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1 # Infant Transmitted/Founder HIV-1 Viruses from Peripartum Transmission
2 are Neutralization Resistant to Paired Maternal Plasma

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20 **Abstract**

21 Despite extensive genetic diversity of HIV-1 in chronic infection, infant HIV-1 infection
22 involves selective transmission of a single or few maternal virus variants. These
23 transmitted/founder (T/F) variants are of particular interest, as a maternal or infant HIV vaccine
24 should raise envelope (Env)-specific IgG responses capable of blocking this group of viruses.
25 However, the maternal or infant factors that contribute to selection of infant T/F viruses are not
26 well understood. In this study, we isolated HIV-1 *env* genes by single genome amplification from
27 16 mother-infant transmitting pairs from the U.S. pre-antiretroviral era Women Infant
28 Transmission Study (WITS). Infant T/F and representative maternal non-transmitted Env
29 variants from plasma were identified and used to generate pseudoviruses for paired maternal
30 plasma neutralization sensitivity analysis. Eighteen out of 21 (85%) infant T/F Env
31 pseudoviruses were neutralization resistant to paired maternal plasma. Yet, all infant T/F viruses
32 were neutralization sensitive to a panel of HIV-1 broadly neutralizing antibodies and variably
33 sensitive to heterologous plasma neutralizing antibodies. Moreover, infant T/F pseudoviruses
34 were overall more neutralization resistant compared to maternal non-transmitted plasma variants
35 (p=0.012). Altogether, our findings suggest that autologous neutralization of circulating viruses
36 by maternal plasma antibodies select for neutralization-resistant viruses that initiate peripartum
37 transmission, raising the spector that enhancement of this response at the end of pregnancy could
38 further reduce infant HIV infection risk.

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39 **Author Summary**

40 Mother to child transmission (MTCT) of HIV-1 can occur during pregnancy (*in utero*), at the
41 time of delivery (peripartum) or by breastfeeding (postpartum). With the availability of anti-
42 retroviral therapy (ART), rate of MTCT of HIV-1 have been significantly lowered. However,
43 significant implementation challenges remains in resource-poor areas, making it difficult to
44 eliminate pediatric HIV. An improved understanding of the viral population (escape variants
45 from autologous neutralizing antibodies) that lead to infection of infants at time of transmission
46 will help in designing immune interventions to reduce vertical HIV-1 transmission. Here, we
47 selected 16 HIV-1-infected mother-infant pairs from WITS cohort (from pre anti-retroviral era),
48 where infants became infected peripartum. HIV-1 *env* gene sequences were obtained by the
49 single genome amplification method. The sensitivity of these infant Env pseudoviruses against
50 paired maternal plasma and a panel of broadly neutralizing monoclonal antibodies (bNAbs) was
51 analyzed. We demonstrated that the infant T/F viruses were more resistant against maternal
52 plasma than non-transmitted maternal variants, but sensitive to most (bNAbs). Signature
53 sequence analysis of infant T/F and non-transmitted maternal variants revealed the potential
54 importance of V3 and MPER region for resistance against to paired maternal plasma. These
55 findings provide insights for the design of maternal immunization strategies to enhance
56 neutralizing antibodies that target V3 region of autologous virus populations, which could work
57 synergistically with maternal ARVs to further reduce the rate of peripartum HIV-1 transmission.

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58 ## Introduction

59 Despite the wide success of antiretroviral therapy (ART) in lowering mother-to-child
60 transmission (MTCT) risk of HIV-1 below 2%, each year more than 150,000 children become
61 infected worldwide [1]. Even if 90% maternal ART coverage is reached, approximately 138,000
62 infant HIV-1 infections will still occur annually [2, 3] due to factors that include: drug non-
63 adherence, breakthrough infections, development of drug resistant viral strains, late presentation
64 of pregnant women to clinical care, and acute infection during late pregnancy or breastfeeding.
65 Vertical transmission of HIV can occur through three distinct modes: antepartum (*in utero)*,
66 peripartum (around the time of delivery), or postpartum (via breastfeeding). Interestingly, only
67 30-40% of infants born to HIV infected mothers acquire HIV-1 in the absence of ART[4]. Thus,
68 maternal factors, such as maternal Env-specific antibodies, may contribute to protecting infants
69 from HIV infection. Maternal factors that are associated with HIV transmission risk include: low
70 maternal peripheral CD4+ T cell count, and high maternal plasma viral load, delivery mode, and
71 infant gestational age [5-7]. Yet, the role of maternal Env-specific antibody responses and their
72 association with reduced MTCT risk still remains unclear. Previous studies have reported an
73 association between the magnitude of maternal antibody responses and reduced risk of MTCT
74 [8-10]. However, this association has not been universally observed [11-15]. Moreover, it has
75 been observed that variants transmitted to infants can be resistant to neutralization by maternal
76 plasma [16], although other studies have failed to replicate these observations [17-19]. These
77 conflicting results may be due to the small number of subjects included in these studies and study
78 designs that inconsistently control for viral and host factors known to impact transmission risk,
79 such as maternal peripheral CD4+ T cell counts, plasma viral load, non-identification of T/F
80 viruses, and ART use. Moreover, isolation of autologous viruses from a large cohort of HIV-

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81 infected, transmitting mothers for assessment of the impact of maternal plasma neutralization
82 activity against her own viruses has not to our knowledge, been investigated. Thus, despite
83 considerable effort, it remains unclear whether maternal antibody responses impact the risk of
84 vertical transmission of HIV.

85 We recently completed a maternal humoral immune correlates of protection analysis to
86 identify maternal humoral immune responses associated with protection against peripartum HIV
87 infection using samples from the US-based Women and Infants Transmission (WITS) study [20].
88 The WITS cohort was enrolled prior to the availability of ART prophylaxis as the clinical
89 standard of care in HIV-infected pregnant mothers and their infants, thereby eliminating the
90 strong impact of ART on vertical HIV transmission risk and outcome [21, 22]. Additionally, we
91 controlled for established maternal and infant risk factors associated with vertical transmission,
92 including maternal peripheral CD4+ T cell count, maternal plasma HIV-1 viral load, infant
93 gestational age, and delivery mode by propensity score matching of transmitting and non-
94 transmitting women. The results of this immune correlate analysis indicated an association
95 between high levels of maternal antibodies against the HIV-1 Env glycoprotein third variable
96 loop (V3) and reduced MTCT risk [20]. In addition, and more surprisingly, the ability of
97 maternal plasma to neutralize tier 1 viruses (easy-to-neutralize), but not tier 2 (difficult to
98 neutralize) viruses, also predicted decreased risk of vertical transmission of HIV-1. Yet,
99 vertically transmitted HIV variants have been characterized as more difficult to neutralize tier 2-
100 like variants [17, 23-26]. Thus, it was surprising that tier 1 virus neutralizing antibodies were
101 associated with decreased transmission risk. More interestingly, maternal V3-specific
102 monoclonal IgG antibodies isolated from a non-transmitting mother neutralized a large
103 proportion of maternal autologous viruses isolated from her plasma[20], leading to the

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104 conclusion that maternal V3-specific non-broadly neutralizing antibodies, which were previously
105 thought to be ineffective at preventing HIV-1 transmission, might indeed play a role in
106 preventing MTCT. In fact, Moody et.al [27] showed that V3 and CD4 binding site (CD4bs)
107 specific monoclonal antibodies isolated from non-pregnant chronically HIV-infected individuals
108 could also neutralize a large proportion of autologous circulating viruses isolated from plasma.
109 These V3 and CD4bs-specific autologous virus-neutralizing mAbs exhibited tier 1 neutralization
110 activity but limited heterologous tier 2 virus neutralization, suggesting that measurement of tier 2
111 heterologous virus neutralization potency of mAbs or plasma does not predict autologous virus
112 neutralization capacity.

113 In contrast to the extensive genetic diversity of HIV-1 variants in a chronically infected host,
114 acute HIV infection in both heterosexual and vertical routes are characterized by a homogeneous
115 viral population [17, 18, 28-31]. This viral genetic bottleneck suggests the selective transmission
116 of a single or homogeneous group of viruses [4]. In the setting of MTCT, maternal or infant
117 immunologic and virologic factors that that drive the selective transmission of one or a few HIV
118 variants are not established [32]. As maternal viruses co-circulate with maternal HIV Env-
119 specific antibodies, it is possible that maternal antibodies play a role in selecting maternal escape
120 viruses that may initiate infection in the infant. Therefore, studying unique features of infant T/F
121 viruses and their neutralization-sensitivity determinants to maternal autologous virus neutralizing
122 antibodies may provide insights of the molecular events that lead to virus escape from maternal
123 humoral responses.

