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1 **Maternal alum-adjuvanted recombinant HIV Env vaccine does not enhance**
2 **autologous virus neutralization in HIV-infected pregnant women**
3

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24 **Abstract**

25 Preventive strategies beyond ART will be required to end the pediatric HIV
26 epidemic. A maternal vaccine capable of boosting neutralizing antibody (nAb)
27 responses against circulating viruses in HIV-infected pregnant women could effectively
28 decrease mother-to-child transmission of HIV. However, it is not known if an HIV
29 envelope (Env) vaccine administered to infected pregnant women can enhance
30 autologous virus neutralization.

31 Here, we assessed autologous virus nAb responses in maternal plasma samples
32 obtained from AIDS Vaccine Evaluation Group (AVEG) Protocols 104 and 102,
33 historical Phase I safety and immunogenicity trials of recombinant HIV Env subunit
34 vaccines in HIV-infected pregnant women (NCT00001041). AVEG 104 participants
35 were randomized to receive 300 μg Env subunit MN recombinant gp120 with alum
36 adjuvant or alum alone. AVEG 102 participants were randomized to receive 640 μg Env
37 subunit recombinant gp160 or placebo. HIV Env-specific maternal plasma binding and
38 neutralizing responses were characterized before and after vaccination in 15 AVEG 104
39 (n=10 vaccinee, n=5 placebo) and 2 AVEG 102 (n=1 vaccinee, n=1 placebo)
40 participants. Single genome amplification (SGA) was used to obtain HIV *env* gene
41 sequences from autologous viruses for pseudovirus production in pre- and post-
42 vaccination plasma of HIV-infected pregnant vaccinees (n=6 gp120, n=1 gp160) and
43 placebo recipients (n=3).

44 We detected an increase in MN gp120-specific IgG binding in the vaccinee group
45 between the first immunization visit and the last visit at delivery (p=0.027, 2-sided
46 Wilcoxon test). However, no difference was observed in the neutralization potency of

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47 maternal plasma collected at delivery against autologous viruses isolated from early or
48 late pregnancy. Thus, maternal vaccination with gp120/160 did not boost maternal
49 autologous virus nAb responses. Immunization strategies capable of more potent B cell
50 stimulation will likely be required to effectively boost autologous virus nAb responses in
51 pregnant women and synergize with ART to further reduce infant HIV infections.
52

53 **Key Words**

54 Human immunodeficiency virus; mother-to-child transmission; maternal vaccination; HIV
55 envelope; autologous virus neutralization
56

57 **Abbreviations**

58 Neutralizing antibody (nAb), envelope protein (Env), AIDS Vaccine Evaluation Group
59 (AVEG), single genome amplification (SGA), envelope gene (*env*), mother-to-child
60 transmission (MTCT), antiretroviral therapy (ART), variable loops 1 and 2 (V1V2),
61 variable loop 3 (V3), membrane-proximal external region (MPER), Women and Infants
62 Transmission Study (WITS), monoclonal antibodies (mAbs), CD4 binding site (CD4bs),
63 recombinant HIV Env gp120 (rgp120), recombinant HIV (rgp160), binding antibody
64 multiplex assay (BAMA), HIV immunoglobulin (HIVIG), mean fluorescent intensity (MFI),
65 zidovudine (ZDV), Toll-like receptor (TLR), antibody dependent cell cytotoxicity (ADCC)
66

67 **Highlights**

68 - Prior maternal HIV Env vaccine trial did not assess autologous virus
69 neutralization

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70 - Circulating viruses isolated from mothers were tested against autologous plasma
71 - Maternal vaccination with HIV Env gp120/160 increased MN gp120-specific IgG
72 binding
73 - Maternal HIV Env vaccine regimen did not boost autologous virus neutralization
74 - More potent B cell stimulation will be required to elicit autologous nAb responses
75

