammation on human immunodeficiency virus type 1 V3
542 populations in blood and semen. *J Virol* 74:8946-8952.
- 543 10. Anderson JA, Ping LH, Dibben O, Jabara CB, Arney L, Kincer L, Tang Y, Hobbs M,
544 Hoffman I, Kazembe P, Jones CD, Borrow P, Fiscus S, Cohen MS, Swanstrom R, Center
545 for HIVAVI. 2010. HIV-1 Populations in Semen Arise through Multiple Mechanisms.
546 *PLoS Pathog* 6:e1001053.
- 547 11. Gupta P, Leroux C, Patterson BK, Kingsley L, Rinaldo C, Ding M, Chen Y, Kulka K,
548 Buchanan W, McKeon B, Montelaro R. 2000. Human immunodeficiency virus type 1
549 shedding pattern in semen correlates with the compartmentalization of viral Quasi species
550 between blood and semen. *J Infect Dis* 182:79-87.
- 551 12. Coombs RW, Lockhart D, Ross SO, Deutsch L, Dragavon J, Diem K, Hooton TM,
552 Collier AC, Corey L, Krieger JN. 2006. Lower genitourinary tract sources of seminal
553 HIV. *J Acquir Immune Defic Syndr* 41:430-438.
- 554 13. Diem K, Nickle DC, Motoshige A, Fox A, Ross S, Mullins JI, Corey L, Coombs RW,
555 Krieger JN. 2008. Male genital tract compartmentalization of human immunodeficiency
556 virus type 1 (HIV). *AIDS Res Hum Retroviruses* 24:561-571.

- 557 14. Gianella S, Mehta SR, Strain MC, Young JA, Vargas MV, Little SJ, Richman DD,
558 Kosakovsky Pond SL, Smith DM. 2012. Impact of seminal cytomegalovirus replication
559 on HIV-1 dynamics between blood and semen. *J Med Virol* 84:1703-1709.
- 560 15. Delwart EL, Mullins JI, Gupta P, Learn GH, Jr., Holodniy M, Katzenstein D, Walker BD,
561 Singh MK. 1998. Human immunodeficiency virus type 1 populations in blood and
562 semen. *J Virol* 72:617-623.
- 563 16. Pasquier C, Walschaerts M, Raymond S, Moinard N, Saune K, Daudin M, Izopet J,
564 Bujan L. 2017. Patterns of residual HIV-1 RNA shedding in the seminal plasma of
565 patients on effective antiretroviral therapy. *Basic Clin Androl* 27:17.
- 566 17. Politch JA, Mayer KH, Welles SL, O'Brien WX, Xu C, Bowman FP, Anderson DJ. 2012.
567 Highly active antiretroviral therapy does not completely suppress HIV in semen of
568 sexually active HIV-infected men who have sex with men. *AIDS* 26:1535-1543.
- 569 18. Jabara CB, Jones CD, Roach J, Anderson JA, Swanstrom R. 2011. Accurate sampling
570 and deep sequencing of the HIV-1 protease gene using a Primer ID. *Proc Natl Acad Sci*
571 *U S A* 108:20166-71.
- 572 19. Zhou S, Jones C, Mieczkowski P, Swanstrom R. 2015. Primer ID Validates Template
573 Sampling Depth and Greatly Reduces the Error Rate of Next-Generation Sequencing of
574 HIV-1 Genomic RNA Populations. *J Virol* 89:8540-8555.
- 575 20. Cohen MS, Hoffman IF, Royce RA, Kazembe P, Dyer JR, Daly CC, Zimba D, Vernazza
576 PL, Maida M, Fiscus SA, Eron JJ, Jr. 1997. Reduction of concentration of HIV-1 in
577 semen after treatment of urethritis: implications for prevention of sexual transmission of
578 HIV-1. AIDSCAP Malawi Research Group. *Lancet* 349:1868-1873.

