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Frequent development of broadly neutralizing antibodies in early life in a large cohort of HIV-infected children

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25 **Abstract**

26 Recent studies conducted in small cohorts of children have indicated that broadly neutralizing
27 antibodies (bnAbs) may develop earlier after HIV infection compared to adults. To define the
28 frequency and kinetics of bnAb responses in a larger pediatric cohort, we evaluated plasma from
29 212 ART-naïve, children living with HIV aged 1 to 3 years. Neutralization breadth and potency
30 was assessed using a panel of 10 tier-2 viruses and compared to those of adults with chronic
31 HIV. Further, the magnitude, epitope specificity and IgG subclass distribution of Env-specific
32 antibodies were also assessed using a binding antibody multiplex assay. We found that 1-year-
33 old children demonstrated neutralization breadth comparable to that of chronically-infected adults,
34 and breadth continued to increase with age such that the pediatric cohort overall exhibited
35 significantly greater neutralization breadth than adults ($p= 0.014$). Similarly, binding antibody
36 responses increased with age, and the levels in 2 to 3 year-old children were comparable to those
37 of adults. Overall, there was no significant difference in antibody specificities or IgG subclass
38 distribution between the pediatric and adult cohorts. Interestingly, the neutralization activity was
39 mapped to a single epitope (CD4 binding site, V2 or V3 glycans) in only 5 of 38 pediatric broadly
40 neutralizing samples, suggesting a polyclonal neutralization response may develop in most
41 children. These results contribute to a growing body of evidence suggesting that the early life
42 immune system may present advantages for the development of an effective HIV vaccine.

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44

45 **Author summary**

46 Understanding the development of broadly neutralizing antibodies during natural HIV infection is
47 critical to provide insights for the development of an effective vaccine. In this study, we used a
48 large cohort (n=212) of HIV infected children aged 1-3 years old to define the frequency and
49 kinetics of broad neutralization in comparison to adults with chronic HIV. We also evaluated the

50 magnitude and epitope specificity of HIV Env-specific antibodies in children and adults. We
51 observed that both the binding and neutralizing antibody responses increased with age.
52 However, by one year of age, children had neutralization breadth comparable to that of
53 chronically infected adults; and overall, 1-3 year-old children demonstrated higher neutralization
54 breadth than adults. Only slight differences in epitope-specificity and IgG subclass distribution
55 were observed between children and adults, suggesting that these factors are not major
56 contributors to the observed age-related difference in neutralization breadth. The epitope
57 specificity of the neutralizing antibodies could not be mapped in the majority of children,
58 suggesting that their response may be polyclonal. Our study contributes to the body of work
59 indicating a potential advantage of the early life immune system for the elicitation of broad
60 neutralization.

61

62 INTRODUCTION:

63 The global HIV pandemic persists despite increased availability of antiretroviral (ARV)
64 treatment. In 2019, 1.5 million people aged 15 years and above became newly infected with HIV
65 and 460,000 of these infections occurred in young adults aged 15-24 [1]. Furthermore,
66 adolescents represent the only age group that has experienced increased numbers of AIDS-
67 related deaths over the last decade [2]. While the need for an HIV vaccine is clear, it is critical
68 that this vaccine be designed for administration prior to sexual debut to both protect during the
69 vulnerable window of adolescence, as well as generate lifelong immunity.

70 Passive immunization studies in animal models have demonstrated that antibodies
71 capable of neutralizing HIV-1 can protect from virus acquisition [3]. Elicitation of broadly
72 neutralizing antibodies (bnAbs) is therefore believed to be crucial for vaccine efficacy. However,
73 traditional vaccine approaches have failed to induce bnAb responses [4]. Furthermore, only a
74 subset of chronically HIV-infected adults (10-30%) develops bnAbs and they only develop them
75 after several years of infection [5]. Understanding how neutralization breadth develops during
76 natural infection will be necessary to guide novel vaccine development strategies.

77 In HIV-infected individuals, bnAbs target epitopes in conserved areas of the Env
78 including the CD4-binding site, V1V2-glycan region, and V3-glycan region on gp120; and the
79 gp120-gp41 interface and fusion domain and membrane proximal external region (MPER) on
80 gp41 [6]. Immunogenetic characterization of bnAbs identified in adults has demonstrated
81 unusual traits such as high levels of somatic hypermutation (SHM), nucleotide insertions and
82 deletions, long complementary determinant region 3 (CDR3) lengths, and restricted variable
83 gene use [7]. Recently, studies have evaluated the presence of bnAbs in HIV-infected children.
84 Notably, Goo et al. (2014) reported that children develop cross-clade neutralization by two years
85 of age [8]. Moreover, Meunchhoff et al. (2016) observed that 75% of HIV-infected
86 nonprogressor children aged \geq five years but only 19% of chronically infected adults

87 demonstrated neutralization breadth [9]. Altogether, these previous studies suggest that HIV-
88 infected children develop neutralization breadth earlier and more frequently than infected adults.

89 Epitope mapping of neutralizing Abs in pediatric cohorts has suggested that bnAbs from
90 children target the same key epitopes on HIV-1 Env glycoproteins as bnAbs from adults.
91 However, in contrast to adults, where broadly neutralizing activity is typically attributed to a
92 single or a few distinct epitope specificities [10] plasma neutralization in children appears to be
93 polyclonal [11]. Furthermore, distribution of these bnAb specificities differed from corresponding
94 maternal plasma, with a higher prevalence of MPER-specific Abs and decreased titers of V2-
95 glycan specific Abs. Thus, it is possible that the mechanism by which children achieve broad
96 plasma neutralization is distinct.

97 Despite these recent findings, our knowledge of the ontogeny of Env-specific Ab
98 responses in pediatric settings remains incomplete. While several studies have investigated the
99 ontogeny and IgG subclass distribution of HIV-specific Abs in HIV-infected adults [12], few
100 studies have been conducted in young children [13]. Further characterization of Env-specific
101 binding responses and IgG subclass distribution in infants and young children might guide the
102 design of vaccines targeting the early life period.

103 Importantly, previous studies investigating neutralization breadth development in
104 children have several inherent limitations such as small cohort size [8] or focus on the specific
105 population of nonprogressor children [9]. To define the frequency and kinetics of bnAb in HIV-
106 infected children, we acquired plasma samples from 212 ART-naïve, clade B HIV-infected
107 children infected either *in utero* or at the time of delivery from the International Maternal,
108 Pediatric, Adolescent AIDS Clinical Trials (IMPAACT) repository. In addition, we obtained
109 plasma samples from 44 ART-naïve HIV chronically-infected adults from the Neutralization
110 Serotype Discovery Project (NSDP) study [14] for comparison. Using these two cohorts, we
111 compared the magnitude, specificity, and IgG subclass distribution of HIV-1 Env-specific Abs
112 between HIV-infected children and adults. In addition, we compared neutralizing antibody

113 responses in children to those of adults using a global panel of HIV-1 strains [15] and key
114 epitopes targeted by pediatric neutralizing antibodies were identified. To our knowledge, this
115 study represents the most comprehensive analysis of HIV neutralizing antibody responses in a
116 large cohort of young children conducted to date.

117

118 **RESULTS:**

119 **HIV-1 Env-specific Ab binding responses in the pediatric cohort in comparison to adults.**

120 A panel of 17 HIV-1 antigens was used to assess the breadth and epitope-specificity of HIV-1
121 Env-specific IgG in cross sectional samples of children with HIV, aged one to three years,
122 (n=212) and in clade B chronically infected adults (n=44). Because these children acquired
123 infection *in utero* or around birth, their age reflects the duration of infection. The breadth of the
124 HIV-specific binding antibodies was comparable between children and adults and we observed
125 no difference in the magnitude of binding to cross-clade specific HIV-1 gp120 and gp140
126 antigens between the two groups (**Figure 1**). More than 90% of children and adults had
127 antibodies that bind to all the Env glycoproteins tested, except for A244 gp120 (Supplemental
128 Table 1). The frequency of responders against A244 gp120 was higher in adults than in children
129 (86 vs 70%, $p=0.032$), but comparable frequencies were observed for the other antigens.
130 Interestingly, the magnitude of the IgG binding against most antigens increased between one
131 and two years of age, but was comparable between two and three year-old children
132 (Supplemental Figure 1). Thus, Env-specific antibody levels progressively increase during the
133 first two years of life in perinatally infected children and, by two years of age, their antibody
134 levels are comparable to that of chronically infected adults.

135 Env-specific antibodies from adults and children generally bound to the same epitopes,
136 but slight differences were observed between the two groups for some of the tested epitopes.
137 Notably, children had higher levels of antibodies against the constant region 1 (C1) and the
138 MPER region whereas adults had higher levels of antibodies against the constant region 5 (C5).

139 Similarly, a higher percentage of children than adults had detectable antibodies against the
140 MPER (79% vs 61%, $p=0.017$) and the C1 peptide (58% vs 11%, $p<0.001$) whereas the
141 frequency of antibodies against the C5 peptide was higher in adults than in children (77% vs
142 42%, $p<0.001$). Thus, overall, HIV-infected children demonstrated robust Env-specific antibody
143 responses that largely target the same Env regions as adult antibodies.

144

145 **HIV-1 Env-specific IgG subclass distribution in HIV-1 infected adults and children.** We
146 then measured the magnitude and frequency of Env-specific IgG1, 2, 3 and 4 in the pediatric
147 and adult samples. Children tended to have a lower magnitude gp120-specific IgG1 responses
148 as compared to adults ($p=0.042$) and both adults and children had very high magnitude of gp41-
149 specific antibodies (**Figure 2**). Children also had lower magnitude gp41-specific IgG3
150 ($p=0.006$), and lower levels of Env glycoprotein-specific IgG4 than adults ($p<0.001$ for gp140,
151 gp120 and gp41). In contrast, children had higher magnitude gp41-specific IgG2 antibodies
152 ($p=0.004$) as compared to adults. Similar to total IgG, the levels of gp120-specific IgG1
153 increased with age, whereas low levels of antibodies from the other IgG subclass were
154 observed across the age groups (Supplemental Figure 2).