76 **Introduction**

77

78 Despite widespread efforts to eliminate pediatric HIV infections, mother-to-child
79 transmission (MTCT) of HIV continues to pose a significant global health challenge.
80 With the wide availability of antiretroviral therapy (ART) for HIV-infected women during
81 pregnancy and breastfeeding, as well as for infant prophylaxis, the rate of new HIV
82 infections among infants have decreased by 41% from 2010 to 2018 [1]. Although
83 approximately 82% of HIV-infected pregnant women across the globe had access to
84 ART in 2018, there were still 160,000 newly acquired pediatric HIV infections in the
85 same year [1]. Some of the factors contributing to these new infections are the
86 emergence of drug-resistant HIV strains, late maternal diagnosis or presentation for
87 prenatal care, acute infection during pregnancy or breastfeeding, and poor
88 implementation of ART in resource-limited areas.
89 In the absence of ART prophylaxis during pregnancy, the MTCT transmission
90 rate is 30-40% and can occur antepartum (*in utero*), intrapartum (during labor and
91 delivery), or postpartum (during breastfeeding) [2]. Even with optimal implementation of
92 antenatal triple-drug ART, breakthrough transmission can occur, with rates as high as

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93 5% [3, 4]. In addition, recent studies have demonstrated that while ART can effectively
94 reduce the rate of MTCT, this reduction comes at the expense of a notable increase in
95 preterm birth and neonatal death, particularly for protease inhibitor-based regimens [4-
96 6]. Moreover, recent reports of increased prevalence of neural tube defects in newborns
97 associated with maternal exposure to dolutegravir-based ART at conception have
98 raised concerns regarding toxicity of ART and highlight an urgent need for additional
99 preventative approaches [7-10]. Thus, due to issues of ART access, adherence,
100 incomplete efficacy, and toxicity, further strategies will be required to eliminate MTCT.
101 Prior studies have implicated HIV Env-specific antibody responses as being
102 potentially protective against HIV-1 transmission. In fact, the partially effective RV144
103 vaccine trial of a recombinant gp120 vaccine indicated that vaccine-elicited IgG against
104 variable loops 1 and 2 (V1V2) of gp120 was associated with decreased risk of HIV-1
105 heterosexual transmission [11-14]. While this particular epitope has not been implicated
106 in protection against MTCT, maternal antibodies against both the variable loop 3 (V3) of
107 Env gp120 and the gp41 membrane-proximal external region (MPER) have been shown
108 to correlate with reduced risk of MTCT [15, 16]. In addition, studies have demonstrated
109 that heterologous virus HIV-neutralizing antibodies are found more frequently or in
110 higher titers in non-transmitting compared to transmitting mothers [17, 18]. However,
111 there is conflicting data regarding the role of maternal antibodies in preventing vertical
112 transmission, as other studies have failed to confirm this association between maternal
113 Env-specific neutralizing antibodies and decreased transmission risk [2, 19]. Moreover,
114 some studies have observed the opposite trend, reporting that transmitting women had
115 higher concentrations of maternal IgG against the Env V3 region compared to non-

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116 transmitting women [20]. Clearly, further investigation is needed to elucidate the
117 relationship between maternal antibody responses and risk of transmission to the infant.
118 We previously investigated immune correlates of vertical HIV-1 transmission in
119 pregnant, in a large cohort of HIV clade B-infected US women from the Women and
120 Infants Transmission Study (WITS) [21]. The results demonstrated that maternal IgG
121 against V3, plasma neutralization of clade-matched tier 1 but not tier 2 HIV-1 variants,
122 and the potency of the maternal plasma to block CD4 from binding to clade B HIV-1
123 Envs predicted reduced risk of MTCT. Interestingly, these responses were co-linear in
124 their prediction of MTCT risk, suggesting that they may be surrogate measures for the
125 same underlying mechanism of virus neutralization that influences infant transmission.
126 In fact, isolated V3-specific monoclonal antibodies (mAbs) that could neutralize tier 1,
127 but not tier 2 heterologous viruses, were able to neutralize most autologous viruses
128 isolated from maternal plasma [21]. Moreover, it has also been demonstrated that
129 autologous V3 and CD4 binding site (CD4bs) mAbs isolated from chronically HIV-1-
130 infected individuals can neutralize autologous, but not heterologous, tier 2 viruses [22].
131 This indicates that non-broadly neutralizing antibodies can potently neutralize
132 autologous circulating viruses, which is especially pertinent in the unique setting of
133 MTCT, as maternal circulating viruses are the source of the vertically transmitted virus.
134 In recent study, we also characterized vertically transmitted and non-transmitted
135 maternal HIV Env variants in 16 mother-infant transmitting pairs from the WITS cohort,
136 and found that, compared to maternal non-transmitted variants, the infant transmitted
137 virus variants were significantly more neutralization-resistant to paired maternal plasma
138 [23]. This finding suggests that autologous neutralizing antibody sensitivity may define