- 579 21. Slatkin M, Maddison WP. 1989. A cladistic measure of gene flow inferred from the
580 phylogenies of alleles. *Genetics* 123:603-613.
- 581 22. Ping LH, Joseph SB, Anderson JA, Abrahams MR, Salazar-Gonzalez JF, Kincer LP,
582 Treurnicht FK, Arney L, Ojeda S, Zhang M, Keys J, Potter EL, Chu H, Moore P, Salazar
583 MG, Iyer S, Jabara C, Kirchherr J, Mapanje C, Ngandu N, Seoighe C, Hoffman I, Gao F,
584 Tang Y, Labranche C, Lee B, Saville A, Vermeulen M, Fiscus S, Morris L, Karim SA,
585 Haynes BF, Shaw GM, Korber BT, Hahn BH, Cohen MS, Montefiori D, Williamson C,
586 Swanstrom R, Study CAI, the Center for HIVAVIC. 2013. Comparison of viral Env
587 proteins from acute and chronic infections with subtype C human immunodeficiency
588 virus type 1 identifies differences in glycosylation and CCR5 utilization and suggests a
589 new strategy for immunogen design. *J Virol* 87:7218-7233.
- 590 23. Joseph SB, Lee B, Swanstrom R. 2014. Affinofile Assay for Identifying Macrophage-
591 Tropic HIV-1. *Bio Protoc* 4.
- 592 24. Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW. 1999. Quantification of CD4,
593 CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially
594 conditioned monocyte-derived macrophages. *Proc Natl Acad Sci U S A* 96:5215-5220.
- 595 25. Joseph SB, Arrildt KT, Swanstrom AE, Schnell G, Lee B, Hoxie JA, Swanstrom R. 2014.
596 Quantification of entry phenotypes of macrophage-tropic HIV-1 across a wide range of
597 CD4 densities. *J Virol* 88:1858-1869.
- 598 26. O'Doherty U, Swiggard WJ, Malim MH. 2000. Human immunodeficiency virus type 1
599 spinoculation enhances infection through virus binding. *J Virol* 74:10074-10080.
- 600 27. Schnell G, Joseph S, Spudich S, Price RW, Swanstrom R. 2011. HIV-1 replication in the
601 central nervous system occurs in two distinct cell types. *PLoS Pathog* 7:e1002286.

- 602 28. Bednar MM, Hauser BM, Ping LH, Dukhovlina E, Zhou S, Arrildt KT, Hoffman IF,
603 Eron JJ, Cohen MS, Swanstrom R. 2015. R5 Macrophage-Tropic HIV-1 in the Male
604 Genital Tract. *J Virol* 89:10688-10692.
- 605 29. Paranjpe S, Craigo J, Patterson B, Ding M, Barroso P, Harrison L, Montelaro R, Gupta P.
606 2002. Subcompartmentalization of HIV-1 quasiespecies between seminal cells and
607 seminal plasma indicates their origin in distinct genital tissues. *AIDS Res Hum*
608 *Retroviruses* 18:1271-1280.
- 609 30. Chaillon A, Gianella S, Wertheim JO, Richman DD, Mehta SR, Smith DM. 2014. HIV
610 migration between blood and cerebrospinal fluid or semen over time. *J Infect Dis*
611 209:1642-1652.
- 612 31. Chaillon A, Smith DM, Vanpouille C, Lisco A, Jordan P, Caballero G, Vargas M,
613 Gianella S, Mehta SR. 2017. HIV Trafficking Between Blood and Semen During Early
614 Untreated HIV Infection. *J Acquir Immune Defic Syndr* 74:95-102.
- 615 32. Cheret A, Durier C, Melard A, Ploquin M, Heitzmann J, Lecroux C, Avettand-Fenoel V,
616 David L, Pialoux G, Chennebault JM, Muller-Trutwin M, Goujard C, Rouzioux C, Meyer
617 L, group AOs. 2017. Impact of early cART on HIV blood and semen compartments at the
618 time of primary infection. *PLoS One* 12:e0180191.
- 619 33. Boeras DI, Hraber PT, Hurlston M, Evans-Strickfaden T, Bhattacharya T, Giorgi EE,
620 Mulenga J, Karita E, Korber BT, Allen S, Hart CE, Derdeyn CA, Hunter E. 2011. Role of
621 donor genital tract HIV-1 diversity in the transmission bottleneck. *Proc Natl Acad Sci U*
622 *S A* 108:E1156-63.
- 623 34. Meyerhans A, Vartanian JP, Wain-Hobson S. 1990. DNA recombination during PCR.
624 *Nucleic Acids Res* 18:1687-1691.

- 625 35. Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn
626 CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J, Anderson JA, Swanstrom R,
627 Haynes BF, Athreya GS, Korber BT, Sharp PM, Shaw GM, Hahn BH. 2008. Deciphering
628 human immunodeficiency virus type 1 transmission and early envelope diversification by
629 single-genome amplification and sequencing. *J Virol* 82:3952-3970.
- 630 36. Liu SL, Rodrigo AG, Shankarappa R, Learn GH, Hsu L, Davidov O, Zhao LP, Mullins
631 JI. 1996. HIV quasispecies and resampling. *Science* 273:415-416.
- 632 37. Houzet L, Matusali G, Dejuq-Rainsford N. 2014. Origins of HIV-infected leukocytes
633 and virions in semen. *J Infect Dis* 210 Suppl 3:S622-30.
- 634 38. Houzet L, Perez-Losada M, Matusali G, Deleage C, Dereuddre-Bosquet N, Satie AP,
635 Aubry F, Becker E, Jegou B, Le Grand R, Keele BF, Crandall KA, Dejuq-Rainsford N.
636 2018. Seminal SIV in chronically-infected cynomolgus macaques is dominated by virus
637 originating from multiple genital organs. *J Virol* doi:10.1128/JVI.00133-18.
- 638 39. Ganor Y, Real F, Sennepin A, Dutertre CA, Prevedel L, Xu L, Tudor D, Charmeteau B,
639 Couedel-Courteille A, Marion S, Zenak AR, Jourdain JP, Zhou Z, Schmitt A, Capron C,
640 Eugenin EA, Cheynier R, Revol M, Cristofari S, Hosmalin A, Bomsel M. 2019. HIV-1
641 reservoirs in urethral macrophages of patients under suppressive antiretroviral therapy.
642 *Nat Microbiol* 4:633-644.
- 643 40. Haizlip J, Isbey SF, Hamilton HA, Jerse AE, Leone PA, Davis RH, Cohen MS. 1995.
644 Time required for elimination of *Neisseria gonorrhoeae* from the urogenital tract in men
645 with symptomatic urethritis: comparison of oral and intramuscular single-dose therapy.
646 *Sex Transm Dis* 22:145-148.