155 While the majority of adults and children had detectable levels of Env glycoprotein-
156 specific IgG1 (94 to 100%), slight differences in the frequency of the other subclasses were
157 observed between adults and children (Supplemental Table 2). Most adults and children had
158 IgG3 and IgG4 against gp41 but a higher proportion of children had gp41-specific IgG2 when
159 compared to adults (87% vs 48%, $p<0.001$). In contrast, IgG4 antibodies against gp120 were
160 detected more frequently in adults than in children (48% vs 22%, $p=0.002$). Approximately half
161 of the adults and children had gp120-specific IgG3 and approximately 20% had gp120-specific
162 IgG2. The proportion of adults and infants with detectable levels of IgG subclass antibodies
163 against the variable loop 2 (V2) and the variable loop 3 (V3) was comparable.

164

165 **HIV neutralization responses in HIV-infected children as compared to infected adults.**

166 The ability of pediatric samples to mediate broad neutralization was assessed against a panel of
167 10 HIV-1 pseudoviruses from the global neutralization panel. This panel was selected from 219
168 pseudoviruses to be representative of the global diversity of circulating HIV-1 strains [15]. The
169 neutralization potency and breadth in children was compared with previously reported data of
170 117 chronically infected adults from the NSDP [14]. Overall 69% of the children and 68% of
171 adults neutralized at least 50% of the viruses in the panel with an ID50 \geq 50. The frequency of
172 broad neutralization slightly increased with age, as only 60% of one-year old children but 76% of
173 two-year old children were able to neutralize 50% of the viruses. Comparable percentage of
174 two-year old and three-year old children (76% vs 75%) neutralized 50% of the viruses with an
175 ID50 \geq 50. Using a more stringent ID50 cutoff of 100, we observed that 26% of children but only
176 19% of adults neutralized 50% of the viruses with an ID50 \geq 100. The percentage of children
177 neutralizing 50% with ID50 >100 was comparable across the age groups (23% for one-year old,
178 29% for two and three-year old). There was no statistical difference in the percentage of
179 children and adults that were able to neutralize 5/10 viruses (**Table 2**). A higher percentage of
180 children than adults were able to neutralize 4/10 viruses (HIV TRO11: 78% vs 68%, $p=0.048$;
181 HIV X2278: 86% vs 75%, $p=0.015$; HIV 246F3: 71% vs 48%, $p<0.001$; HIV CH119: 88% vs
182 67%, $p<0.001$), whereas a higher percentage of adults neutralized one virus (HIV X1632: 63%
183 vs 28%, $p<0.001$). Thus, overall, the pediatric cohort demonstrated comparable or superior
184 neutralization frequency as compared to adults for 9/10 viruses tested. Children also
185 demonstrated comparable or superior neutralization potency (**Figure 3**). The median
186 neutralization titer was higher in children than in adults for 4/10 viruses tested whereas adult
187 samples demonstrated higher neutralization potency against one virus.

188 To assess the totality of the virus neutralization activity, neutralization scores were
189 generated for each pediatric and adult sample. The neutralization score was defined as the
190 average area under the magnitude-breadth curve (AUC) of the neutralization curve for all tested

191 viruses. Overall, the neutralization score was higher in children than in adults ($p=0.014$, **Figure**
192 **4a**). As with other antibody measurements, the neutralization score increased with age across
193 the one, two, and three-year old age groups ($p=0.014$, **Figure 4b**), but importantly, the
194 neutralization score in one-year old children was comparable to that of adults ($p=0.44$, **Figure**
195 **4c**). Thus, by one year of age, the plasma neutralization activity in perinatally infected children is
196 comparable to that of chronically infected adults.

197 Because the neutralization score is influenced both by the proportion of the viruses
198 neutralized as well as the potency with which these viruses are neutralized, magnitude-breadth
199 curves were generated to determine the relative contribution of breadth and potency on the
200 superior neutralization breadth score of children (**Figure 5**). The average ID₅₀ of the pediatric
201 cohort was significantly greater than that of the adult cohort with a similar proportion of viruses
202 neutralized, indicating that the higher neutralization score observed in children is mostly driven
203 by a superior neutralization potency with comparable number of viruses neutralized.

204

205 **Association between neutralization breadth and clinical factors in HIV-infected children.**

206 To explore the potential association between neutralization breadth and patient clinical factors,
207 we performed linear regression analyses between pediatric neutralization breadth scores and
208 patient CD4 T cell percentage and absolute counts provided by the IMPAACT repository
209 (Supplemental Figure 4). We found a weakly positive association between neutralization
210 breadth score and CD4+ T cell percentage (slope=0.01, $p=0.002$) as well as with CD4+ T cell
211 counts (slope=0.07, $p=0.003$). We also explored the association between viral load and
212 neutralization breadth and we did not observe a significant association (slope 0.10, $p=0.28$).
213 However, this analysis was limited by the small number of children with available viral load in
214 the IMPAACT database ($n=15$). The impact of other clinical factors such the timing of infection
215 (intrauterine versus perinatal) could not be evaluated due to the paucity of information in the

216 IMPAACT database. Nevertheless, our results suggest that factors other than CD4 T cell could
217 likely contribute to drive neutralization breadth development in children.

218 We also examined associations between neutralization and binding antibody responses
219 (supplemental table 3). We observed a weak, but statistically significant correlation between
220 total gp120 and gp140 IgG and neutralization score. Moreover, gp140 IgG4 and gp41 IgG4
221 levels were weakly associated with neutralization score. There was no association between
222 antibody specificity that differ between adults and children (such as C1 or C5-specific IgG) and
223 neutralization score. Overall this data suggest a possible weak association between IgG
224 subclass distribution and breadth development in young children.

225

226 **Epitope specificity of neutralizing antibodies in HIV infected children.** Pediatric samples
227 that were able to neutralize ≥ 5 viruses with an ID₅₀ ≥ 100 (n=38) were utilized to map the
228 epitope specificity of neutralizing antibodies. Neutralization assays were performed against a
229 virus from the global panel that the plasma sample neutralized potently (either HIV TRO11,
230 25710 or BJOX002) and against a panel HIV-1 pseudoviruses with selective mutations to
231 abrogate the neutralization potency of a specific class of broadly neutralizing antibodies [16]
232 (Supplemental Table 3). In addition, the samples were tested against a mutant pseudovirus
233 TRO11.W672A to assess the presence of MPER-specific antibodies. A total of 5/38 plasma
234 samples demonstrated at least a 3-fold reduction in ID₅₀ against one mutant as compared to
235 the parent virus (**Table 4**). Three samples demonstrated substantial reduction of neutralization
236 against a mutant that abrogates activity of V3 glycan-specific bnAbs, whereas one sample
237 demonstrated reduced neutralization of a mutant that abrogates activity of CD4 binding site
238 bnAbs, and the last showed reduced activity against a mutant that abrogates the activity of V2
239 glycan-specific bnAbs. None of the plasma samples demonstrated a dominant MPER reactivity.
240 Thus, the epitope specificity of the neutralizing antibodies could not be mapped in 33/38 HIV-
241 infected children with strong bnAb activity.

242

243 **Association between binding antibody responses and neutralization epitope specificity**

244 We conducted analysis to determine if there is an association between the levels of epitope-
245 specific binding antibodies and the neutralization specificity. PTD 2 (from Table 4) in which
246 neutralization specificity mapped to V2-glycan epitopes demonstrated moderately high V1V2-
247 specific IgG and IgG1 response ranking at the 65% and 56% percentile in the distribution of
248 these responses. No V1V2-specific IgG2, IgG3 or IgG4 was detected in this child (supplemental
249 Figure 5). The three children (PTD 3-5) in which neutralization specificity mapped to V3 glycan
250 dependent epitopes demonstrated high V3-specific IgG binding ranking at the 75, 85, and 83%
251 percentile of the IgG distribution. They also demonstrated high V3-specific IgG1 ranking at the
252 61, 79, and 75% percentile for the IgG1 distribution, whereas the levels of the other IgG
253 subclass were variable. Thus, while high levels of V1V2 or V3-specific IgG or IgG 1 antibodies
254 are not indicative of V2 or V3-specific neutralizing antibody responses, when children exhibited
255 neutralizing antibodies to a particular site they tended to have high binding antibodies to the
256 same site.

257

258 **DISCUSSION**

259 While induction of a broad neutralizing antibody response is a critical goal for HIV
260 vaccine, current immunization approaches have failed to achieve this goal [17]. Recent reports
261 indicating that: 1) HIV vaccination can elicit more robust and durable antibody responses in
262 infants than in adults [18] [19]; and 2) that children may develop broad neutralization more
263 frequently and earlier than adults [9], suggest that the early life immune system may present
264 advantages to overcome the unique immunologic challenges posed by the induction of
265 neutralization breadth through vaccination. The purpose of this study was to assess the
266 presence and characteristics of broadly neutralizing antibodies in a large cohort of HIV-infected,
267 ART-naïve young children and compare the kinetics of the development of bnAb responses

268 after infection to that of chronically-infected adults. Our results are in concordance with prior
269 reports indicating that children are able to develop broad neutralizing antibody responses and,
270 importantly, that the mechanism of breadth development may differ between adults and children
271 [8, 9].