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139 infant transmitted/founder variants, and, therefore, boosting autologous neutralizing
140 antibody responses in HIV infected pregnant women could be a viable immune strategy
141 to decrease vertical transmission.

142 Yet, it is unknown whether vaccination of HIV-infected pregnant women with an
143 Env vaccine would even temporarily enhance autologous virus neutralizing antibody
144 responses. In two historic vaccine trials completed in 1993-1995 by the AIDS Vaccine
145 Evaluation Group (AVEG) Protocols 104 and 102, safety and immunogenicity of
146 recombinant HIV Env gp120 and gp160 (rgp120, rgp160) respectively as antigens, were
147 tested in HIV-infected pregnant women [24]. While the Env vaccine was safe and well
148 tolerated, there was limited enhancement of maternal immune responses against
149 heterologous viruses in vaccinees compared to placebo recipients [24]. Importantly,
150 because of the immunological phenomenon of original antigenic sin, it is possible that
151 immunization with an heterologous Env vaccine may recruit memory immune cells in
152 HIV-infected pregnant women, leading to an enhancement of their autologous virus
153 neutralizing immune responses. In the setting of MTCT, evocation of original antigenic
154 sin for enhancement of autologous virus neutralization could be an effective strategy to
155 impede perinatal virus transmission. Nevertheless, whether maternal rgp120/160
156 vaccination enhanced neutralizing antibody responses against the maternal autologous
157 circulating viruses remained unknown.

158 In this study, we sought to assess whether immunization of HIV-infected
159 pregnant women with an alum-adjuvanted recombinant Env vaccine elicited maternal
160 antibody responses that improved autologous virus neutralization responses.
161 Additionally, we characterized representative maternal virus population diversity from

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162 pre- and post-immunization time points in 7 vaccinees and 3 placebo recipients and
163 assessed the antibody binding kinetics and ability of maternal plasma to neutralize
164 these autologous viruses. This work offers novel insights into the feasibility of enhancing
165 maternal autologous virus neutralization and antibody responses through maternal HIV
166 Env vaccination as an adjunctive strategy to protect the infant against HIV-1 acquisition.
167
168 **Materials & Methods**
169
170 *Study Subjects*
171 Maternal plasma samples were obtained from the AIDS Vaccine Evaluation
172 Group (AVEG) Protocols 104 and 102, a Phase I study of safety and immunogenicity of
173 MN rgp120 and rgp160 HIV-1 vaccines in HIV-infected pregnant women
174 (ClinicalTrials.gov; NCT00001041). In the AVEG 104 Protocol, 26 HIV-infected pregnant
175 women with CD4+ T cell counts >400/mm^3 were enrolled in the second trimester of
176 healthy pregnancy and randomized to receive either 300 µg of MN rgp120 (Genentech)
177 with alum (n=17) as an adjuvant or alum with diluent (n=9) between 16 and 24 weeks of
178 gestation [24]. Booster immunizations were administered monthly, until delivery, for a
179 minimum of 3 vaccine doses and a maximum of 5 vaccine doses (Figure 1). Similarly, 2
180 HIV-infected pregnant women were enrolled with the same criteria in the AVEG 102
181 Protocol, though instead these women received either 640 µg of rgp160 (VaxSyn,
182 MicroGeneSys) (n=1) or a placebo (n=1). Maternal plasma samples from multiple visit
183 time points were available for 15 AVEG 104 participants (n=10 MN rgp120 vaccine, n=5