- 647 41. Mansky LM, Temin HM. 1995. Lower in vivo mutation rate of human immunodeficiency
648 virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol*
649 69:5087-5094.
- 650 42. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C,
651 Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH,
652 Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart
653 EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee
654 HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw
655 GM. 2008. Identification and characterization of transmitted and early founder virus
656 envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 105:7552-7557.
- 657 43. Joseph SB, Swanstrom R, Kashuba AD, Cohen MS. 2015. Bottlenecks in HIV-1
658 transmission: insights from the study of founder viruses. *Nat Rev Microbiol* 13:414-425.
- 659 44. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Gnanou VV, Keele
660 BF, Learn GH, Turnbull EL, Salazar MG, Weinhold KJ, Moore S, B CCC, Letvin N,
661 Haynes BF, Cohen MS, Hraber P, Bhattacharya T, Borrow P, Perelson AS, Hahn BH,
662 Shaw GM, Korber BT, McMichael AJ. 2009. The first T cell response to
663 transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J*
664 *Exp Med* 206:1253-1272.
- 665 45. Maldarelli F, Wu X, Su L, Simonetti FR, Shao W, Hill S, Spindler J, Ferris AL, Mellors
666 JW, Kearney MF, Coffin JM, Hughes SH. 2014. HIV latency. Specific HIV integration
667 sites are linked to clonal expansion and persistence of infected cells. *Science* 345:179-
668 183.

- 669 46. Wagner TA, McLaughlin S, Garg K, Cheung CY, Larsen BB, Styrchak S, Huang HC,
670 Edlefsen PT, Mullins JI, Frenkel LM. 2014. HIV latency. Proliferation of cells with HIV
671 integrated into cancer genes contributes to persistent infection. *Science* 345:570-573.
- 672 47. Cohn LB, Silva IT, Oliveira TY, Rosales RA, Parrish EH, Learn GH, Hahn BH,
673 Czartoski JL, McElrath MJ, Lehmann C, Klein F, Caskey M, Walker BD, Siliciano JD,
674 Siliciano RF, Jankovic M, Nussenzweig MC. 2015. HIV-1 integration landscape during
675 latent and active infection. *Cell* 160:420-432.
- 676 48. Simonetti FR, Sobolewski MD, Fyne E, Shao W, Spindler J, Hattori J, Anderson EM,
677 Watters SA, Hill S, Wu X, Wells D, Su L, Luke BT, Halvas EK, Besson G, Penrose KJ,
678 Yang Z, Kwan RW, Van Waes C, Uldrick T, Citrin DE, Kovacs J, Polis MA, Rehm CA,
679 Gorelick R, Piatak M, Keele BF, Kearney MF, Coffin JM, Hughes SH, Mellors JW,
680 Maldarelli F. 2016. Clonally expanded CD4+ T cells can produce infectious HIV-1 in
681 vivo. *Proc Natl Acad Sci U S A* 113:1883-1888.
- 682 49. Wang Z, Gurule EE, Brennan TP, Gerold JM, Kwon KJ, Hosmane NN, Kumar MR, Beg
683 SA, Capoferri AA, Ray SC, Ho YC, Hill AL, Siliciano JD, Siliciano RF. 2018. Expanded
684 cellular clones carrying replication-competent HIV-1 persist, wax, and wane. *Proc Natl*
685 *Acad Sci U S A* 115:E2575-E2584.
- 686 50. Vanpouille C, Introini A, Morris SR, Margolis L, Daar ES, Dube MP, Little SJ, Smith
687 DM, Lisco A, Gianella S. 2016. Distinct cytokine/chemokine network in semen and
688 blood characterize different stages of HIV infection. *AIDS* 30:193-201.
- 689 51. Lisco A, Munawwar A, Introini A, Vanpouille C, Saba E, Feng X, Grivel JC, Singh S,
690 Margolis L. 2012. Semen of HIV-1-infected individuals: local shedding of herpesviruses
691 and reprogrammed cytokine network. *J Infect Dis* 205:97-105.