272 Only a few studies have assessed broad neutralizing antibody responses in HIV infected
273 children. Notably Goo et al, investigated broad neutralization in a small cohort (n=28) of clade A
274 infected children from Kenya and reported that broad neutralization could be detected in infants
275 as early as one year after infection [8]. Subsequently, Muenchhoff et al., investigated broad
276 neutralization in a cohort of children from South Africa (aged >5 years) and reported higher
277 frequency of broad neutralization in children as compared to chronically infected adults [9]. In
278 contrast to these previous studies, our study focused on a large cohort of clade B HIV-infected
279 children. Moreover, we focused on children aged 1 to 3 to specifically define the kinetics of the
280 development of broad neutralization in children, as HIV-infected adults usually develop
281 neutralization breadth after several years of infection [44]. This age range excluded the possibility
282 that passive maternal antibodies contributed to the measured neutralization as before one year
283 of age it would be difficult to decipher the contribution of maternal versus infant antibodies. The
284 uniqueness of this historical set of pediatric ART naïve samples is worth noting as the WHO
285 now recommends initiation of ART at the time of diagnosis. To assess neutralization breadth,
286 we used viruses from the global neutralization panel [18]. We observed that one year old HIV-
287 infected children can demonstrate broad neutralization, corroborating the findings of Goo et al
288 [8] that HIV-neutralization can develop rapidly in early life. Yet, in contrast to the findings from
289 Muenchhoff et al., (2016) who reported more frequent bnAb development in children than in
290 adults (75% vs 19%, $p < 0.0001$) [9], we found that the proportion of children and adults who
291 neutralized 50% of the viruses was comparable (69% vs 68%). Comparable frequencies
292 between the groups were still observed when we only considered children and adults who
293 neutralize 50% of viruses with ID50 >100 (26% vs 19%, $p = 0.13$). Interestingly, Makhdoomi et

294 al., reported that the neutralization breadth in HIV- infected children from India aged 5 to 17
295 years [45] increased over time. In our study, although we did not test longitudinal samples, we
296 compared the neutralization activity across the children's age groups. We found that the
297 neutralization scores increased with age (Fig 4), but by one year of age, the breadth potency
298 neutralization score was comparable to that of chronically infected adults. Thus, the fact that our
299 cohort was younger than that of Muenchhoff et al., may contribute to explain the differences in
300 observed results. Furthermore, whereas our cohort was US- based (predominant clade B
301 infections), their cohort was South African based (predominant clade C infections). The South
302 African cohort also focused on the specific subpopulation of nonprogressors [9] and the virus
303 panels used in the two studies to assess neutralization breadth were different. Finally, it is
304 possible that differences in transmission modes between the two cohorts contributed to the
305 slightly divergent results [20]. In our US-based cohort, transmission occurred in utero and
306 perinatally, whereas breast milk transmission is also an important mode of transmission in
307 South Africa. Despite these differences, it is worth noting that our results combined with those of
308 previous studies establish that children are able to rapidly develop broad neutralizing antibody
309 responses after HIV infection.

310 Several studies have attempted to understand immune differences between children and
311 adults, with particular focus on the first 12 months of life and the development of B cell
312 populations [21-26]. However, although several studies have investigated the kinetics of HIV-
313 specific binding antibodies in adults, less is known about pediatric HIV-specific antibody
314 responses. We found that although the magnitude of HIV-specific binding Ab responses against
315 key Env epitopes was lower in one-year-old children, by two years of age it was comparable to
316 that of adults (Supplemental Figure 1). While we found no difference in binding breadth between
317 adults and children, we observed some slight differences in IgG binding epitope specificities
318 between the two groups. Notably, MPER and C1-specific antibodies were higher in children
319 than in adults whereas C5-specific antibodies were higher in adults. It has also been previously

320 reported that infants develop antibodies against the HIV gp160 precursor glycoprotein first, in
321 contrast to adults who first develop anti-gp41 antibodies, which suggests possible differences in
322 the kinetics of HIV-specific Abs between these two populations [13, 27]. Overall, the difference
323 in epitope specificities between adults and children suggests that similar immunogens could
324 elicit distinct responses in these two populations, an observation that may be relevant for
325 vaccine development and monitoring.

326 Previous studies indicated that increased levels of HIV-specific IgG2 and IgG4 during early
327 HIV infection in adults correlate with development of broad neutralization [28]. Moreover, an
328 association between high levels of IgG3 and neutralization have been reported [29]. We
329 therefore investigated if a distinct IgG subclass distribution in children as compared to adults
330 may contribute to explain the early development of broad neutralization. Overall, we found that
331 the IgG subclass profiles of the pediatric and adult cohorts were grossly similar with only a few
332 statistically significant differences. Most notably, we observed that the majority of children
333 mounted a detectable gp41-specific IgG2 response, whereas this response was seen in less
334 than half of chronically infected adults. The clinical significance of this difference is unclear as
335 there was no association between this response and neutralization breadth. The ability of
336 children to produce IgG2 is usually delayed compared to other subclasses [30], thus the
337 observation that young children develop more robust IgG2 responses than adults is somewhat
338 surprising. Nevertheless, as an association between low levels of gp41-specific IgG2 are usually
339 associated to later stage disease [31], the higher levels of gp41-specific IgG2 in children may
340 simply be a marker of a more recent infection. Interestingly, it was recently reported that IgG3
341 enhances the neutralization activity of a HIV V2-specific bnAb [16]. Thus, we were interested to
342 define if higher HIV-specific IgG3 were observed in children. For most antigens, there was no
343 difference in the magnitude of IgG3 responses between adults and children, except for gp41
344 and V1V2 that were higher in adults than in children. Thus, overall, our results suggest that IgG

345 subclass distribution may not contribute to the early development of broad neutralization in
346 children.

347
348 Previous studies have suggested that in contrast to adults in which a single epitope
349 specificity frequently mediate neutralization breadth [32, 33], plasma broad neutralization in
350 children may be mediated by a polyclonal response. For example, Goo et al, were not able to
351 map the epitope specificity of broad neutralization in the majority of the children from their
352 cohort suggesting that either the pediatric neutralizing antibodies target novel neutralization
353 epitopes that are distinct from adults or broad neutralization is mediated by polyclonal
354 antibodies. Ditse et al, [11] analyzed the epitope specificity of neutralizing antibodies in 16 clade
355 C infected nonprogressor children from South Africa. They observed that the majority of children
356 had antibodies targeting multiple known neutralization epitopes, and that most children had
357 antibodies against V2 and V3-glycan dependent epitopes whereas MPER antibodies rarely
358 confer breadth to children. Similarly, Mishra et al [46] reported that plasma bnAbs targeting the
359 V2-apex were predominant in a small cohort of 10 elite neutralizers infants from India. In their
360 study, only two children demonstrated responses against multiple neutralizing epitopes. In
361 contrast to these previous studies, we were only able to map neutralization to a dominant
362 epitope in five out of 38 samples tested. In three of these, broad neutralization was mediated by
363 V3-glycan dependent antibodies, whereas V2-glycan dependent and CD4 binding site antibody
364 were the dominant neutralizing epitopes in the other two samples, respectively. While it is
365 possible that our selection of mutant pseudoviruses did not capture an existing dominant
366 epitope, the most common bnAb targets- V2 glycan, V3 glycan, CD4bs, MPER and gp120-gp41
367 interface [11]- were represented in our panel, and thus it is likely that the fact that we could not
368 map the dominant specificity in 33/38 samples indicate that breadth was mediated either by a
369 polyclonal response or that these pediatric nAbs target a novel neutralization epitope. A
370 dominant polyclonal response in children may indicate that the mechanisms by which the

371 pediatric and adult immune systems generate plasma broad neutralization could differ. In adults,
372 it has been proposed that the development of bnAbs results from cooperation between multiple
373 B cell lineages; “cooperative” lineages react to and select for escape mutants, which allows the
374 bnAb lineage to be continually stimulated by the founder virus to the extent of sustained affinity
375 maturation [7]. Our findings may suggest that the abundance of naïve B cells in children allows
376 for activation of multiple independent B cell lineages upon HIV infection leading to a polyclonal
377 response. Thus, plasma broad neutralization in children could be mediated by the additive
378 activity of a collection of antibodies with limited to moderate neutralization.

379 Elucidating the mechanisms through which children achieve broad neutralization will be
380 critical to guide vaccine development. Importantly, analysis of two V3-glycan dependent bnAbs
381 isolated from children indicated that breadth was acquired through a pathway distinct from that
382 of adult antibodies from the same class [34, 35] with notably lower levels of somatic
383 hypermutation and early accumulation of critical improbable mutations [36]. Identifying and
384 characterizing more Abs from children is imperative to determine if low levels of SHM is a
385 consistent unique feature of pediatric nAbs. This knowledge will ultimately guide HIV vaccine
386 strategies aiming at inducing broad neutralization. Such strategies could involve initiation of
387 vaccination early in life (following the immunization schedule for the under 5 year olds) and
388 boosting through pre-adolescence in order to achieve protective immunity prior to sexual debut.

389

390 **MATERIALS AND METHODS:**

391 **Samples:**

392 Pediatric plasma samples from ART-naïve children aged one, two and three-years old (n=212)
393 who were enrolled in the completed IMPAACT studies ACTG 152, 300, 382 and 390 [37-40]
394 were obtained from the IMPAACT biospecimen repository. All these children were assumed to
395 be infected with clade B HIV-1 at birth or *in utero* as they were born to women who acquired HIV
396 in the US. Adult plasma samples (n=44) were obtained from ART-naïve adults with chronic

397 clade B HIV-1 infection of \geq three years who participated in the Neutralization Serotype
398 Discovery Project [14]. A summary of the clinical characteristics of the included patients in
399 provided in Table 1.