124 The use of broadly neutralizing antibodies as a treatment and/or prevention strategy is
125 currently being explored in adult and infant clinical trials [33, 34]. Among the new generation of
126 bNAbs, VRC01 (antibody recognizing CD4bs region) has been able to neutralize about 80% of

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127 diverse HIV-1 strains [35, 36]. This has lead to studies of VRC01 impact on HIV-1 infection in
128 adults and infants when infused passively, with a phase I study of pharmacokinetics and safety of
129 VRC01 in HIV-exposed newborns currently underway [37]. However, the susceptibility of infant
130 T/F viruses to bNAbs like VRC01 does not seem to define infant T/F viruses from maternal non-
131 transmitted viruses [17] and administration of a bNAb to chronically-infected mothers is likely to
132 lead to rapid development of resistant viruses [34, 38]. Thus, defining the role of autologous
133 neutralization in MTCT is critical to establishing the utility of active maternal vaccination to
134 further reduce and eliminate infant HIV infections.

135 In this study, we characterized maternal non-transmitting and infant T/F viruses from 16
136 HIV-1 clade B infected peripartum transmission mother-infant pairs from the WITS cohort and
137 defined the role of concurrent maternal autologous virus neutralizing antibodies in selecting for
138 infant T/F viruses. We sought to define if neutralization resistance to paired maternal plasma was
139 a defining feature of infant T/F viruses compared to other circulating non-transmitted maternal
140 variants, which will inform the development of maternal or infant vaccination strategies to
141 further reduced MTCT risk to achieve an HIV-free generation.

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142 # Results

143 ## Maternal and infant sample characteristics

144 We selected sixteen, HIV-1 infected mother-infant transmission pairs from the WITS cohort that
145 met the inclusion criteria of peripartum transmission (infants tested negative for HIV-1 infection
146 at birth by HIV-1 DNA PCR, yet had HIV-1 DNA detectable at one week of age or older; see
147 Table1). These HIV-exposed infants had not reportedly been breastfed [22]. Infant plasma
148 samples available for sequencing were between 16 - 74 days of age. Five HIV-infected infants
149 with heterogeneous virus populations were excluded from the study due to our inability to
150 confidently infer the infant T/F virus. The maternal plasma viral load of the selected transmitting
151 women ranged from 4,104 to 3,68,471 copies/mL, and peripheral blood $CD4^+$ T-cell counts
152 ranged from 107 to 760 cell/mm^3. Infant plasma viral loads varied between 11,110 and 2,042,124
153 copies/mL, and $CD4^+$ T-cell counts were between 1,872 and 7,628 cell/mm^3. All infants were
154 born via vaginal delivery except for three infants (100014, 100155, 100307) born via cesarean
155 section, thus potentially representing late *in utero* transmission. Four infants (100014, 100155,
156 100307, 102149) were born prematurely (31, 34, and 36 weeks respectively), and the remaining
157 infants were born between 37 and 40 weeks of gestation.

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158 **Table 1. Maternal and infant pair clinical characteristics around delivery.**

Maternal and Infant ID	Year	Maternal Visit of sample collection	Mode of Transmission	Maternal plasma Viral Load (copies/mL)	Maternal Peripheral CD4+ T-cell count (cells/mm^3)	Mode of Delivery	Weeks Gestation	Infant Age at sample Collection (days)	Infant Viral Load (copies/mL)	Infant Peripheral CD4+ T-cell count (cells/mm^3)
100002	1993	Delivery	Peripartum	305,213	221	Vaginal	38	16	1,230,000	3326
100014	1993	Delivery	Peripartum	21,918	692*	C-section	31	66	120,000	2012
100046	1993	Delivery	Peripartum	12,635	707	Vaginal	40	63	1,025,458	2509
100052	1993	Delivery	Peripartum	23,750	450	Vaginal	40	34	333,142	3219
100155	1992	Delivery	Unknown	87,193	318	C-Section	36	60	NA	3609
100307	1991	Delivery	Unknown	139,053	467*	C-section	36	32	905,079	2308
100383	1991	Delivery	Peripartum	358,602	244	Vaginal	37	74	338,000	2071‡
100504	1993	NA	Unknown	68287	1049	C-Section	39	30	NA	2742
100711	1990	Delivery	Unknown	4,104*	571*	Vaginal	39	64	341,691	1872‡
100890	1991	Delivery	Peripartum	368,471	409	Vaginal	39	26	192,000‡	2598
100997	1991	Delivery	Peripartum	175,526	413	Vaginal	38	20	46,289‡	7048
101421	1991	Delivery	Peripartum	17,370	760	Vaginal	37	29	48,783	7628
101984	1991	Delivery	Peripartum	94,087	107	Vaginal	36	30	317969	1980
102149	1993	Delivery	Unknown	253,906	373	Vaginal	34	33	2,042,124	3181
102407	1993	Delivery	Peripartum	104,922	422	Vaginal	40	35	11,100	4677
102605	1992	NA	Unknown	5684	337	Vaginal	39	60	156639	1360

159 *Peripheral CD4+ T cell count and/or viral load reported from next available visit, within 2 months
160 from the sample that was obtained for sequencing. NA =Not Available.
161 ‡Peripheral CD4+ T cell count and/or viral load reported from 25 weeks gestation, as CD4+ T cell
162 count and/or viral load was not available at time of delivery.

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163 **Characterization of the complete *env* gene sequences from paired mother-infant plasma**

164 A total of 463 *env* genes were obtained from 16 maternal plasma samples (collected at time of
165 delivery) from transmitting mothers as previously described [24]. Paired infant plasma samples were
166 used to obtain 465 *env* gene sequences (Table 2). Neighbor-joining phylogenetic trees and
167 highlighter plots of the *env* sequences from each infant were used to define infant T/F viruses. These
168 analyses showed within-lineage low diversity populations in infant Env isolates and chronic-like
169 diversity in maternal *env* sequences (Fig.1, A-C and Fig. S1 A-B). In 6 out of 16 (37%) infants we
170 detected 2 or 3 (in case of 100002) genetically distinct T/F variants, one of which was present at
171 higher frequency (primary T/F), while the second one was present at lower frequency (secondary
172 T/F). In the other 10 infants demonstrated we observed only one T/F virus (67%). With the
173 exception of two infants, all of our samples had over 20 infant sequences, giving us a 90%
174 confidence that we were able to sample all variants with a population frequency of at least 10%. For
175 the two infant samples for which we only had 15 and 18 sequences respectively, we were 90%
176 confident that we were able to sample all variants with a population frequency of 15% or more [24].
177 Using an algorithm described in the Methods, out of these 463 maternal *env* variants, we selected
178 134 SGA variants for *env* pseudovirus production (5-12 per mother) to represent the *env* genetic
179 diversity found in the plasma of each transmitting mother at the time of delivery.

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180 **Table 2. Number of sequences, T/F viruses, and estimated days since most common recent**
181 **ancestor (MRCA) in infants.**

Maternal and Infant pair ID	Number of Infant envelope gene SGAs	Estimated Number of infant T/F viruses*	Infant age at sample collection (days)	Estimated days Since Most Recent Common Ancestor (95% CI)‡	Notes
100002	43	3	16	21 (14,28)	Fits a Poisson after removing recombinants.
100014	26	1	66	35 (28,42)	Fits a Poisson.
100046	33	1	63	77 (65,89)	Fits a Poisson after removing hypermutation.
100052	27	1	34	58 (41,75)	Fits a Poisson after removing hypermutation.
100155	23	1	60	63 (50,76)	Fits a Poisson.
100307	31	2	32	27 (18,36)	Fits a Poisson after removing recombinants.
100383	24	1	74	80 (67,92)	Fits a Poisson.
100504	44	1	30	13 (8,18)	Fits a Poisson after removing hypermutation.
100711	26	1	64	27 (17,37)	Fits a Poisson after removing hypermutation.
100890	18	2	26	35 (17,54)	Fits a Poisson after removing recombinants.
100997	32	2	20	19 (13,25)	Fits a Poisson after removing recombinants.
101421	22	2	29	20 (11,29)	Fits a Poisson.
101984	34	2	30	14 (10,19)	Fits a Poisson after removing hypermutation.
102149	27	1	33	43 (31,55)	Fits a Poisson after removing hypermutation.
102407	15	1	35	15 (9,22)	Fits a Poisson.
102605	40	1	60	NA	Does not fit a Poisson, evidence for selection.

182 *Number of T/Fs were estimated through visual analysis of highlighter plots and phylogenetic
183 trees after eliminating recombinants and hypermutated sequences.
184 ‡Days since most recent common ancestor (MRCA) were calculated using the LANL tool
185 Poisson Fitter https://www.hiv.lanl.gov/content/sequence/POISSON_FITTER/pfitter.html

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186 **Confirming the timing of infant HIV-1 infection**

187 As the infants were selected for peripartum transmission, their age at sampling (in days) was also
188 the post-infection time. To confirm the time of infection, all infant alignments were analyzed
189 using the LANL Poisson Fitter tool [39]. For infants that had more than one T/F, only the
190 sequences in the major T/F lineage were used for this analysis. When recombinants and
191 APOBEC enrichment were detected, the timing was calculated after removing recombinants
192 and/or positions enriched for hypermutation [24, 39].