- 692 52. Osborne BJW, Marsh AK, Huibner S, Shahabi K, Liu C, Contente T, Nagelkerke NJD,
693 Kovacs C, Benko E, Price L, MacDonald KS, Kaul R. 2017. Clinical and Mucosal
694 Immune Correlates of HIV-1 Semen Levels in Antiretroviral-Naive Men. *Open Forum*
695 *Infect Dis* 4:ofx033.
- 696 53. Kinter AL, Poli G, Fox L, Hardy E, Fauci AS. 1995. HIV replication in IL-2-stimulated
697 peripheral blood mononuclear cells is driven in an autocrine/paracrine manner by
698 endogenous cytokines. *J Immunol* 154:2448-2459.
- 699 54. Pasquereau S, Kumar A, Herbein G. 2017. Targeting TNF and TNF Receptor Pathway in
700 HIV-1 Infection: from Immune Activation to Viral Reservoirs. *Viruses* 9.
701

	Before STI Treatment		After STI Treatment		CD4 Count ^a	Diagnosed STI ^b
	Plasma Viral Load (log ₁₀ copies/mL)	Semen Viral Load (log ₁₀ copies/mL)	Plasma Viral Load (log ₁₀ copies/mL)	Semen Viral Load (copies/mL)		
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

Table 1. Relevant clinical information for the participants analyzed. ^aCells per microliter of blood. ^bDiagnosed STI. Gon, gonorrhea; Tri, trichomonas; Ulc, genital ulcers; NPI, no pathogen identified. ND, not done.

	Before STI Treatment				After STI Treatment			
	# HIV <i>env</i> V1/V3 sequences	Structured Slatkin- Maddison value	Standard Slatkin- Maddison value	Visual Inspection	# HIV <i>env</i> 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	Superinfection	306	0	0	Superinfection
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

Table 2. Summary of the methods used to determine the relationship between blood-derived and semen-derived HIV-1 *env* V1/V3 sequences. Bolded values indicate statistically significant compartmentalization as determined by the indicated method.

Figure 1. Representative neighbor-joining *env* V1/V3 phylogenetic trees depicting compartmentalization between the blood and semen-derived lineages (A) and equilibration between blood and semen-derived lineages (B). Blood-derived sequences are shown in red, semen-derived sequences are shown in blue. Circles indicate compartmentalized nodes.

Figure 2. HIV-1 population dynamics between blood and semen remain unchanged after STI treatment. Neighbor-joining phylogenetic trees depicting blood-derived *env* V1/V3 sequences (shades of red) and semen-derived *env* V1/V3 sequences (shades of blue). Sequences from before and after STI treatment are shown. In (A) and (B), the same compartmentalized lineage appears in the semen before and after STI treatment (circled nodes). In (C), a different semen-derived, compartmentalized lineage is observed in the pre and post STI-treatment time points.

Figure 3. Clonal amplification of identical sequences is observed in both blood and semen-derived viruses in men with and without urethritis. An analysis of the percent of V1/V3 sequences that are not identical, derived from blood (A), or semen (C). The percent of unique sequences remains stable in overtime in both the blood (B) and semen (D). An unpaired t test was used to generate the indicated p values.

Figure 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. Boxed sequences represent those that are identical in the V1/V3 amplicon, and nearly identical over the full envelope amplicon.

Figure 5. SGA-derived HIV-1 envelopes from the semen are T-cell tropic. (A-C) Neighbor-joining trees of *env* V1/V3 blood (red) and semen (blue) derived sequences. The graphs below depicts 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 represent the average of three biological replicates.

Figure 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 and after STI treatment. The values were compared to a group of HIV+ men without a concurrent STI (labeled as “control”, and depicted by the grey bars). A one-way ANOVA was used to generate the indicated p value.

Figure 7. Characterization of a super-infection. A) Pre and post-STI treatment neighbor-joining phylogenetic trees depicting a distinct semen-only lineage from participant S031. Semen-derived sequences are shown in blue, blood-derived sequences are shown in red. The percent of all semen-derived sequences that cluster independently from the blood is shown at the bottom. B) A neighbor-joining phylogenetic tree containing blood and semen-derived sequences from five participants. Sequences from each participant are shown in a different color, sequences from S031 are purple. C) Highlighter plot depicting recombination between blood and semen-derived sequences from S031.