400 **Binding antibody multiplex assay for the measurement of Env-specific IgG:**

401 A previously described binding antibody multiplex assay (BAMA) [27] was used to measure IgG
402 binding to a panel of 17 HIV-1 antigens. These included: 1) cross-clade gp120s (Con6 gp120/B,
403 MNgp120, and A244 gp120); 2) cross-clade gp140s (B.con_env03 gp140, ConS gp140 CFI,
404 A1.Con gp140, and 1086C gp140); 3) gp70 scaffold Env constructs (gp70_B.CaseA_V1V2,
405 gp70 MNV3); 4) a clade B recombinant gp41 (RecMN gp41); 5) peptides representing the V2
406 (Bio-V2.B), the V3 (Bio-V3.B), the MPER (MPER656) the C5 (RV144 C5.2B) and the C1 (C1
407 Biotin) regions of the HIV Env; and finally 6) YU2 Core, YU2 Core D368R mutant to assess
408 antibodies against the CD4 binding site. A pilot study was conducted to determine the optimal
409 testing dilution for the samples. This dilution was 1:100 dilutions for all antigens except for the
410 gp140s, Bio-V3B and recMNgp41 that were tested at 1:2000. Total antigen-specific IgG was
411 then detected with a mouse anti-human IgG phycoerythrin-conjugated Ab (Southern Biotech) at
412 2 $\mu\text{g}/\text{mL}$. Binding was measured using a Bio-Plex 200 instrument (Bio-Rad Laboratories, Inc.).
413 HIV-1 human hyperimmune immunoglobulin (HIVIG) derived from the plasma of HIV-1-infected
414 donors was used as a positive control and normal human serum was used as a negative
415 control. IgG responses were expressed as mean fluorescence intensity (MFI). All MFI values
416 were adjusted for nonspecific binding by subtracting the MFI of blank beads, except for
417 gp70_B.CaseA_V1V2 and gp70 MNV3, which were adjusted by subtracting the MFI of beads
418 coupled with a control gp70 construct (MuLVgp70). An HIV Env-specific Ab response was
419 considered positive if it had MFI values above a positivity cutoff determined as highest of either
420 the mean plus 3 standard deviations of the MFI of a panel of 20-30 HIV negative plasma, or the
421 lower detection limit of 100 MFI. To ensure consistency between assays, 50% effective

422 concentration and maximum MFI values of HIVIG control were tracked by Levey-Jennings
423 charts [41].

424 **Binding antibody multiplex assay for the *measurement of IgG subclasses*:**

425 Measurement of IgG subclass-specific HIV-1 binding Ab response was conducted as described
426 above with some modifications. All plasma samples were tested against a limited panel of 5
427 HIV-1 antigens: B.con_env03 gp140, MNgp120, gp70_B.CaseA_V1V2, gp70 MNV3, and Rec
428 MN gp41 at a 1:50 dilution. Antigen-specific subclass response was detected using biotin-
429 conjugated mouse anti-human IgG1 (BD Pharmingen, 4µg/L), IgG2 (Southern Biotech, 5ug/mL),
430 IgG3 (Calbiochem, 2µg/mL), or IgG4 (BD Pharmingen, 2µg/mL) and tertiary detection agent
431 streptavidin-phycoerythrin (BD Biosciences) at 5 µg/mL.

432 **Neutralization Assays:**

433 Neutralization was measured as the ability of plasma samples to reduce virus infection of TZM-
434 bl cells as previously described [42] against a panel of 10 viruses from the global neutralization
435 panel (deCamp et al., 2014) [15] (Table 2). Briefly, plasma samples were incubated with an HIV-
436 1 pseudovirus for 45-90 min at 37°C, then TZM-bl cells were added and plates were incubated
437 for 48 hr. A luciferase substrate (Bright-Glo; Promega) was added, and luminescence was
438 measured. Results were reported as the 50% inhibitory dilution (ID50), which is the dilution of
439 plasma resulting in 50% reduction in luminescence compared to that of virus control wells.

440 **Mapping of Neutralizing Epitopes:**

441 Plasma neutralization was assayed against a mutant panel of HIV-1 strains BJOX002, 25710, or
442 TRO.11 as described above. Panel choice was dependent on which parent virus was most
443 potently neutralized by the plasma sample. Each panel consisted of 6-11 HIV-1 Env epitope
444 knockout pseudoviruses (Supplemental Table 4). Significant reductions in ID50 between parent
445 and knockout virus suggests that epitope contributes to the neutralization ability of that sample.
446 In addition, samples were tested for MPER reactivity using TRO.11.W672A pseudotyped virus.

447

448 **Statistical analysis**

449 All statistical analyses were performed in the R statistical computing and graphics environment.
450 For two sample tests we used Mann-Whitney rank-based tests [47]. Neutralization scores were
451 computed as the area under the magnitude-breadth (M-B) curve, which describes the
452 magnitude (NAb titer) and breadth (number of isolates neutralized) of an individual plasma
453 sample assayed against all tested viruses, and equals the average log₁₀ titer over the targets
454 [48]. All p-values are two-sided. Boxplots were used to graphically display distributions of log₁₀
455 NAb titers to individual isolates. Positive response rates were compared between groups by
456 Fisher's exact tests [49]. Median regression was conducted using the quantreg package [50].
457 Titers of NABs among responders were compared between groups by 95% CIs about the ratio
458 of GMTs. Equality of the overall distribution of log₁₀ NAb titers between two groups was tested
459 as described (46), using 10,000 permuted data-sets to compute a p-value. The false discovery
460 rate (FDR) was used to determine tests that remained statistically significant after adjustment
461 for the multiple hypothesis tests. The FDR method was performed at level 0.05. Assessment of
462 magnitude and breadth of neutralization of a panel of isolates.

463

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465
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Figures & Tables

	Adult Cohort	Pediatric Cohort		
Cohort Size	n = 117	1-year-old	2-year-old	3-year-old
		n = 91	n = 62	n = 59
Gender	N/A	?	?	?
Clade Infected	Multiple Clade-Infected (with subset of 44 Clade B-Infected)	Clade B-Infected		
Duration of Infection	Chronically Infected for ≥ 3 years	Infected <i>in utero</i> or at birth		
ART Exposure	ART-naïve	ART-naïve		

Table 1. Cohort Clinical Summary

Pediatric cohort samples were provided by completed IMPAACT studies ACTG 152, 300, 382, and 390. Adult clade B-infected plasma samples were provided by the Neutralization Serotype Discovery Project.

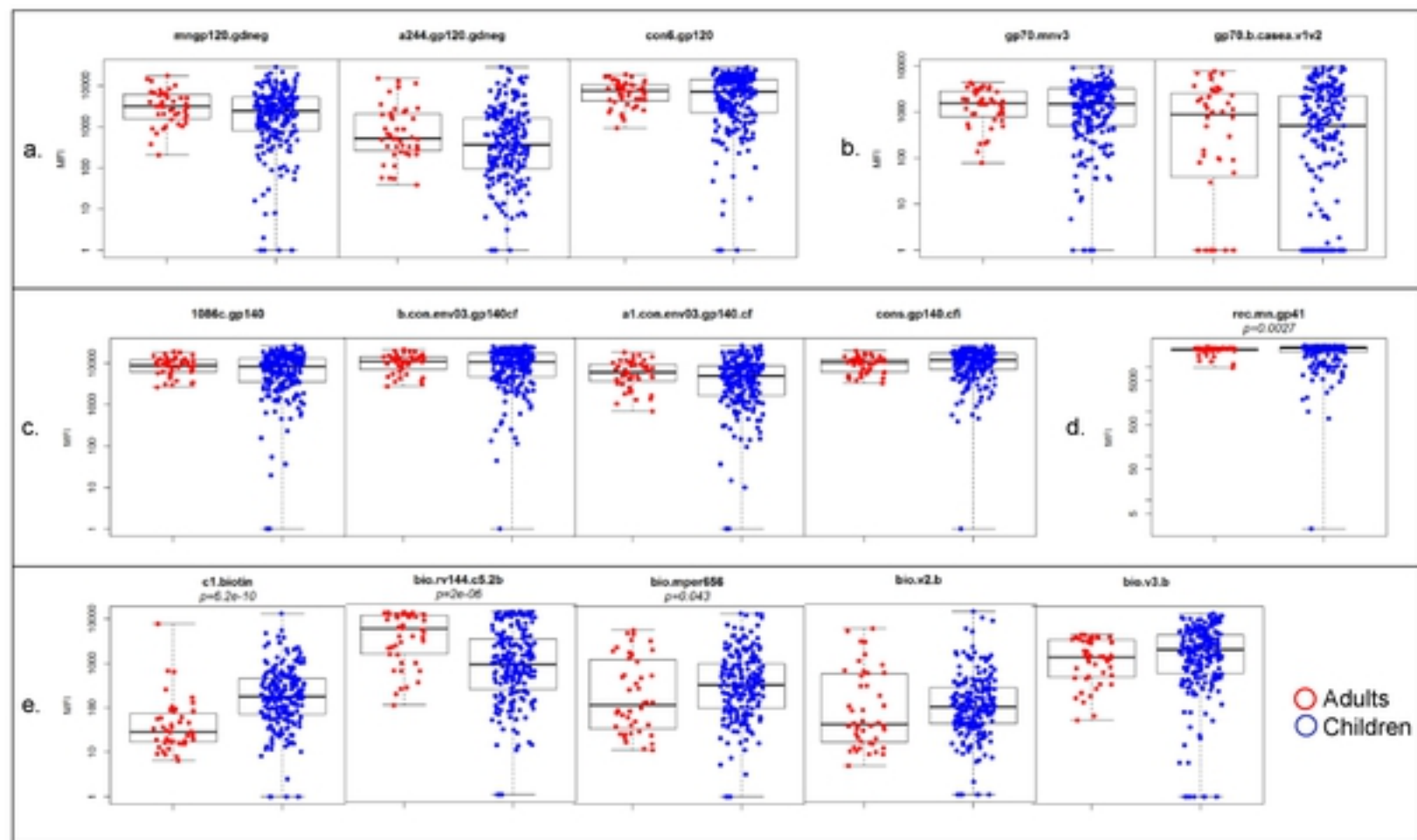


Figure 1. HIV-1 Env-Specific Total IgG

Total IgG for select HIV-1 Env epitopes were measured by binding antibody multiplex assay in adult and pediatric cohorts. Epitopes include gp120 (a), variable loops (b), gp140 (c), gp41 (d) and peptides (e). Significant difference between adult and pediatric cohorts by Wilcoxon as noted.