193 All but one infant (102605) yielded a good Poisson fit, indicating that the amount of diversity
194 found in these samples was compatible with a random accumulation of mutations as observed in
195 acute infections. Four infants had detected recombinants and 6 infants yielded a good Poisson fit
196 after removing positions enriched for hypermutation (Table 2). The time since the most common
197 ancestor was consistent with transmission at delivery in 9 out of 16 pairs (57%), within the 95%
198 confidence interval of the Poisson Fitter time estimate (Table 2). For three infants, Poisson Fitter
199 estimated the time since infection to be younger than the reported infant age, and for one the
200 infection showed more diversity than expected by the reported infant age. Discrepancies between
201 actual vs. predicted transmission timing could be due to a number of factors, including late *in*
202 *utero* infection, postpartum infection from unreported breastfeeding, and the model being
203 designed to evaluate an adult rather than infant HIV-1 evolution, which could gather mutations
204 more rapidly due to more robust T cell responses.

205 Infant 102605 *env* SGAs did not yield a good Poisson fit due to non-random accumulation of
206 non-synonymous mutations (which breaks the model assumption of random accumulation of
207 mutations) at HXB2 positions 752-754 (Fig. S2). We looked at this region in the LANL
208 immunology database (https://www.hiv.lanl.gov/content/immunology/index), and found five

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209 different human CTL epitopes that have been documented in the literature, confirming that the
210 non-random mutations found in infant 102605 were likely due to selection pressure by T cell
211 responses.

212 **Neutralization sensitivity and tier classification of the infant T/F viruses**

213 Twenty-one infant T/F *env* amplicons including 16 primary T/Fs and 5 secondary T/Fs were used
214 to generate pseudoviruses and their neutralization sensitivity to paired maternal plasma and a
215 panel of bNAbs was assessed. None of the mothers with the exception of two (100014 and
216 100504) had non-specific neutralization activity as assessed by neutralization activity against a
217 murine leukemia virus (MLV). Eighteen out of 21 infant T/F Env pseudoviruses (86%) were
218 resistant to paired maternal serum (ID_{50}<40). Sensitivity of 2 Infant T/F pseudoviruses' (100014
219 and 100504) against paired maternal plasma could not be determined with confidence due to
220 higher plasma reactivity against MLV. T/F virus of infant 100046 was sensitive against paired
221 maternal plasma (Fig. 2).

222 When Infant T/F pseudoviruses were tested against the autologous plasma, only 2 infants
223 T/F (100046 and 100155) showed some sensitivity as per our criteria (ID_{50}> 3X that of MLV)
224 while others were completely resistant. Some infant T/F pseudoviruses did show sensitivity
225 against their own plasma but were not considered as sensitive due to high reactivity against
226 MLV.

227 To determine whether these infant T/F *env* variants were globally resistant to heterologous
228 plasma neutralization, we performed neutralization tier phenotyping using a standardized panel
229 of heterologous plasma of HIV-1 infected individuals [40]. Thirteen (62%) of 21 infant T/F Env
230 pseudoviruses tested were classified as tier 2 neutralization phenotype while 3 (14%) of 21 were

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231 classified as tier 3 neutralization phenotype, as expected for infant T/F viruses (Fig. 2).
232 Remarkably, the remaining 5 (24%) tested were classified as the easier-to-neutralize tier 1b or
233 tier 1a sensitivity, possibly because that these variants were uniquely resistant to their paired
234 maternal plasma (Fig. 2).

235 In contrast to the relative resistance to paired maternal plasma neutralization, all the infant
236 T/F viruses were relatively sensitive to second-generation HIV-1 broadly neutralizing antibodies,
237 such as VRC-01, (IC_{50} range 0.12-5.0 μg/ml), PGT121 (IC_{50} range 0.01-0.13 μg/ml), NIH 45-46
238 (IC_{50} range 0.01-0.28 μg/ml) and first generation bNAb 10E8 (IC_{50} range 0.05-0.76 μg/ml) (Fig.
239 3 and Fig. S3). Not surprisingly, the infant T/F Env pseudoviruses were less neutralization
240 sensitive to the less potent first generation broadly neutralizing antibodies b12 (IC_{50} range 2.97-
241 25 μg/ml), 4E10 (IC_{50} range 1.31-18.73 μg/ml) and 2F5 (IC_{50} range 1.01-19.09 μg/ml) (Fig. 3).
242 Importantly, all the infant T/F viruses were neutralization sensitive to VRC-01, a bNAb currently
243 being evaluated in clinical trials for use in HIV exposed infants. However, V3 glycan-specific
244 bNAbs, which clustered together in neutralization sensitivity, and NIH45-46 mediated the most
245 neutralization breadth and potency against the infant T/F viruses (Fig. 3).

246 We next calculated the geometric means of both the breadth and potency of the panel of bNAbs
247 against the infant T/F viruses and compared with their potency against other HIV variants as
248 documented in CATNAP (Compile, Analyze and Tally NAb Panels) [41], the Los Alamos
249 National Laboratory (LANL) interface that collects all published immunological data. In general,
250 potency and breadth of the bNAbs against infant T/F viruses followed the potency and breadth
251 calculated in CATNAP (p=0.013 and 0.02 respectively, Spearman correlation test), with one
252 exception: bNAb 2G12 displayed more potent and broad responses in the infants than in the
253 CATNAP collective data (Fig. 3).

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254 **Neutralization sensitivity of non-transmitted maternal *env* variants compared to infant T/F**
255 ***env* variants**

256 Pseudoviruses were prepared from a total of 134 non-transmitted maternal *env* variants using the
257 promoter PCR method [42] and assessed for neutralization sensitivity against paired maternal
258 plasma, including the isolated maternal non-transmitted variant that was most closely related to
259 the infant T/F variant. Variable neutralization sensitivity to paired maternal plasma was observed
260 in non-transmitted maternal variants, with some of the variants exhibited neutralization
261 sensitivity while others showed complete neutralization resistance (Fig. 4). Comparison of
262 neutralization sensitivity between infant T/F Env variants and the identified closest maternal
263 variant within each mother-infant pair revealed no consistent pattern. Over a 2-fold increase in
264 sensitivity was observed for Env pseudoviruses of non-transmitted maternal variants that were
265 most closely related to infant T/F for 6 infants (100002, 100307, 100052, 102149, 102407 and
266 102605). In contrast, infant T/F Env pseudoviruses from 3 infants (100014, 100046 and 100504)
267 were more sensitive to maternal plasma than their most closely related maternal variants.

268 Yet, the infant T/F viruses were generally more resistant to the maternal plasma at
269 delivery than the non-transmitted viruses from mothers within each maternal-infant pair, with the
270 exception of 100046. However, since there were only 1 or 2 T/F viruses in each infant, we could
271 not perform statistical analysis to determine if the differences are statistically significant within
272 each pair. To determine whether infant T/F viruses were overall more resistant to maternal
273 plasma than the paired non-transmitted maternal viruses, we employed a 1-sided permutation test
274 to compare the neutralization sensitivity of maternal non-transmitted variants to the infant T/F
275 Env variants. Remarkably, infant T/F Env variants were overall significantly more resistant to
276 paired maternal plasma collected at delivery than non-transmitted maternal Env variants

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277 (p=0.01). Even when excluding the mother-infant pairs with high MLV neutralization (100014
278 and 100504), the infant T/F Env variants remained more resistant to neutralization than non-
279 transmitted maternal variants (p=0.005).

280 To assess whether any particular epitope-specific neutralization sensitivity was distinct in
281 infant T/Fs compared to matched maternal variants, we determined the neutralization sensitivity
282 of 4 bNAbs targeting distinct vulnerable epitopes on HIV-1 Env: VRC-01 (CD4bs-specific)
283 (VRC-01), PG9 (V2 glycan-specific), DH429 (V3 glycan-specific), and DH512 (membrane
284 proximal external region – MPER-specific) (Fig. 5). We used the same 1-sided permutation test
285 described above to assess for differences in neutralization sensitivity to these bNAbs in infant
286 T/Fs vs non-transmitted maternal sequences. Interestingly, we found that infant T/F viruses were
287 significantly more resistant to DH512 (MPER-specific) compared to non-transmitted maternal
288 sequences (p = 0.025 by 1-sided permutation test; p=0.045 when excluding the two mothers with
289 non-specific neutralization), while all other comparisons yielded no statistical significance (Fig.
290 5).