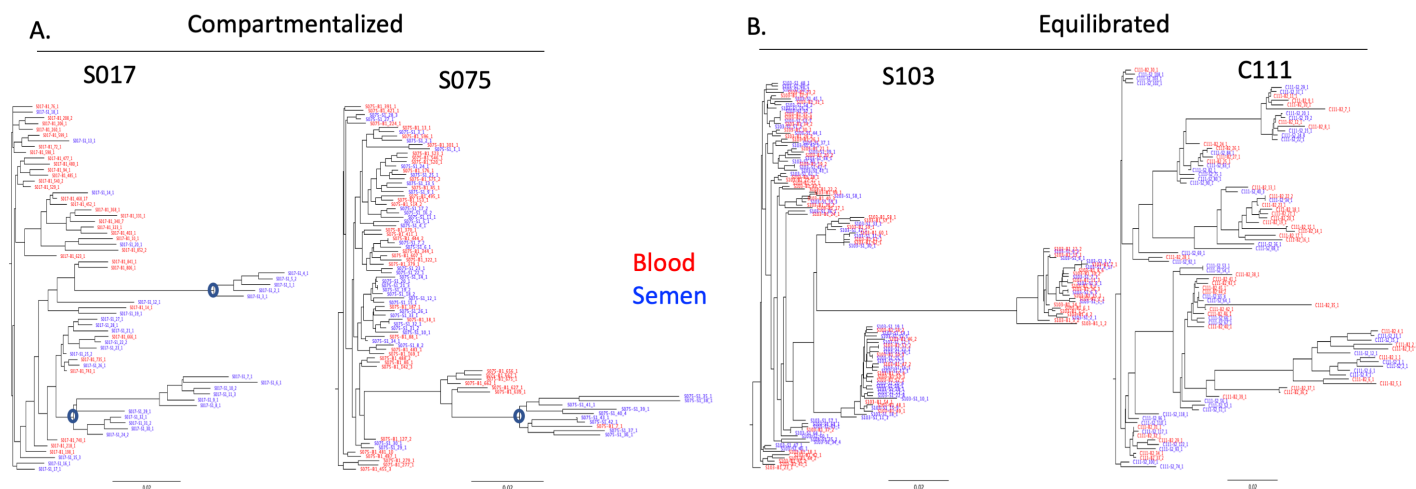


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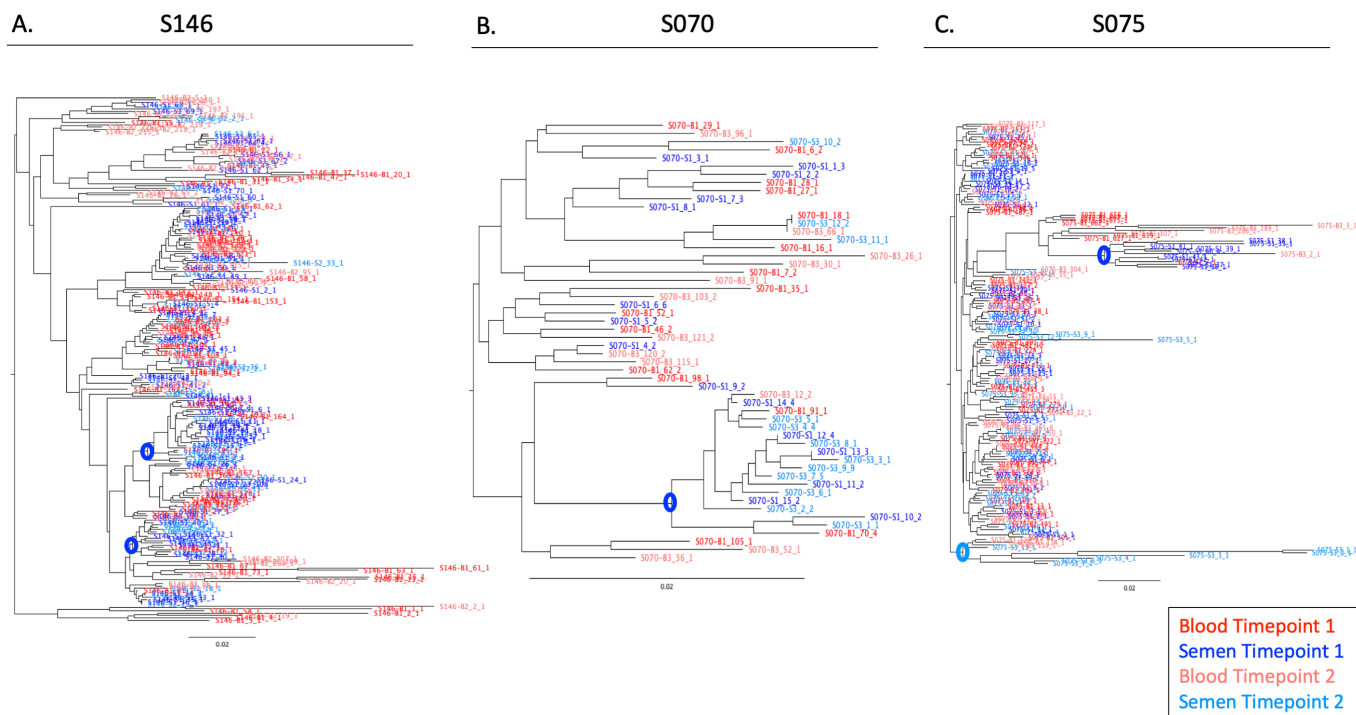