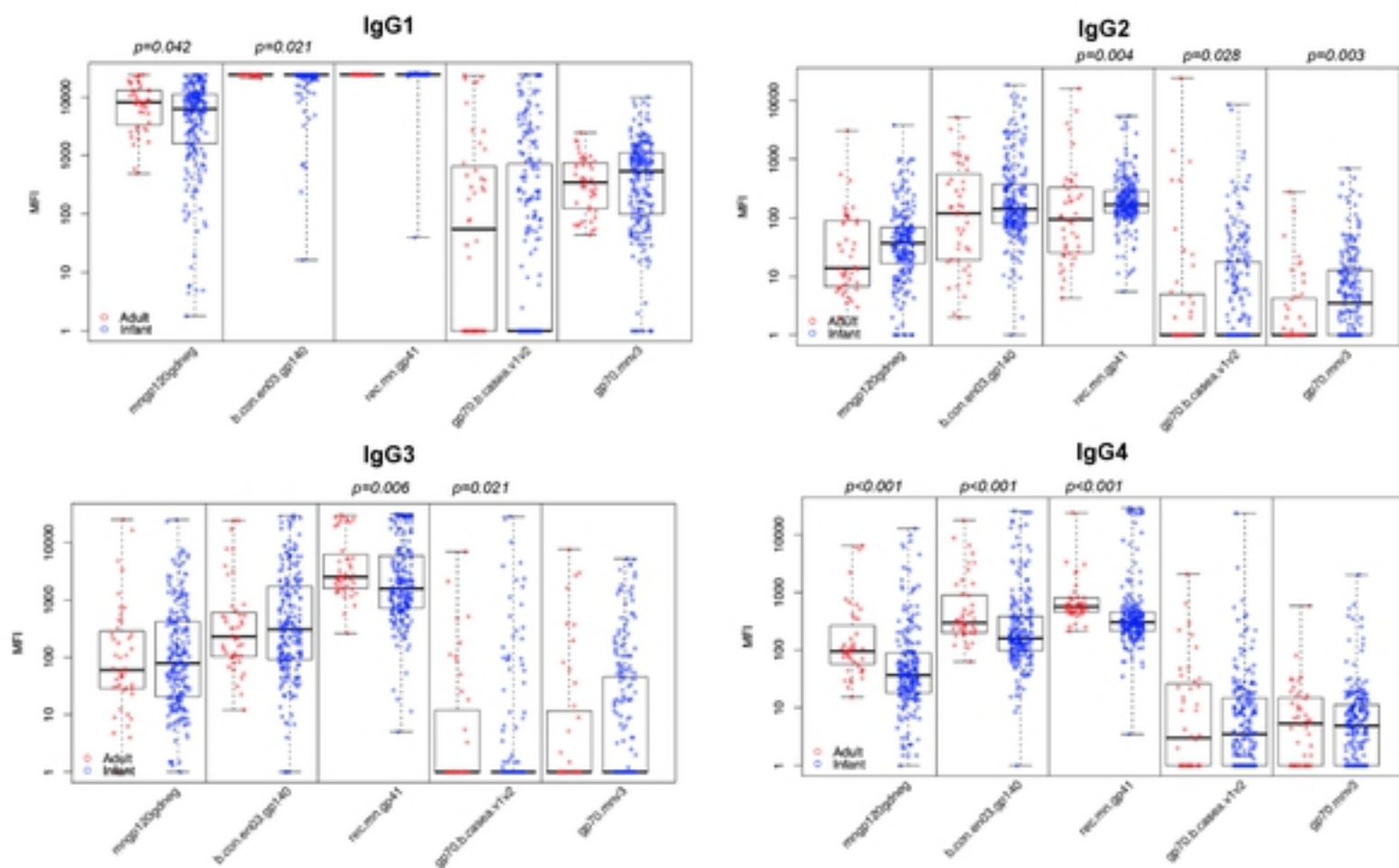


Figure 2. HIV-1 Env-Specific IgG Subclass. Individual IgG subclasses for select HIV-1 Env epitopes were measured by binding antibody multiplex assay in adult and pediatric cohorts. Significant difference between adult and pediatric cohorts by Wilcoxon as noted.

Virus	Clade	Adults (%)	Children (%)	
25710	C	91	90	
TRO11	B	68	78	p=0.048
X2278	B	75	86	p=0.015
BJOX2000	CRF07_BC	60	55	
X1632	G	63	28	p<0.001
CE1176	C	62	51	
246F3	AC	48	71	p<0.001
CH119	CRF07_BC	67	88	p<0.001
CE0217	C	46	40	
CNE55	CRF01_AE	34	41	

Table 2. Frequency of neutralization response in adults vs. children

Proportion of adult and pediatric samples with ID₅₀>100 for each virus. The pediatric cohort demonstrated comparable or superior neutralization frequency to adults in 9/10 viruses tested. The adult cohort had higher neutralization frequency against only one virus, X1632. Statistically significant p-values included as determined by Fisher test.

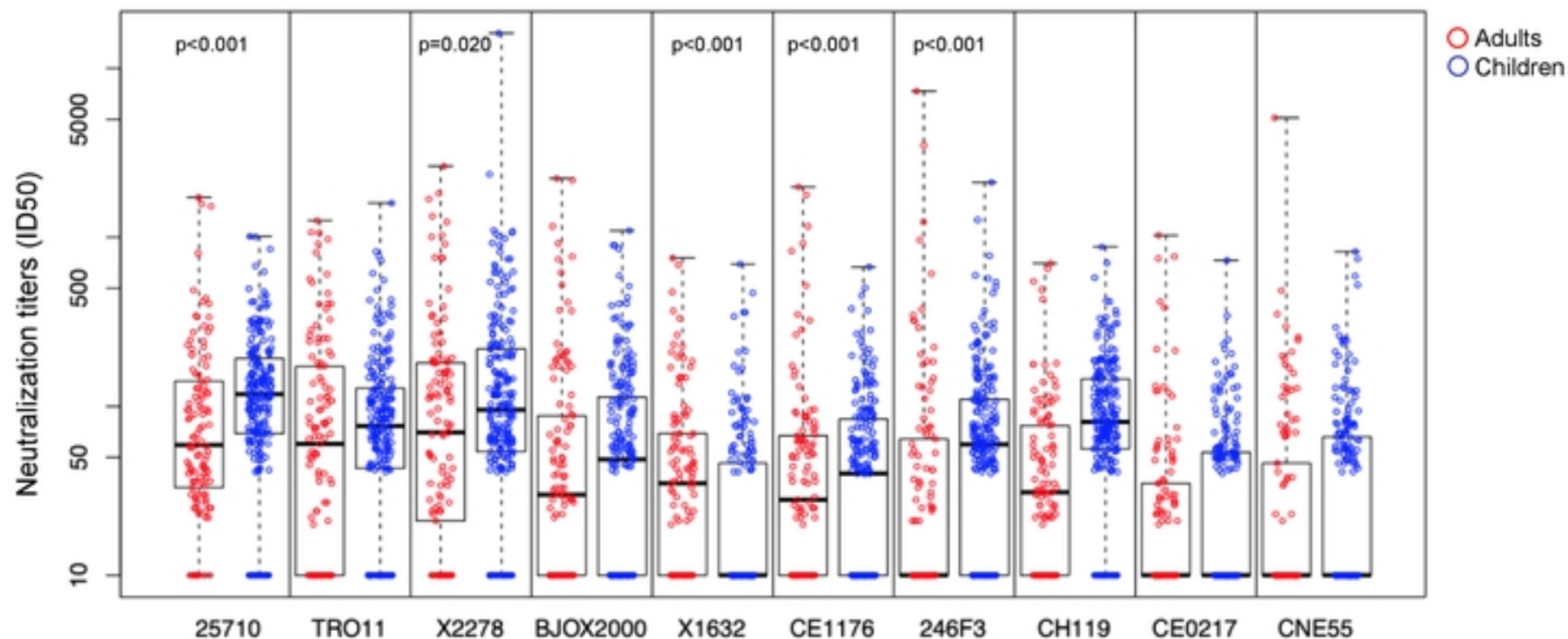


Figure 3. Neutralization potency in adults vs. children

The neutralization titers of the pediatric samples were calculated for each virus and compared to data from the historical adult cohort. Children demonstrated higher neutralization potency against 4/10 viruses and adults demonstrated higher neutralization potency against 1/10 viruses.

Statistical significance determined by Wilcoxon test as noted.