291 **Signature sequence analysis of infant T/F variants to predict neutralization resistance**

292 Because DH512 binds to the MPER region, we investigated the amino acid positions within
293 this epitope (positions 662-683) and identified 4 positions that were either associated with higher
294 maternal plasma neutralization (position 662, amino acid A, K, Q, or S were significantly more
295 resistant than the wild type E, p=9.9e-05, 1-sided permutation test), lower DH512 IC_{50} (position
296 667 and 676, p=9.9e-04 and 0.003, respectively by 1-sided permutation test), or both (position
297 683, amino acid R was significantly more resistant than the wild type K (most frequent AA at
298 this position), p<1e-04 by 1-sided permutation test; Fig. 7). However, when we looked at the Env

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299 sequences in individual mother-infant pairs, these amino acid residues that associated with
300 DH512 neutralization resistance were equally distributed across non-transmitted maternal
301 sequences and infant T/F viruses. Therefore, we could not to determine if T/F variants enriched
302 for neutralization resistance-conferring amino acids were more apt to be transmitted compared to
303 non-transmitted maternal variants. This could be due to the low sequence number within pairs, or
304 it could suggest that the wild type amino acids at these positions are associated with DH512
305 neutralization resistance but not necessarily transmission.
306

307 **V3 loop amino acid signature sequence analysis and neutralization sensitivity to paired**
308 **maternal plasma**

309 As maternal V3-specific IgG binding and tier 1 virus neutralizing responses were predictive of
310 MTCT risk in this cohort [20], we explored possible signatures of neutralization resistance to
311 paired maternal plasma in the V3 region. We examined the highly variable N and C-terminal
312 region amino acid residues K305Q, I307T, H308T, R315Q, F317L, A319T, and D322R (Fig. 7),
313 3 of which (K305Q, I307T, and H308T) have previously been identified critical targets of the
314 V3-specific IgG responses associated with reduced MTCT risk [43]. Comparing non-transmitted
315 maternal sequences and infant T/F viruses, we found position K305R to be significantly
316 associated with higher sensitivity to paired maternal plasma ($p<0.001$ by 1-sided permutation
317 test). At position 308, sequences carrying mutations from the consensus amino acid H (N, P, S or
318 T) were significantly associated with higher neutralization resistance to paired maternal plasma
319 ($p<0.001$ by 1-sided permutation test). However, as for the MPER residues, we observed that
320 these amino acids were equally distributed across non-transmitted maternal and infant T/F virus
321 sequences, indicating that none of these amino acid residues were directly involved with

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322 transmission risk. This discrepancy could partially be due to the heterogeneity at amino acid
323 residue position 308. Yet, 6 out of 14 transmitting mother-infant pairs exhibited distinct amino
324 acid residues at position 308 from the more frequently occurring histidine (Fig. 7), suggesting
325 variability at this position could be overrepresented in transmitting pairs.

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326 **Discussion**

327 While maternal and infant ART has considerably reduced rates of MTCT, pediatric HIV
328 infection remains a significant public health problem in areas of high HIV prevalence, with up to
329 16% of HIV-infected women still transmitting the virus to their infant globally [1]. It is likely
330 that a maternal or infant HIV-1 vaccine will be required to eliminate pediatric HIV [44].
331 However, a better understanding of factors that may drive the genetic bottleneck of virus
332 populations in the setting of MTCT of HIV will be required to develop vaccination strategies that
333 can block HIV transmission. Recent findings published by our group demonstrated that maternal
334 V3 loop-specific and tier 1 virus-neutralizing antibody responses both correlated and were
335 independently associated with of reduced MTCT risk. Moreover, we established that V3-specific
336 antibodies in maternal plasma could neutralize maternal autologous viral variants circulating in
337 plasma [20, 27]. To examine the potential role of maternal Env-specific responses in driving the
338 viral genetic bottleneck of MTCT, we aimed to define if neutralization resistance to maternal
339 autologous virus neutralizing antibodies is a defining feature of infant T/F variants compared to
340 circulating maternal non-transmitted plasma variants.

341 In this cohort, 6 out of 16 (37%) peripartum-infected infants were infected by at least 2 T/F
342 viruses. Interestingly, infection with multiple T/F viruses occurs in approximately 19-24% of
343 heterosexual HIV infections [24, 45, 46], and 12-38% of homosexual infections [47-49], whereas
344 up to 60% of infections that occur through intravenous drug use involve multiple T/Fs [50].
345 Thus, the rate of multiple T/F transmissions in this mother-infant cohort is in line with or slightly
346 higher than sexual transmission modes, but lower than that of transmission via intravenous drug
347 use. While it is well established that a genetic bottleneck occurs in the setting of MTCT, the
348 determinants that drive the selection of 1 or multiple T/F viruses are less clear [4]. Importantly,

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349 the lack of maternal ART prophylaxis around the time of delivery in the WITS cohort could
350 contribute to the observed high rate of multiple T/F viruses, potentially stemming from a larger
351 virus inoculum in this cohort compared to ARV-treated mothers. Regardless, of the impact of
352 maternal ART on the bottleneck of infant T/F viruses, maternal or infant immunization strategies
353 will likely need to generate Env-specific responses that can block the diverse pool of maternal
354 viruses circulating in plasma. Notably, these infant T/F viruses uniformly represented a minor
355 variant of the maternal viral population Env variants, indicating that maternal antibodies that can
356 block infant virus transmission will need to target minor circulating variants.

357 A greater understanding of virologic characteristics of infant T/F viruses will also be
358 important to developing immune-based strategies to prevent MTCT. As maternal V3-specific
359 IgG responses predicted reduced risk of transmission in this cohort, we investigated V3 loop
360 residues in the maternal and infant viruses and how they related to paired maternal plasma
361 neutralization sensitivity. Despite the association between maternal V3-specific IgG responses
362 targeting the C terminal region and reduced MTCT risk in this cohort [43], we did not find amino
363 acid residues within the C terminal region to be associated neutralization resistance to paired
364 maternal plasma. Instead, we found that maternal non-transmitted and infant T/F viruses carrying
365 N, P, S or T at the N terminal region amino acid residue position 308 were more neutralization
366 resistant to paired maternal plasma. These seemingly disparate findings could partly be explained
367 by several reasons. Firstly, N terminal amino acid residues 308 and 309 have been shown to
368 interact with C terminal amino acid residue 317, and this interaction leads to the stabilization of
369 the V3 loop [51, 52]. Thus, the disruption of intra-peptide interactions at either the N and or C
370 terminal region could lead to altered neutralization sensitivity of viruses to paired maternal V3-
371 specific IgG plasma responses. Secondly, it should be noted that in this study, we compared

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372 maternal non-transmitted circulating viruses to infant T/F viruses in 16 transmitting mother
373 infant pairs, whereas we previously defined the potentially-protective role of maternal V3-
374 specific IgG binding and neutralizing responses by comparing transmitting and non-transmitting
375 women in the larger (n=248) WITS cohort [20, 43]. Finally, V3 loop accessibility to maternal
376 neutralizing antibodies may be modulated by amino acid residues by distal amino acid residues
377 within gp120 or gp41 [15]. For example, specific glycosylation sites within the V1 loop may
378 alter V3 loop accessibility to V3-specific neutralizing antibodies [53]. Moreover, interactions
379 between C2 and V3 may stabilize the structure of the HIV-1 Env [54], as demonstrated with the
380 recent elucidation of the SOSIP trimer [55].

381 In contrast to previous studies that examined the neutralization sensitivity of randomly
382 selected or non-paired infant or maternal virus isolates, our study defined the neutralization
383 sensitivity of paired infant T/F viruses and maternal non-transmitted variants. Moreover, our
384 study accounted for phylogenetic relationships of infant T/F viruses and maternal non-
385 transmitted variants to represent the diverse maternal virus lineage pools. Furthermore, our study
386 carefully controlled for analysis confounders such as transmission mode, disparate maternal and
387 infant sample testing. Moreover, as the WITS cohort was enrolled and followed prior to the
388 availability of ART to prevent MTCT, virus variant selection in this cohort is not influenced by
389 ART selection pressures. With this robust study design, our analysis demonstrated that infant T/F
390 viruses are mostly resistant to concurrent autologous maternal plasma, suggesting that infant T/F
391 viruses are defined by neutralization resistance to maternal autologous virus neutralizing
392 antibodies. This work confirms previous studies that have made this prediction based on smaller
393 studies or with less well-defined maternal and infant virus variants [16, 56, 57]. Yet, Miligan *et.*
394 *al* [58] recently showed that neutralization resistant viruses do not predict MTCT risk in a

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395 breastfeeding transmission setting. However, as our analysis focused on peripartum transmission
396 only, there may be distinct virologic or immunologic determinants in peripartum and postpartum
397 HIV transmission. Yet an important, a novel observation gleaned this study is that infant T/F
398 viruses' neutralization resistance to maternal plasma is not predictive of neutralization resistance
399 to heterologous plasma. Remarkably, the tiered categorization of infant T/F viruses ranged from
400 easy to neutralize tier 1 a viruses, to very difficult to neutralize tier 3 viruses, suggesting that
401 heterologous plasma neutralization resistance is not a defining feature of infant T/F viruses.
402 Specifically, 24% of infant T/F viruses isolated in this study were classified as "easy to
403 neutralize" tier 1b or tier 1a variants by a standard panel of heterologous plasma [40], consistent
404 with the hypothesis that these infant T/F viruses may be specifically resistant to maternal
405 antibodies that co-evolved with the transmitted variants.