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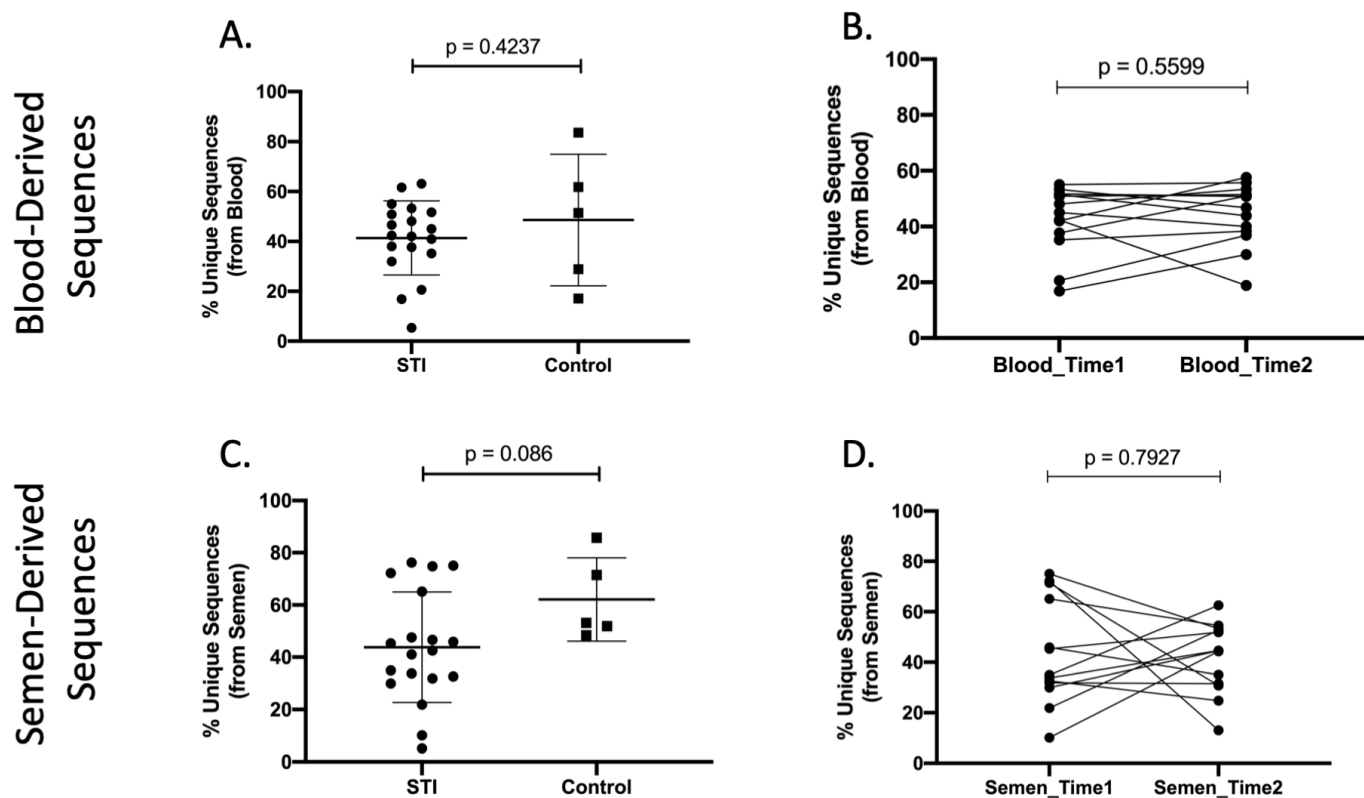