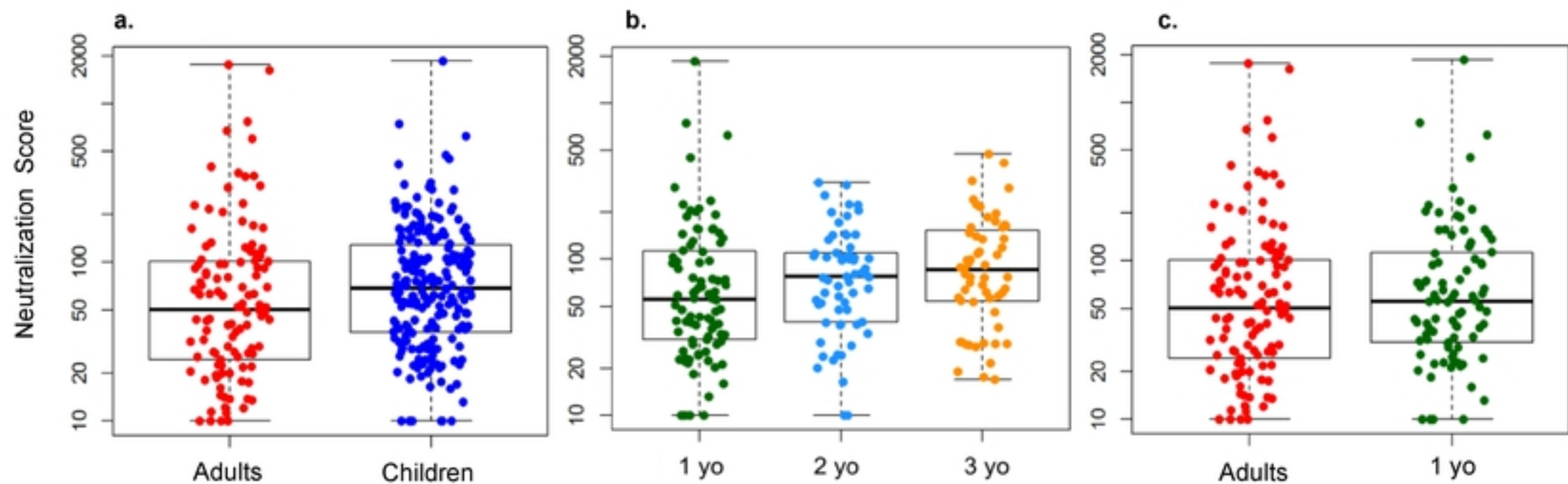


Figure 4. Neutralization score in adults vs. children

Neutralization score was determined as the AUC of the neutralization curve for all tested viruses (a) Overall, the neutralization score was greater in children than in chronically infected adults ($p=0.014$, Wilcoxon). (b) Neutralization score increased with age ($p=0.014$, median regression analysis) (c) The neutralization score of the 1 year-old cohort was comparable to that of adults ($p=0.44$, Wilcoxon).

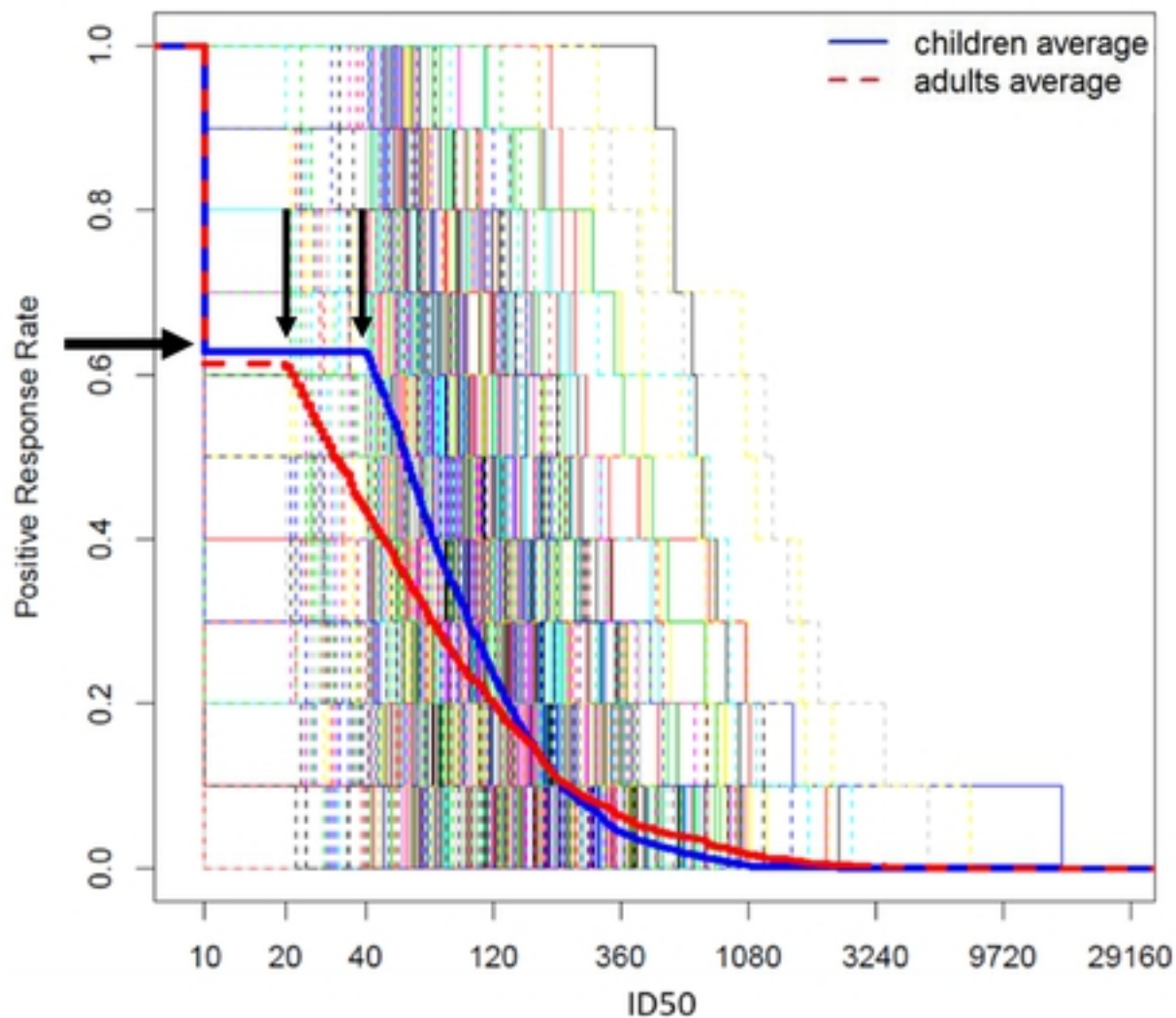


Figure 5. Magnitude-breadth curve comparing adults vs. children

The average ID50 value for the pediatric cohort was significantly greater than that of the adult cohort ($p = 0.013$, Mann Whitney U) as indicated by the vertical arrows whereas a similar proportion of viruses were neutralized in the two cohorts as indicated by the horizontal arrow.

			Epitope targeted by mutant										putative epitope specificity
PTD	Age	WT ID50	CD4bs	CD4bs	CD4bs	CD4bs	2G12 Sensitive	V3 Glycan	V2 Glycan	gp120-gp41 (35022)	gp120-gp41 (35022)	gp120-gp41 (PGT151)	
			N279A	N280D	G458Y	N276Q	N295V	N332A	N160K	N88A	N625A	N611A	
1	1	331	1550	2531	2428	<50	NA	476	474	628	745	NA	CD4 bs
2	1	1268	2111	3516	2108	754	1100	1083	370	1444	928	923	V2 glycan
3	2	415	NA	NA	NA	NA	171	80	362	322	338	328	V3 glycan
4	3	471	1486	5411	938	196	167	120	605	397	260	427	V3 glycan
5	3	890	1571	4097	1223	503	1954	115	1098	1420	677	972	V3 glycan

c.

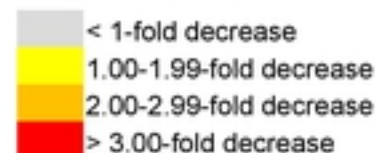
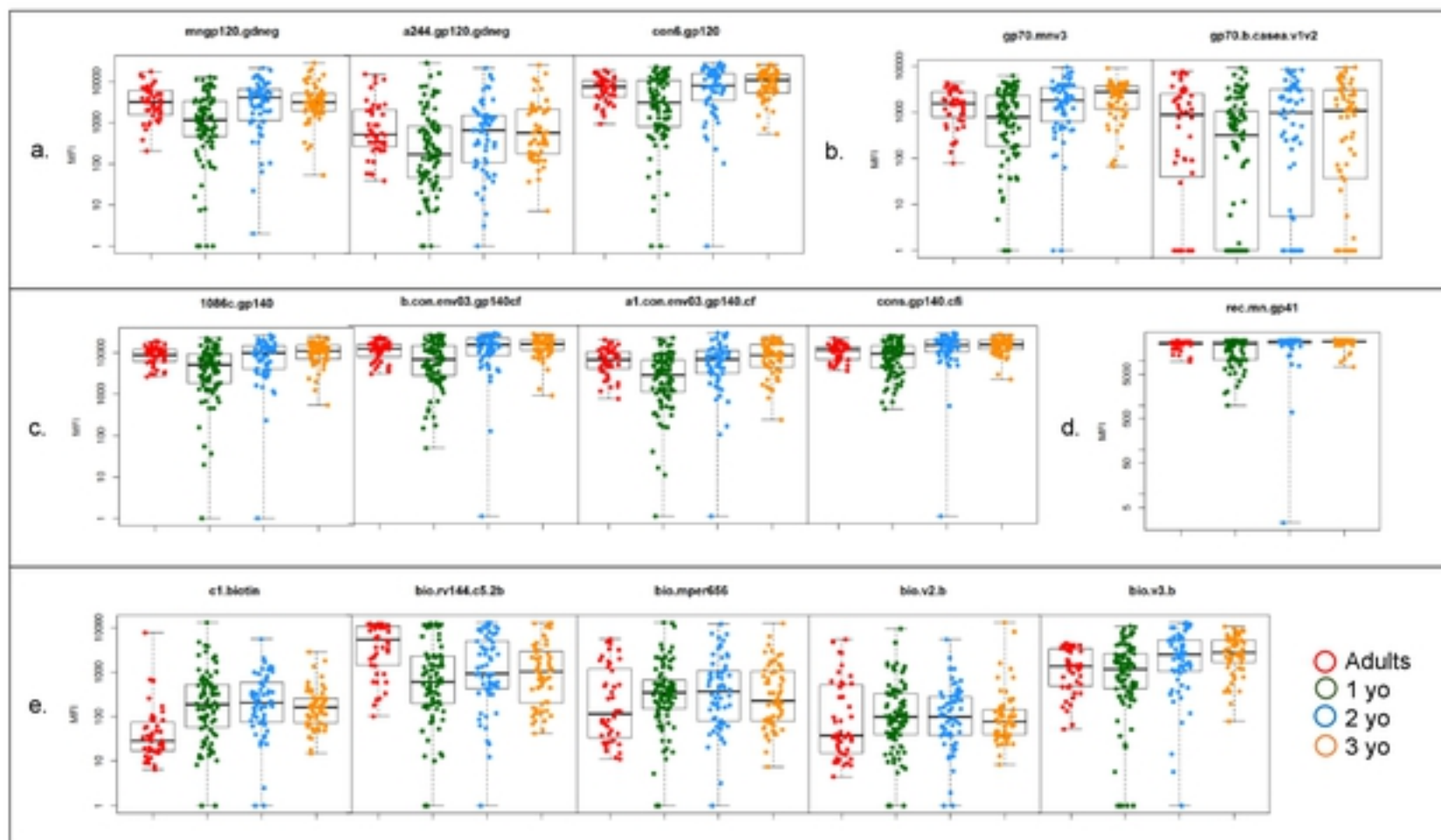