406 Not unsurprisingly, the majority of infant T/F viruses were neutralization sensitive to a
407 number of second generation broadly neutralizing antibodies (Fig. 4). This finding is clinically
408 relevant, as it suggests that infant passive immunization with second generation broad and potent
409 bNAbs to prevent HIV-1 transmission could be an effective strategy to block MTCT.
410 Interestingly, there is an ongoing passive immunization clinical trial of high-risk, HIV-exposed
411 infants with VRC01 (https://clinicaltrials.gov/ct2/show/record/NCT02256631). The uniform
412 sensitivity of these clade B infant T/F viruses isolated in our study to VRC01 neutralization
413 suggests that these viruses would be effectively neutralized by VRC01, suggesting that clade B
414 infant virus transmission may be blocked by VRC01.

415 To our knowledge, this is the largest study that has characterized infant T/F and maternal
416 viruses and their neutralization sensitivity to maternal autologous virus neutralizing responses.

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417 Our study specifically addresses whether infant T/F viruses are defined by their neutralization
418 sensitivity to maternal autologous virus neutralizing antibodies in peripartum MTCT of HIV.
419 MTCT is a unique setting in which protective antibodies only need block autologous virus
420 variants circulating in blood to which the infant is exposed. The observation that infant T/F
421 viruses are neutralization resistant compared to non-transmitted maternal variants suggests that
422 the development of a maternal vaccine that boosts maternal autologous virus neutralizing
423 responses may be a viable strategy to further reduce MTCT risk. Maternal Env immunization
424 regimens with closely related, but not identical, Envs to maternal circulating virus populations
425 may elicit antibodies that target her autologous virus pool through the well-described immune
426 phenomenon of 'original antigenic sin' [59, 60]. Our central finding that maternal autologous
427 virus neutralization shapes the genetic bottleneck of peripartum transmission has important
428 implications in designing maternal Env vaccination strategies that can synergize with current
429 maternal ART treatment strategies help achieve an HIV-free generation.

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430 # Materials and Methods

431 **Study Subjects and sample collection**

432 Maternal and infant pairs from the WITS cohort that met the following criteria were selected:
433 peripartum transmission, infant plasma samples from < 2.5 months of age, and maternal samples
434 available from around delivery. Peripartum transmission was defined by negative a negative
435 PCR result or negative culture from peripheral blood samples collected within 7 days of birth
436 with subsequent a positive result 7 days after birth (Table 1).

437

438 **Ethics Statement**

439 Samples used in this study were obtained from an existing cohort named as Women Infant
440 Transmission Study (WITS). WITS cohort samples were received as de-identified material and
441 were deemed as research not involving human subjects by Duke University Institutional Review
442 Board (IRB). The reference number for that protocol and determination is Pro00016627.

443 **Viral RNA Extraction and SGA isolation**

444 Viral RNA was purified from the plasma sample from each patient by the Qiagen QiaAmp viral
445 RNA mini kit and subjected to cDNA synthesis using 1X reaction buffer, 0.5 mM of each
446 deoxynucleoside triphosphate (dNTP), 5 mM DTT, 2 U/mL RNaseOUT, 10 U/mL of
447 SuperScript III reverse transcription mix (Invitrogen), and 0.25 mM antisense primer 1.R3.B3R
448 (5'-ACTACTTGAAGCACTCAAGGCAAGCT TTATTG-3'), located in the *nef* open reading
449 frame. The resulting cDNA was end-point diluted in 96 well plates (Applied Biosystems, Inc.)
450 and PCR amplified using Platinum Taq DNA polymerase High Fidelity (Invitrogen) so that <
451 30% of reactions were positive in order to maximize the likelihood of amplification from a single

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452 genome. A second round of PCR amplification was conducted using 2µl of the first round
453 products as template. 07For7 (5'-AAATTAYAAAAATTCAAAATTTTCGGGTTTATTACAG-
454 3') and 2.R3.B6R (5'- TGA AGCACTCAAGGCAAGCTTTATTGAGGC -3') were used as
455 primer pair in the first round of PCR amplification step, followed by a second round with
456 primers VIF1 (5'- GGGTTTATTACAGGGACAGCAGAG -3')(nt 5960–5983 in the HXB2 tat
457 coding region) and Low2c (5'- TGAGGCT TAAGCAGTGGGTT CC -3') (nt 9413–9436 in
458 HXB2 nef). PCR was carried out using 1X buffer, 2 mM MgSO4, 0.2 mM of each dNTP, 0.2µM
459 of each primer, and 0.025 U/µl Platinum Taq High Fidelity polymerase (Invitrogen) in a 20µl
460 reaction. Round 1 amplification conditions were 1 cycle of 94°C for 2 minutes, 35 cycles of
461 94°C for 15 seconds, 58°C for 30 seconds, and 68°C for 4 minutes, followed by 1 cycle of 68°C
462 for 10 minutes. Round 2 conditions were one cycle of 94°C for 2 minutes, 45 cycles of 94°C for
463 15 seconds, 58°C for 30 seconds, and 68°C for 4 minutes, followed by 1 cycle of 68°C for 10
464 minutes. Round 2 PCR amplicons were visualized by agarose gel electrophoresis and sequenced
465 for envelope gene using an ABI3730xl genetic analyzer (Applied Biosystems). The final
466 amplification 3'-half genome product was ~4160 nucleotides in length exclusive of primer
467 sequences and included all of *rev* and *env* gp160, and 336 nucleotides of *nef*. Partially
468 overlapping sequences from each amplicon were assembled and edited using Sequencher (Gene
469 Codes, Inc). Sequences with double peaks per base read were discarded. Sequences with one
470 double peak were retained as this most likely represents a Taq polymerase error in an early round
471 of PCR rather than multiple template amplification; such sequence ambiguities were read as the
472 consensus nucleotide. Sequence alignments and phylogenetic trees were constructed using
473 ClustalW and Highlighter plots were created using the tool at
474 https://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html.

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475 **Sequence Alignment**

476 All maternal and infant envelope sequences were aligned using the Gene Cutter tool available at
477 the Los Alamos National Laboratory (LANL) website
478 (http://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html) and then refined
479 manually. Full-length envelope sequences were manually trimmed in Seaview [61]. The infant
480 T/F env virus sequences were visually identified looking at phylogenetic trees and highlighter
481 plots, and infant consensus sequences of the major T/F lineage were created using the LANL
482 Consensus Maker tool
483 (http://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html). For infants that were
484 infected by 2 or more distinct T/F viruses, the highlighter plots and phylogenetic trees were
485 rooted on the consensus of the major variant.

486 **Infant T/F Virus Envelope Characterization**

487 Maternal and infant envelope alignments were characterized using Bio-NJ phylogeny (Mega 6
488 Software) and highlighter plot
489 (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/HIGHLIGHT_XYPLOT/highlighter.ht
490 ml). The number of infant T/F viruses was determined by visual inspection of both phylogenetic
491 trees and highlighter plots of infant-maternal env sequence alignments. Hypermutation was also
492 evaluated using the tool Hypermut
493 (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html). Sequences with
494 significant hypermutation ($p<0.1$) were removed from the alignment and not included in further
495 analysis. When a sample was found to be overall enriched for hypermutation [24], positions
496 within the APOBEC signature context were removed (Table 2). All 16 infant infections were
497 acute and we were able to time the infection using the Poisson Fitter method after removing

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498 putative recombinants and/or hypermutated sequences as described above. Days since infant
499 infection were calculated using the Poisson Fitter tool
500 (http://www.hiv.lanl.gov/content/sequence/POISSON_FITTER/ pfitter.html) which estimates the
501 time since infection based on the accumulation of random mutations from the most recent
502 common ancestor (MRCA) [39]. For infants infected with 2 or more T/F viruses, only the major
503 variant was analyzed to obtain the time since the infection. The defined mutation rate was 2.16e-
504 5. Values were reported in days with a 95% confidence interval and a goodness-of-fit p-value.

505 **Infant T/F SGA Cloning**

506 Amplicons from the first round PCR product that matched the infant consensus sequence
507 (T/F virus sequence) were ligated into pcDNA3.1 Directional Topo vectors (Invitrogen) by
508 introducing a –CACC 5' end via a PCR reaction with the primers Rev19 (5'-
509 ACTTTTTGACCACTTGCCACCCAT-3') and Env1A (5'-caccTTAGGCATCTCCT
510 ATGGCAGGAAGAAG-3'). Phusion® High-Fidelity PCR Master Mix with HF Buffer was
511 used according to the manufacturer's instructions (New England BioLabs). Plasmids were then
512 transformed into XL10 gold chemically competent Escherichia coli cells. Cultures were grown at
513 37°C for 16 hours. Colonies were selected for growth, and plasmids were minipreped and quality
514 controlled by restriction enzyme digestion using BamHI and XhoI (New England BioLabs).
515 Plasmids containing an insert of correct size were sequenced to confirm 100% sequence
516 homology with the original env infant consensus sequence. Plasmids were then prepared by
517 Megaprep (Qiagen) kit and re-sequenced to confirm. For three infants, 100046, 100383 and
518 101580, no single genome isolated matched 100% of nucleotides in the consensus sequence.
519 Therefore site-directed mutagenesis on a single nucleotide was performed to create an isolate
520 identical to the consensus sequence. Primers for site directed mutagenesis were designed using

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521 Agilent's QuikChange primer design program and Agilent's QuikChange II XL kit was used.
522 Sequencing of the clones was done to ensure 100% homology with the infant consensus
523 sequence.