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S031 Full-Length Env



S031 Env V1/V3

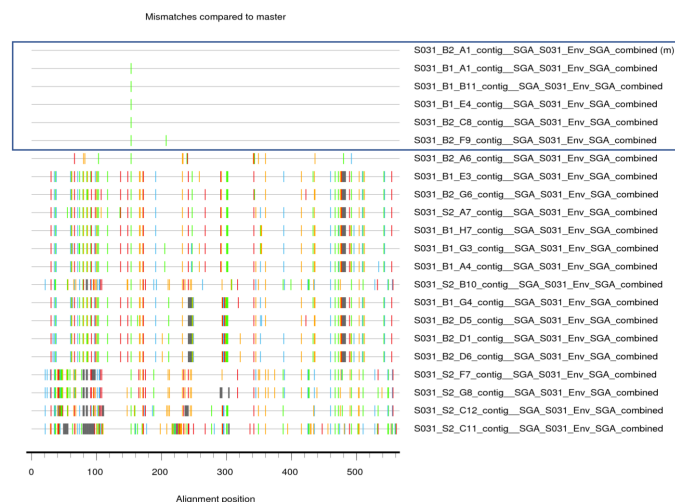


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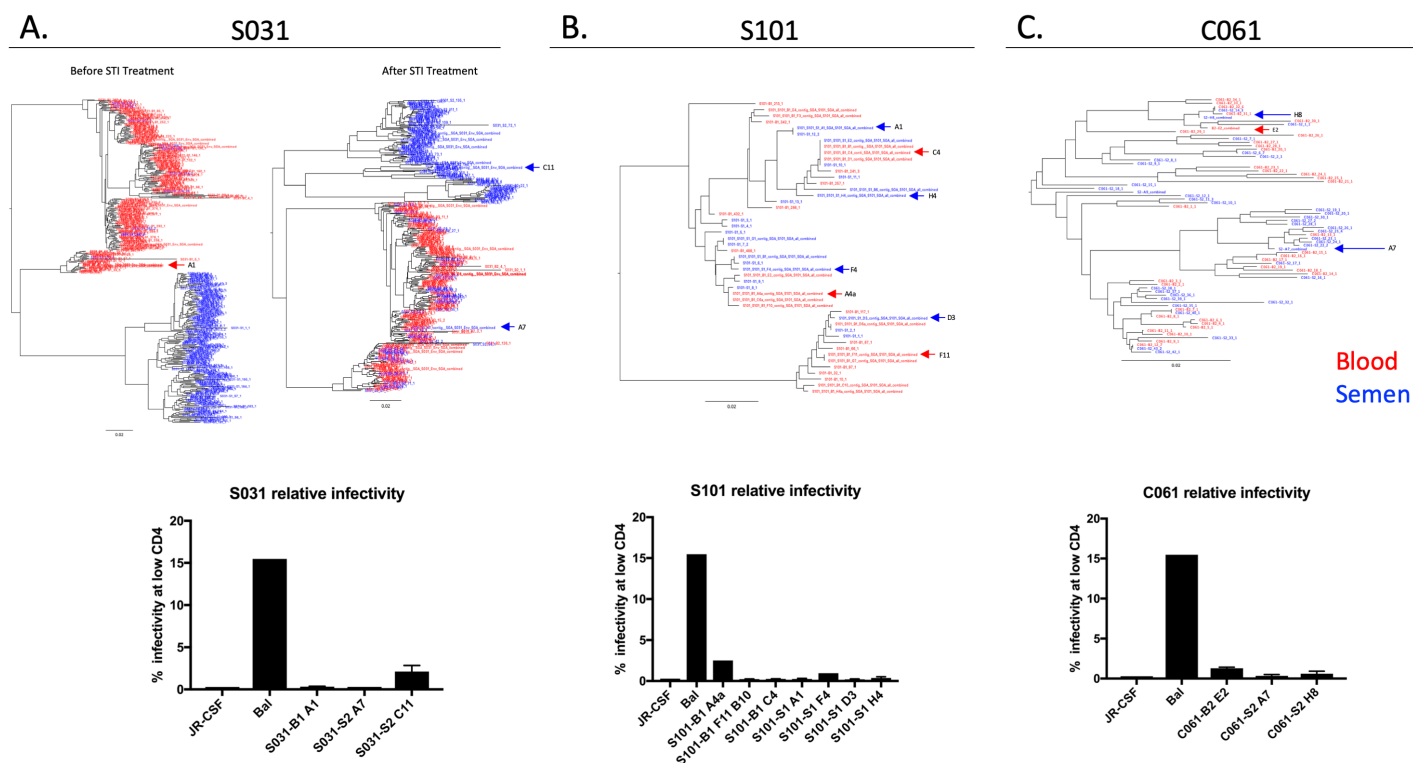