Table 3. Epitope specificity of broadly neutralizing pediatric samples Neutralization of mutant variants in the 5 pediatric samples that neutralized ≥ 5 of the 10-virus global panel with an ID₅₀ ≥ 100 and demonstrated at least a 3-fold reduction in ID₅₀ against one mutant pseudovirus.

Supplemental Figures

Antigen	Adults (%)	Children (%)	
Con6 gp120/B	100	96	
MNgp120	100	92	
A244 gp120	86	70	p=0.032
B.con_env03 gp140 CFI	100	100	
ConS gp140 CFI	100	100	
A1.Con gp140	100	96	
1086C gp140	100	98	
RecMN gp41	100	100	
gp70_B.CaseA_V1V2	73	67	
gp70 MNV3	98	90	
Bio-V2.B	39	53	
Bio-V3.B	95	93	
MPER656	61	79	p=0.017
RV144 C5.2B	77	42	p<0.001
C1 Biotin	11	58	p<0.001
YU2 Core	98	92	
YU2 Core D368R	23	36	

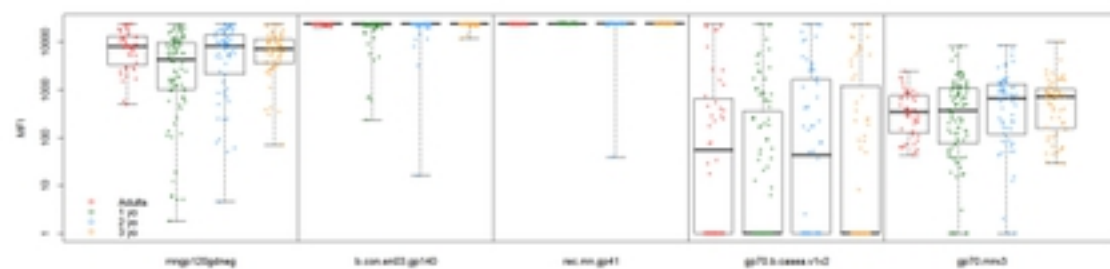
Supplemental Table 1. Frequency of Env-specific IgG binding response in adults vs. children
Proportion of adult and pediatric samples with binding magnitude of MFI >100 for each antigen, as assessed by BAMA.
Statistically significant p-values included as determined by Barnard's test.



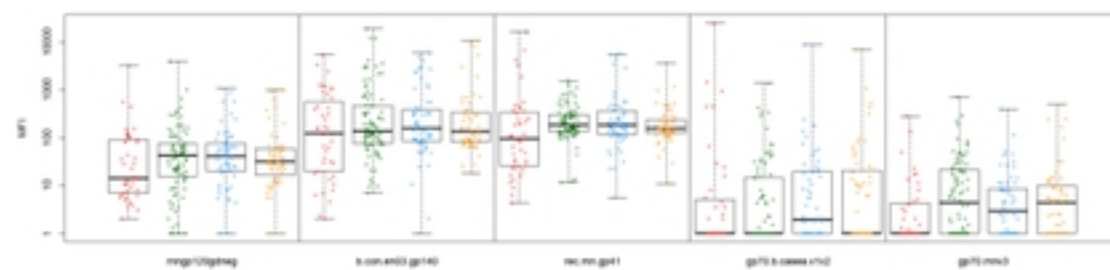
Supplemental Figure 1. HIV-1 Env-Specific Total IgG Extended

Total IgG for select HIV-1 Env epitopes were measured by binding antibody multiplex assay in adult and pediatric cohorts, including age 1, 2, and 3-year old sub-cohorts shown here. Epitopes include gp120 (a), variable loops (b), gp140 (c), gp41 (d) and peptides (e).