524 **Pseudovirus preparation**

525 Env pseudoviruses were prepared by transfection in HEK293T (ATCC, Manassas, VA) cells
526 with 4μg of env plasmid DNA and 4μg of env-deficient HIV plasmid DNA using the FuGene 6
527 transfection reagent (Roche Diagnostics) in a T75 flask. Two days after transfection, the culture
528 supernatant containing pseudoviruses was harvested, filtered, aliquoted, and stored at -80°C. An
529 aliquot of frozen pseudovirus was used to measure the infectivity in TZM-bl cells. 20μl of
530 pseudovirus was distributed in duplicate to 96-well flat bottom plates (Co-star). Then, freshly
531 trypsinized TZM-bl cells were added (10,000 cells/well in Dulbecco's modified Eagle's medium
532 (DMEM)-10% fetal bovine serum (FBS) containing HEPES and 10 μ g/ml of DEAE-dextran).
533 After 48 h of incubation at 37°C, 100μl of medium was removed from the wells. 100μl of
534 luciferase reagent was added to each well and incubated at room temperature for 2 min. 100μl of
535 the lysate was transferred to a 96-well black solid plate (Costar), and the luminescence was
536 measured using the Bright-Glo™ luminescence reporter gene assay system (Promega).

537 **Neutralization Assays**

538 Neutralizing antibody activity was measured in 96-well culture plates by using Tat-regulated
539 luciferase (Luc) reporter gene expression to quantify reductions in virus infection in TZM-bl
540 cells. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent
541 Program, as contributed by John Kappes and Xiaoyun Wu. Assays were performed with HIV-1
542 Env-pseudotyped viruses as described previously [62]. Test samples were diluted over a range of

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543 1:20 to 1:43740 in cell culture medium and pre-incubated with virus (~150,000 relative light unit
544 equivalents) for 1 hr at 37˚C before addition of cells. Following a 48 hr incubation, cells were
545 lysed and Luc activity determined using a microtiter plate luminometer and BriteLite Plus
546 Reagent (Perkin Elmer). Neutralization titers are the sample dilution (for serum/plasma) or
547 antibody concentration (for sCD4, purified IgG preparations and monoclonal antibodies) at
548 which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus
549 control wells after subtraction of background RLU in cell control wells. Serum/plasma samples
550 were heat-inactivated at 56˚C for 1 hr prior to assay. Murine leukemia virus SVA.MLV was used
551 as a negative control [40]. A response was considered positive if the plasma ID_{50} against infant
552 T/F viruses was at least 3 times higher than the ID_{50} versus SVA.MLV.

553 **Env Virus Variant Tier Phenotyping Assay**

554 Neutralization titers (ID50s) were determined essentially as described above using five plasma
555 samples from HIV+ individuals in chronic infection. The geometric mean titer (GMT) was
556 calculated in Microsoft Excel and tier phenotype was determined by comparing these values to
557 the GMTs of standard panels of viruses representing tier 1A, tier 1B and tier 2 viruses [40, 63]
558 using the same five HIV+ plasma samples.

559 **Sequence Selection Algorithm**

560 To select maternal non-transmitted variants and capture the most divergent sequences from the
561 infant T/F, we devised an algorithm as follows. The algorithm finds the most variable positions
562 in the amino acid alignment and ranks all sequences with respect to the frequencies at these
563 positions. Sequences are then selected starting from the most divergent based on motif coverage

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564 as observed in the alignment and in the phylogenetic tree (in other words, if a group of diverging
565 sequences all share the same motif, only one in the group and/or tree node is selected).

566 **Statistical Analysis**

567 To test whether infant transmitted viruses were statistically significantly more resistant to
568 maternal plasma than non-transmitted maternal sequences, we devised a 1-sided permutation
569 test. At each iteration, we randomly assigned the "transmitted" status to any one sequence in
570 each infant-mother pair, and then ranked the remaining sequences in the pair according to
571 maternal plasma responses. All ranks across all pairs were then summed. We repeated this
572 randomization 1,000 times and then calculated the p-value as the percentage of sum of ranks that
573 were above the observed sum of ranks, out of all randomizations performed. This method is
574 robust, as it does not make any underlying assumption of the distribution of the maternal
575 plasmas, and it preserves the within mother-infant correlation of the data. The same algorithm
576 was used to test whether specific amino acid positions conferred resistance to maternal plasma
577 and/or antibodies. This time the "transmitted" status that was reshuffled at each iteration was the
578 wild type amino acid.

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759 ## Supporting Information

760 **Fig. S1 Phylogenetic tree and highlighter plot for all other 12 mother infant pairs.** Infant
761 sequences are labeled in red circles and maternal sequences are labeled in blue squares.
762
763 **Fig. S2 Phylogenetic tree and highlighter plot for mother infant pair 102605.** Infant
764 sequences are labeled in red and maternal sequences are labeled in blue. The non-random
765 accumulation of synonymous mutations in the infant (which caused the Poisson Fitter analysis to
766 fail) is evident on the right as marked by red box.
767
768 **Fig. S3 Neutralization sensitivity of Infant T/F viruses to heterologous broadly neutralizing**
769 **antibodies (bNAbs).** Dark colors represent easily neutralized viruses. Second T/F viruses are
770 marked with an *.

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771 **Figure legends**

772 **Fig. 1 Highlighter plots of maternal and infant viruses**

773 Example of mother-infant pair where the infant was infected with a single T/F virus and no
774 evidence of evolutionary selection in the infant (3/16 infants) (A). Example of mother infant with
775 evidence of evolutionary selection in the infant (B), and Example of mother infant pair where the
776 infant was infected by two distinct T/F viruses (6/16) infants (C). Individual infant and maternal
777 viruses are represented by red dots and blue squares, respectively, on the tree. Colored hash marks
778 on each highlighter plot represent nucleotide differences as compared with the infant consensus
779 sequence at the top and are color-coded according to nucleotide.

780

781 **Fig. 2: Tier phenotyping and neutralization sensitivity of Infant T/F viruses to paired**
782 **maternal plasma and heterologous broadly neutralizing antibodies (bNAbs)**. Dark colors
783 represent easily neutralized viruses. Second T/F viruses are marked with a star (*).

784

785 **Fig. 3 Infant neutralization sensitivity against a panel of bNAbs.**

786 The strength of the responses is color coded from dark to light where darker reds indicate stronger
787 responses. Aquamarine indicates absence of response. T/F viruses from the same infant are labeled
788 using the same color (left columns). The potency (geometric mean of responses) and breadth (%
789 neutralized) of the infant viruses to each bNAb were compared with the geometric means of the
790 bNAb's potency and breadth obtained from published studies using the LANL repository
791 CATNAP (columns on the right). The most potent neutralization against infant viruses was
792 mediated by V3 glycan bNAbs and CD4 binding site specific NIH45-46.

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793 **Fig. 4 Neutralization sensitivity of maternal and infant viruses to paired maternal plasma at**
794 **delivery.** Sensitivity of maternal non-transmitted variants (black dots), infant T/F variants (blue
795 and green triangles) and the closest maternal variant (blue and green dots) to the infant T/F variant
796 against autologous maternal sera. Sequences were selected following an algorithm to represent
797 different motifs that diverge from the infant T/F (see Methods). Black horizontal lines represent
798 the median of the ID_{50} of maternal and infant sequences. The dashed line represents the detection
799 threshold.
800
801 **Fig. 5 Neutralization sensitivity of maternal and infant viruses to paired maternal plasma**
802 **and an MPER specific bNAb with and without identified signature sequences within MPER.**
803 IC_{50} of maternal non-transmitted variants (black dots), infant T/F variants (blue and green
804 triangles) and the closest maternal variant (blue and green dots) to the 4 antibodies PG9, VRC01,
805 DH429, and DH512. Black horizontal lines represent the median of the IC_{50} of maternal and infant
806 sequences. Dashed lines represent the detection threshold.
807
808 **Fig. 6 Comparison of neutralization sensitivity of maternal and infant viruses to epitope**
809 **specific bNAbs.** Comparison of paired maternal plasma and MPER specific bNAb DH512
810 maternal non-transmitted sequences (black dots) and infant T/F sequences (green triangles)
811 between sequences that carry the wildtype amino acid at HXB2 positions 662, 676, 676, and 683,
812 compared with those that carry a mutant. These four positions were chosen because they yielded
813 a significant association with either maternal plasma responses (i.e. sequences carrying the
814 mutant were statistically significantly more resistant to maternal plasma) or with DH512
815 responses (i.e. sequences carrying the mutant were statistically significantly more resistant to DH

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816 512) in the MPER epitope. P-values were obtained using a 1-sided permutation test (see

817 Methods). Gray boxes represent median and quartiles of the responses.

818

819 **Fig. 7 Frequency plot of the V3 region sequences of mother and Infant T/F viruses.**

820 Weblogo plot showing frequency of amino acids in the V3 region was made using the

821 AnalyzAlign tool from the LANL website. Positions were numbered based on HXB2 amino acid

822 sequence. Amino acid positions identified in the signature sequence analysis are marked at the

823 top of the plot.