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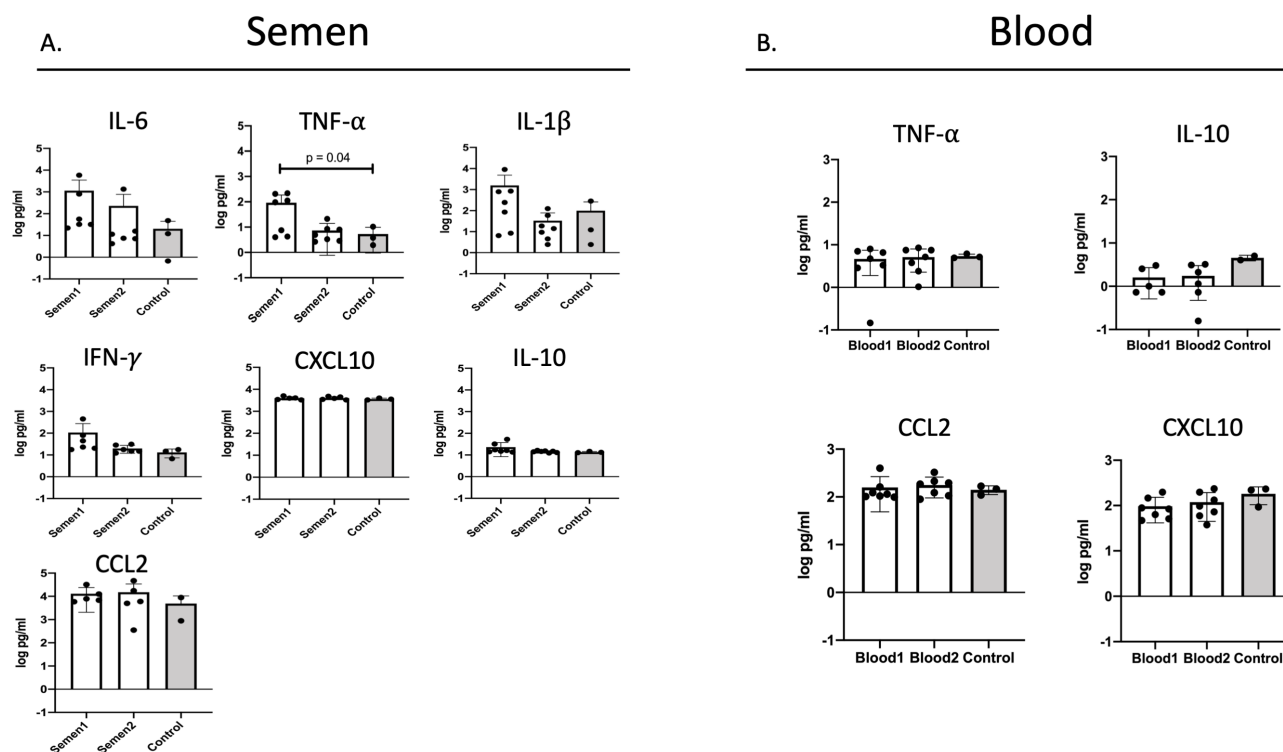


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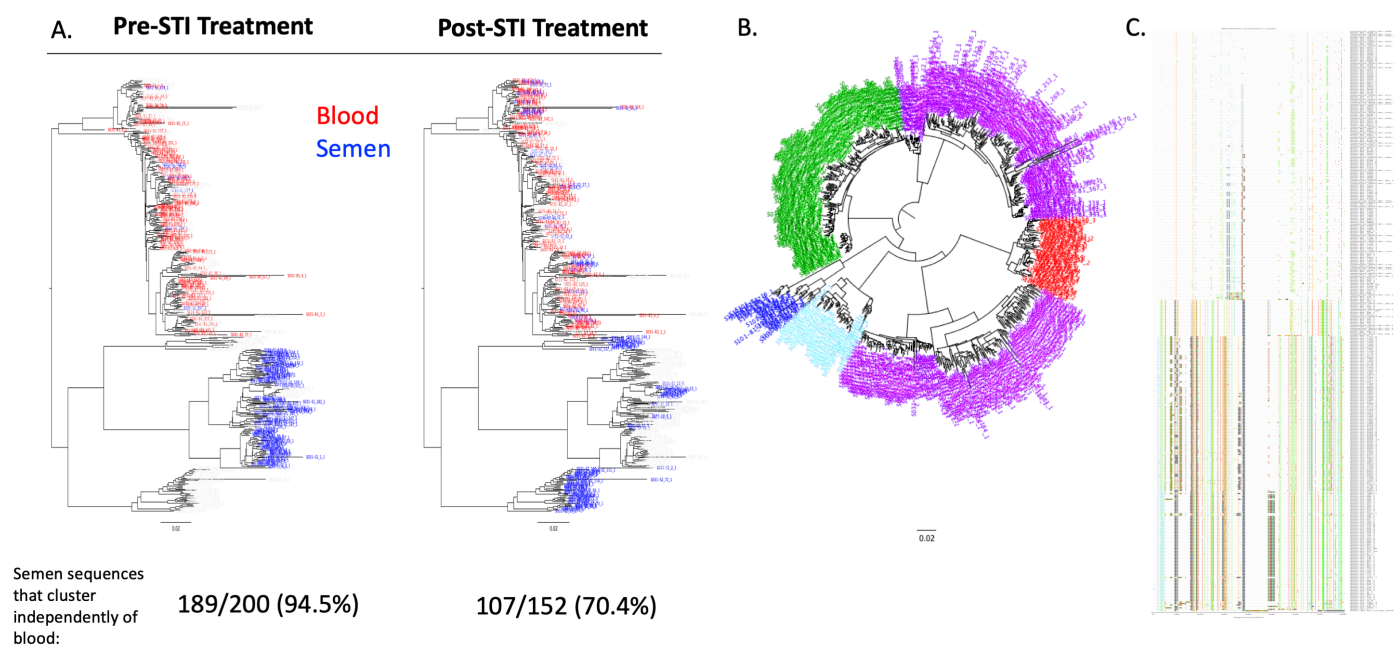


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