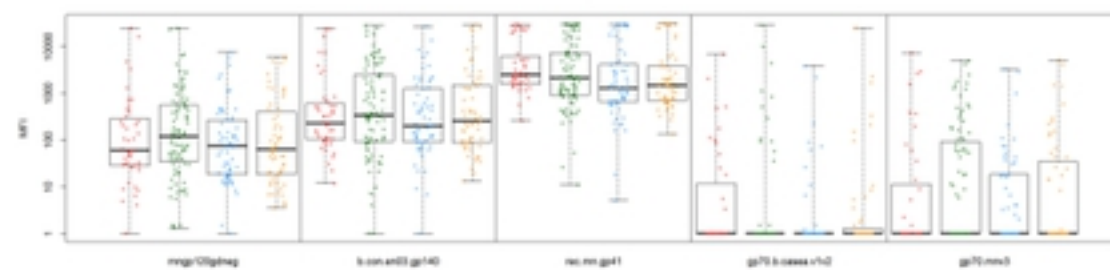
IgG1



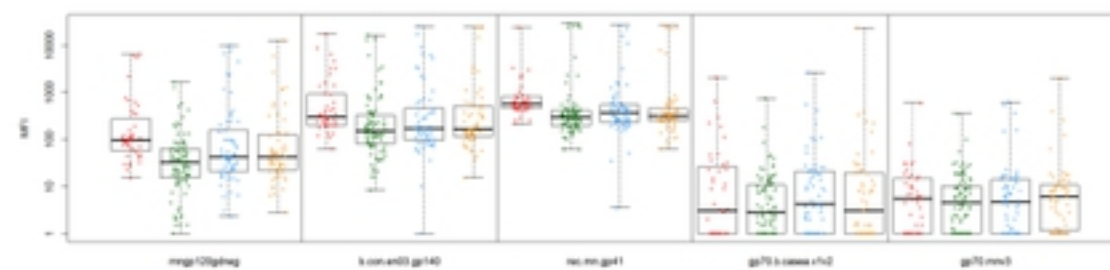
IgG2



IgG3



IgG4

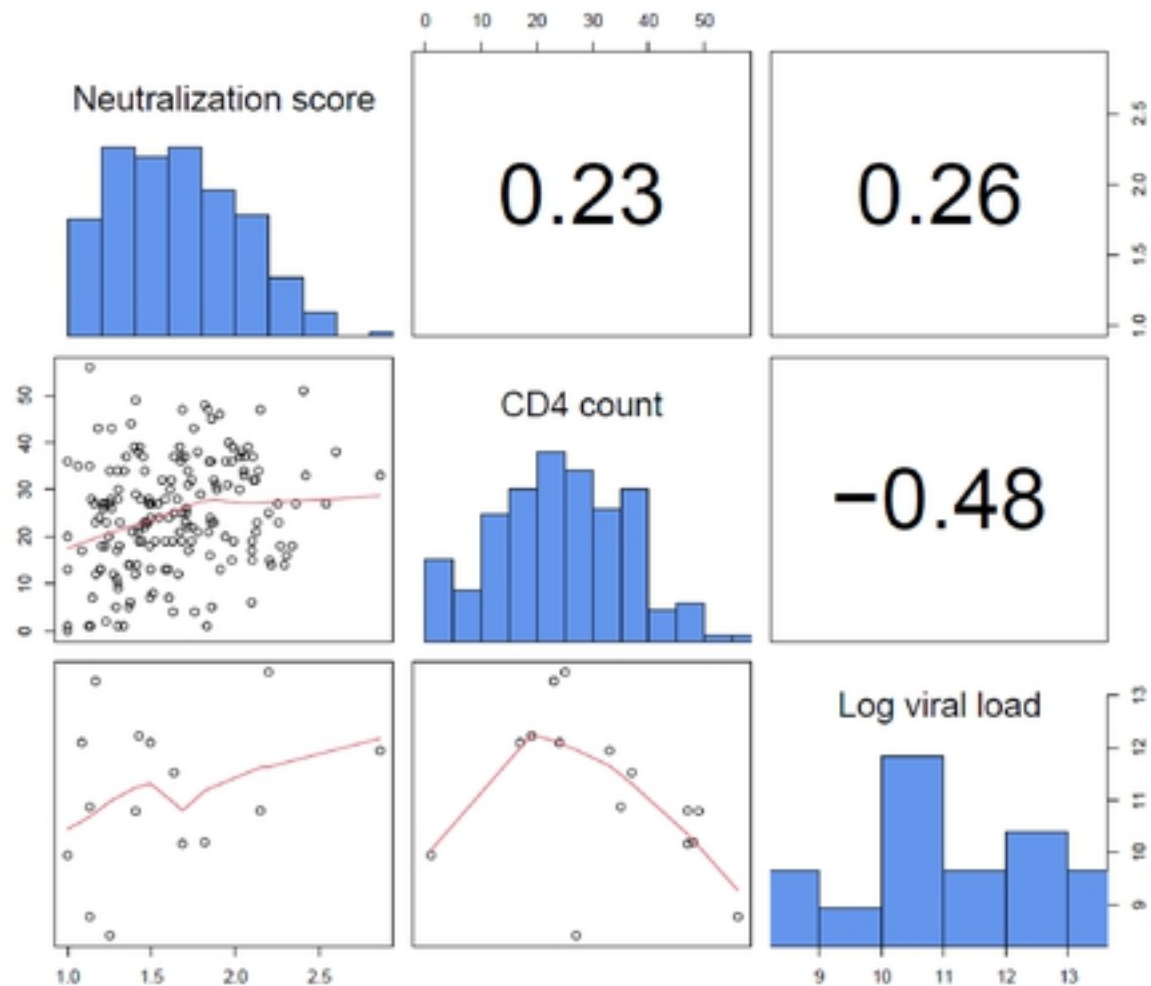


Supplemental Figure 2. HIV-1 Env-Specific IgG Subclass Extended

Individual IgG subclasses for select HIV-1 Env epitopes were measured by binding antibody multiplex assay in adult and pediatric cohorts, including age 1, 2, and 3-year old sub-cohorts shown here.

IgG Subclass	Antigen	Adults (%)	Children (%)	
IgG1	MNgp120	100	94	
	B.con_env03 gp140 CFI	100	100	
	RecMN gp41	100	100	
	gp70_B.CaseA_V1V2	45	38	
	gp70 MNV3	80	75	
IgG2	MNgp120	20	21	
	B.con_env03 gp140 CFI	55	64	
	RecMN gp41	48	87	p<0.001
	gp70_B.CaseA_V1V2	11	8	
	gp70 MNV3	5	5	
IgG3	MNgp120	43	46	
	B.con_env03 gp140 CFI	77	71	
	RecMN gp41	100	98	
	gp70_B.CaseA_V1V2	16	8	
	gp70 MNV3	16	19	
IgG4	MNgp120	48	22	p=0.002
	B.con_env03 gp140 CFI	98	73	p=0.002
	RecMN gp41	100	96	
	gp70_B.CaseA_V1V2	14	7	
	gp70 MNV3	2	5	

Supplemental Table 2. Frequency of Env-specific IgG subclass binding response in adults vs. children
Proportion of adult and pediatric samples with binding magnitude of MFI >100 for each antigen, as assessed by BAMA. Statistically significant p-values included as determined by Barnard's test.



CD4 %~ Neutralization score	CD4 count ~ Neutralization score	VL ~ Neutralization score
0.01 (CI=0.000, 0.001, p=0.002)	0.07 (CI=0.02, 0.11, p=0.003)	0.10 (CI=-0.10, 0.31, p=0.28)

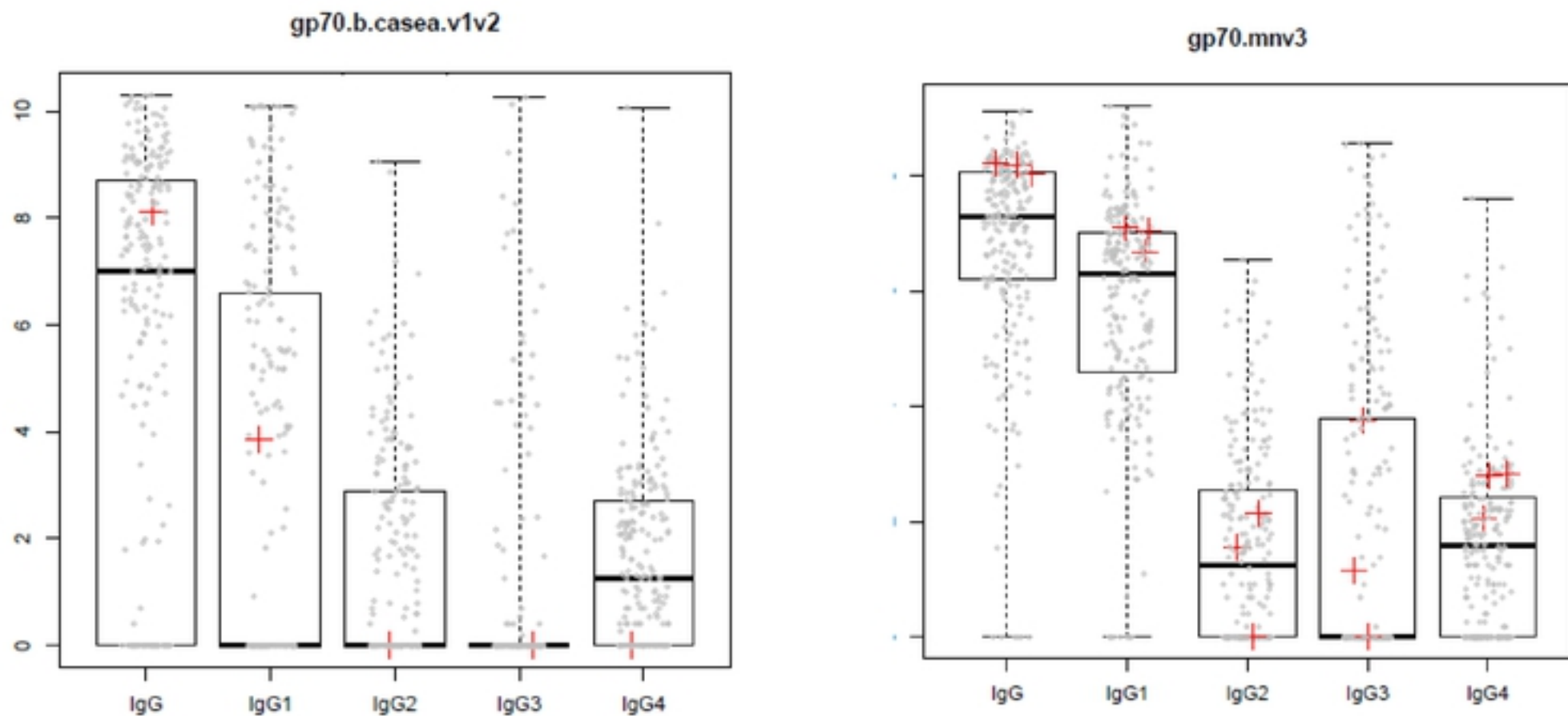
Supplemental Figure 4. Association between neutralization breadth score and clinical factors for pediatric cohort. Estimated slopes and p-values from linear regression are shown below.

Supplemental Table 3: association between binding and neutralizing antibody responses

	Spearman correlation with neutralization score	P value	Holm adjusted p value
IgG MN gp120	0.205	0.003	0.039
IgG1 MN gp120	0.181	0.008	0.101
IgG2 MN gp120	0.141	0.041	0.407
IgG3 MN gp120	-0.048	0.490	1.000
IgG4 MN gp120	0.187	0.006	0.082
IgG B con gp140	0.261	0.000	0.002
IgG1 B con gp140	0.147	0.032	0.354
IgG2 B con gp140	0.085	0.215	1.000
IgG3 B con gp140	0.010	0.889	1.000
IgG4 B con gp140	0.208	0.002	0.034
IgG gp41	0.136	0.049	0.437
IgG1 gp41	0.100	0.148	1.000
IgG2 gp41	0.068	0.322	1.000
IgG3 gp41	0.024	0.728	1.000
IgG4 gp41	0.249	0.000	0.004
IgG C1	0.055	0.436	1.000
IgG C5	0.054	0.433	1.000

Parent Virus	TRO11	25710-2.43	BJOX002000.03.2	Epitope Specificity
Mutants	TRO.11.N279A	25710-2.43.N279A		CD4bs
	TRO.11.N280D	25710-2.43.N280D		
	TRO.11.G458Y	25710-2.43.G458Y		
	TRO.11.N276Q	25710-2.43.N276Q		
	TRO.11.S365P			
	TRO.11.N295V		BJOX002000.03.2.L295N	2G12
	TRO.11.N332A	25710-2.43.N332A	BJOX002000.03.2.N332A	V3 Glycan
	TRO.11.N160K	25710-2.43.N160K	BJOX002000.03.2.N160K	V2 Glycan
	TRO.11.N88A	25710-2.43.N88A	BJOX002000.03.2.N88A	gp120-gp41 Interface
	TRO.11.N625A	25710-2.43.N625A	BJOX002000.03.2.N625A	
	TRO.11N611A	25710-2.43.N611A	BJOX002000.03.2.N611A	

Supplemental Table 4. Mutant pseudovirus panels for epitope mapping



Supplemental Figure 5: Association between binding and neutralization epitope specificity. Distribution of the V1V2-specific (A) and of V3-specific (B) binding responses in the pediatric cohort. The PTDS in whom neutralization specificity mapped to V2 glycan (PTD 1, panel A) or to V3 glycan (PTD 2, 4 and 5, panel B) is indicated with the red cross. Overall, PTD ranks above the 50% percentile for V1V2 specific IgG and IgG1 but not for the other IgG subclasses. PTD 2, 4, and 5 ranked above the 50% percentile for V3-specific IgG, IgG1 and IgG4.