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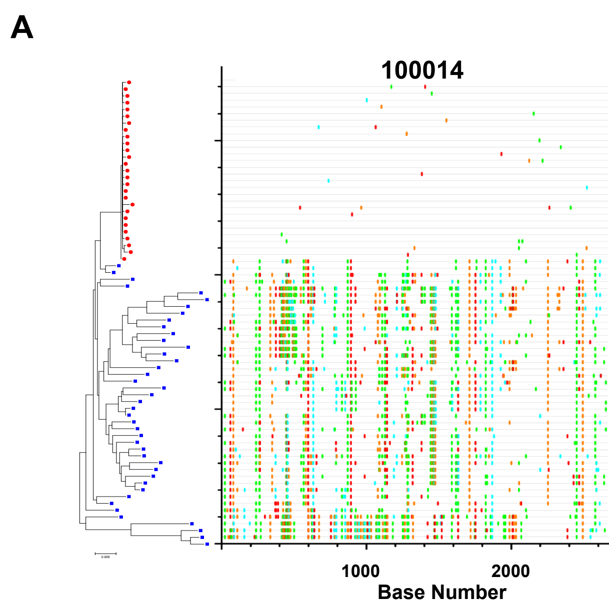
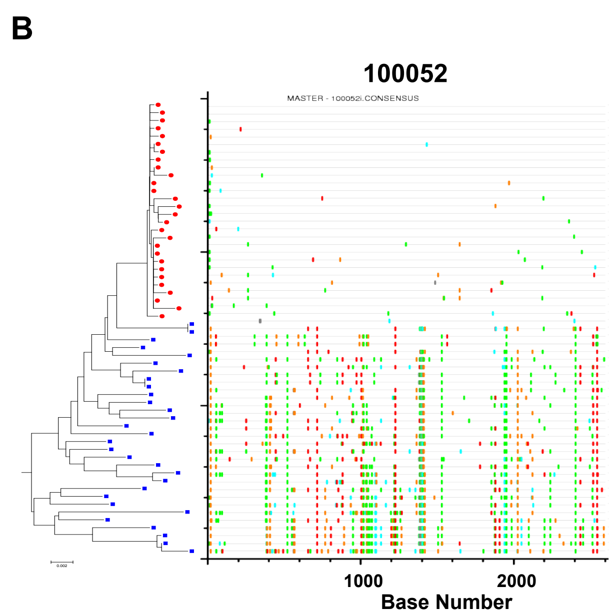
A



B



C

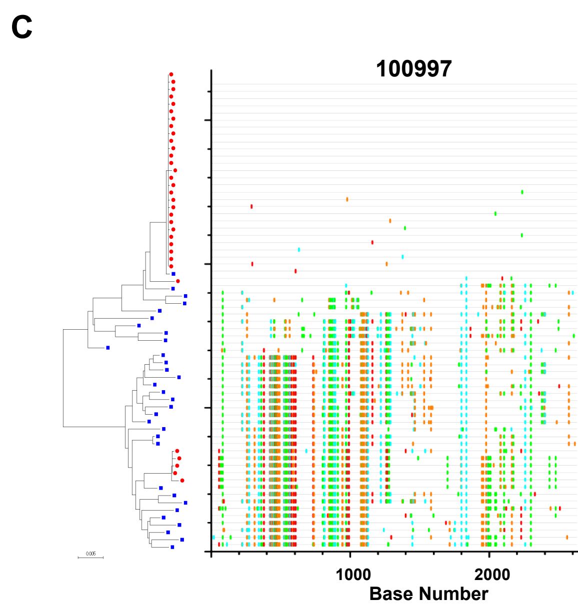


Fig. 1

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Infant T/F	Virus Tier	Maternal plasma	Maternal plasma vs MLV	Infant plasma	Infant plasma vs MLV
100002i.2.1	2	<20	<20	84	110
100014i.2.20	2	89	79	97	106
100046i.3.2	2	98	<20	411	134
100052i.2.15	1B	37	<20	200	175
100307i.2.26	1B	<20	<20	<40	<20
100307i.2.7*	2	28	<20	<20	<20
100383i.3.5	2	<20	<20	310	156
100711i.3.26	2	<20	<20	<40	<20
100890i.2.1	2	<20	<20	<40	<20
100890i.2.4*	1A	<20	<20	<20	<20
100997i.2.2	2	<20	<20	<20	<20
100997i.2.18*	3	<20	<20	<20	<20
101421i.2.23	1B	<20	<20	<40	<20
101421i.2.11*	2	30	<20	<20	<20
102149 i.2.23	2	29	<20	113	88
102407 i.2.1	2	22	<20	109	46
102605i.33	2	25	<20	26	<20
100155i.21	1B	21	<20	550	122
100504i.20	2	126	60	306	134
101984i.10	3	26	<20	<20	<20
101984i.21*	3	<20	<20	<20	<20

ID_{50}
<60
60-100
101-200
201-300
>300

Fig. 2

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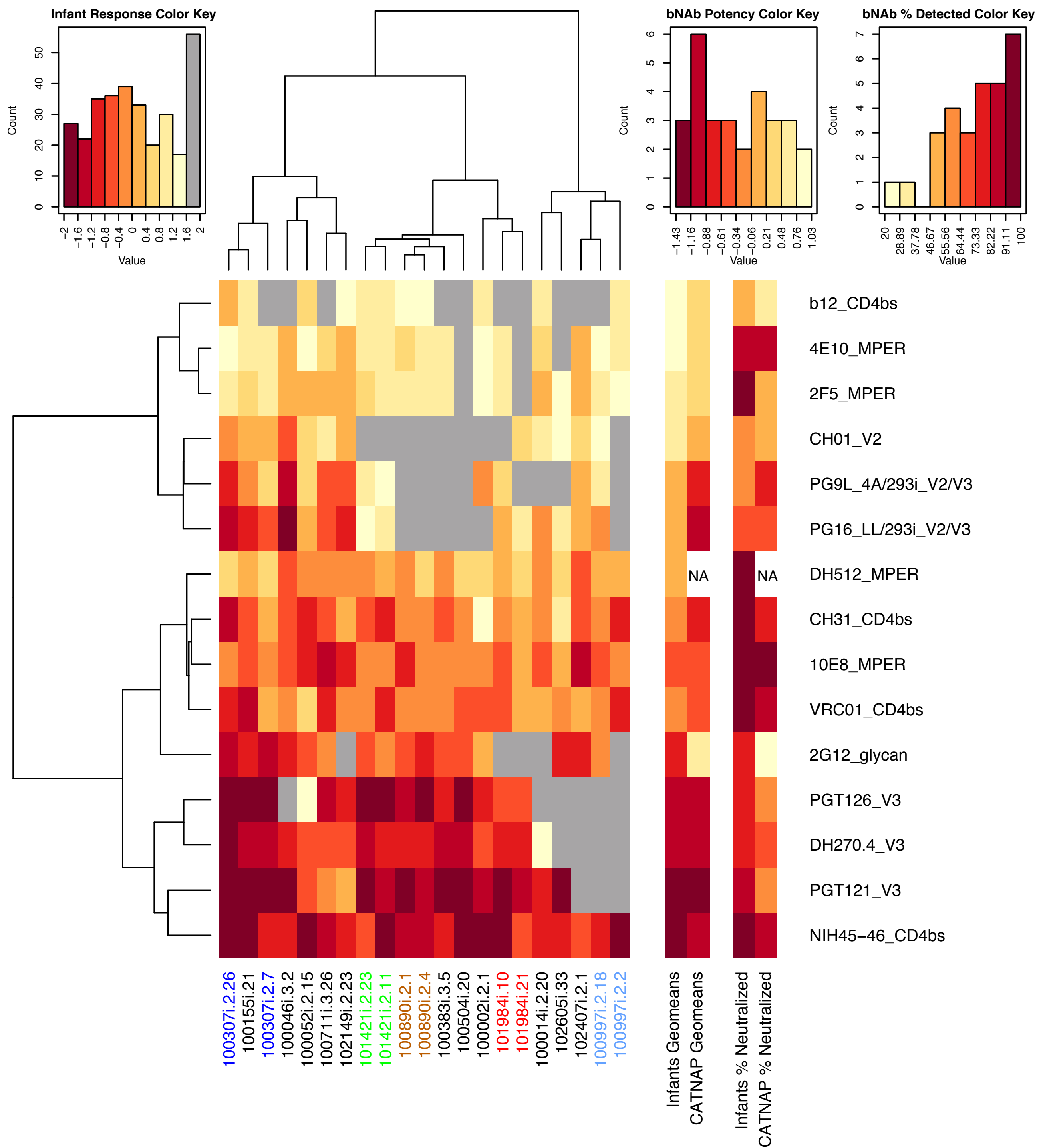

Fig. 3

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Maternal variants
1st Infant T/F
2nd Infant T/F
Maternal variant closest to 1st Infant T/F
Maternal variant closest to2nd Infant T/F
Maternal sequences median

Neutralization ID50
1000
100
10

100002 100014 100046 100052 100307 100383 100711 100890 100997 101421 102149 102407 102605 100155 100504 101984

Mother/Infant Pairs

Fig. 4

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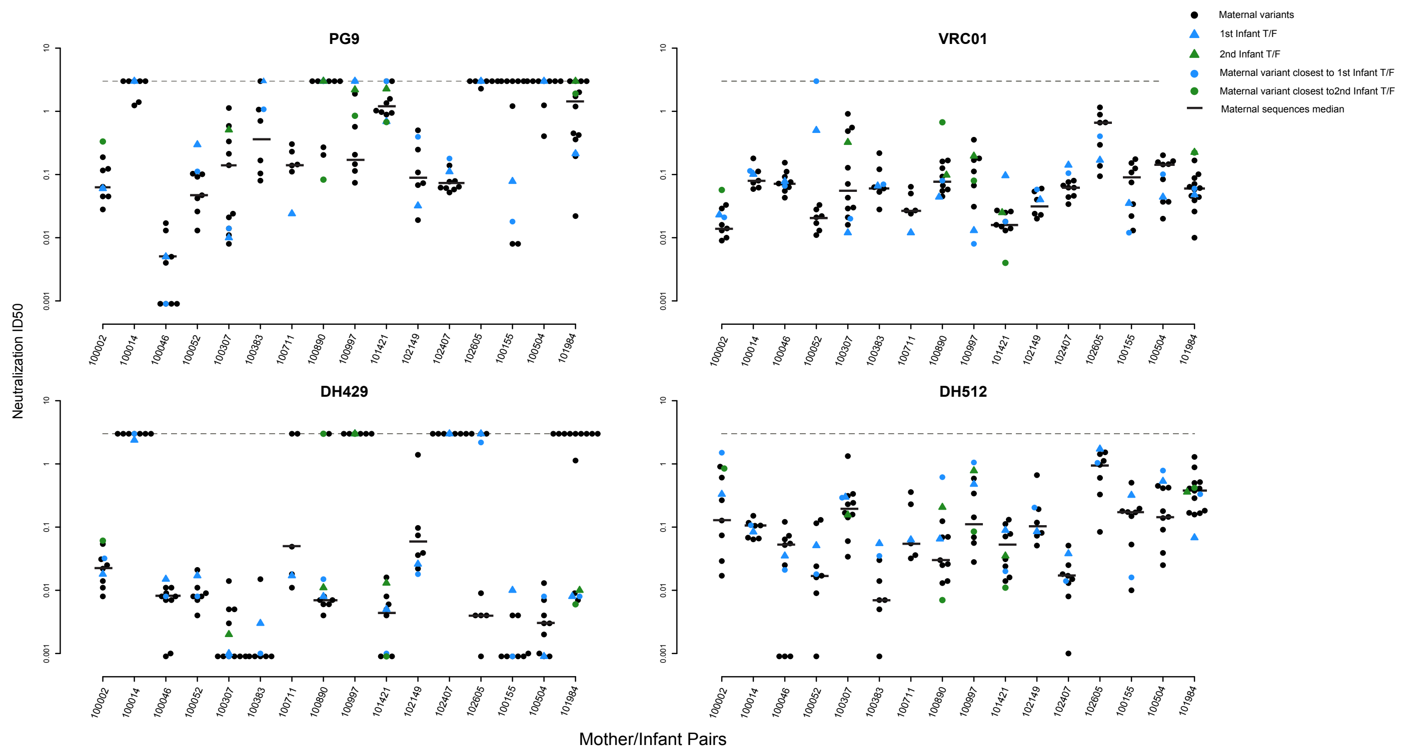

Fig. 5

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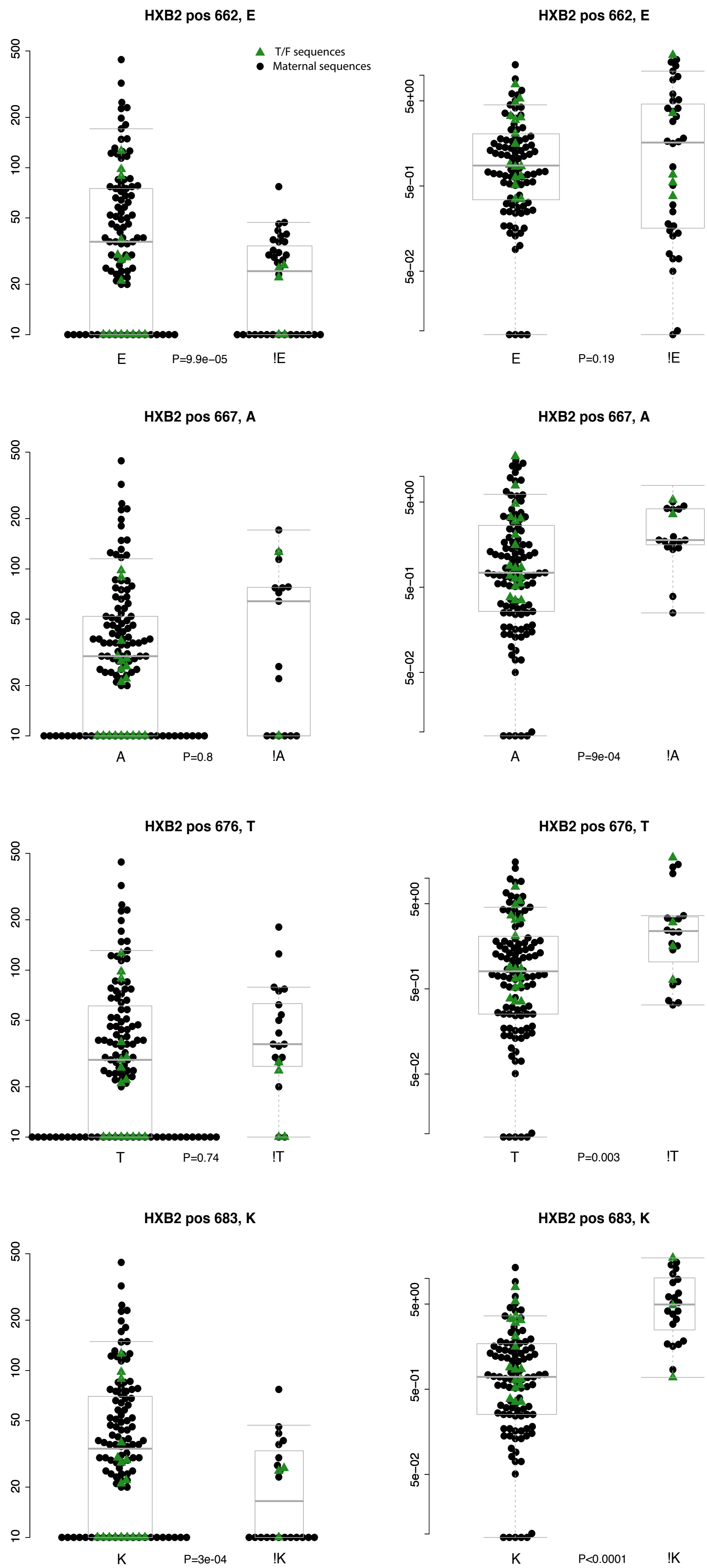

Fig. 6

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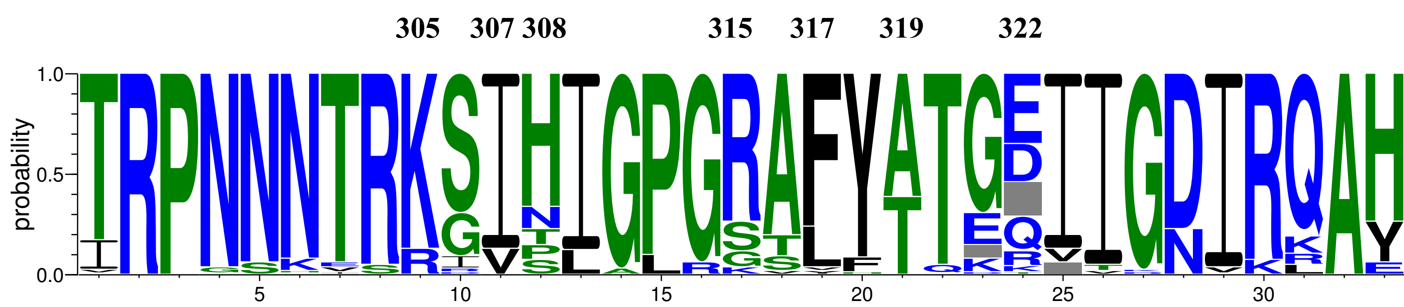

Fig 7