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184 alum placebo) and 2 AVEG 102 participants (n=1 rgp160 vaccine, n=1 placebo) (Table
185 1).
186
187 *Ethics statement*
188 Original study protocols AVEG 102 and 104 were approved by local institutional
189 review boards at the seven sites involved in the original study [24]. Informed consent
190 was obtained from all women, and also from their partners when available. In the
191 present study, the use of de-identified maternal samples from the AVEG 102 and 104
192 protocol cohorts was deemed exempt by the Duke University Institutional Review
193 Board. Moreover, in this study, individual patient identification (PTID) numbers are
194 instead represented by PubID numbers.
195
196 *Viral RNA extraction and cDNA synthesis*
197 Viral RNA was extracted from the plasma sample from each mother with a
198 QIAamp Viral RNA Mini Kit (Qiagen) and subjected to reverse transcription and cDNA
199 synthesis using 1X reaction buffer, 0.5 mM of each deoxynucleoside triphosphate
200 (dNTP), 5 mM DTT, 2 U/mL RNaseOUT, 10 U/mL of SuperScript III reverse
201 transcription mix (Invitrogen), and 0.25 mM antisense primer 1.R3.B3R (5'-
202 ACTACTTGAAGCACTCAAGGCA AGCTTTATTG-3'), located in the HIV-1 *nef* open
203 reading frame.
204
205 *Single genome amplification*

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206 Full-length envelope (*env)* genes were then amplified by nested PCR from
207 diluted viral cDNA, as previously described [23, 25]. Briefly, cDNA was endpoint-diluted
208 in 96-well plates (Applied Biosystems) with the goal of <30% positive amplification, in
209 order to maximize the likelihood of obtaining a single genome. First round PCR was
210 carried out with 1X buffer, 2 mM MgSO4, 0.2 mM of each dNTP, 0.2μM of each primer,
211 and 0.025 U/μl Platinum Taq High Fidelity polymerase (Invitrogen) in a 20μl reaction.
212 For the first round of PCR amplification, primer pairs were Env 5'ex (5'-
213 TAGAGCCCTGGAAGCATCCAGGAAG-3') and Env 3'ex (5'-
214 TTGCTACTTGTGATTGCTCCATGT-3'), Env 5'ex and 2.R3.B6R (5'-
215 TGAAGCACTCAAGG CAAGCTTTATTGAGGC-3'), or 07For7 (5'-
216 AAATTAYAAAAATTCAAAATTTTCGGGTT TATTACAG-3') and 2.R3.B6R. The
217 following PCR conditions were used for Round 1 amplification: 1 cycle of 94°C for 2
218 minutes, 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 3
219 minutes and 30 seconds for the Env 5'ex/3'ex primers (4min and 30s for Env
220 5'ex/2.R3.B6R, 5min and 30s for 07For7/2.R3.B6R), followed by a final cycle of 68°C for
221 10 minutes. Then, a second round of PCR amplification was carried out with 2μl of the
222 first round product as template, 0.2μM of each primer, and the same PCR mixture as in
223 round one, in a 50μl reaction. Primer pairs for second round PCR were Env 5'in (5'-
224 TTAGGCATCTCCTATGGCAGGAAGAAG-3') and Env 3'in (5'-
225 GTCTCGAGATACTGCTCCCACCC-3'), Env 5'in and 2.R3.B6R (5'-TGAA
226 GCACTCAAGG CAAGCTTTATTGAGGC-3'), or Low2c (5'-
227 TGAGGCTTAAGCAGTGGGT TCC-3') and VIF1 (5'-
228 GGGTTTATTACAGGGACAGCAGAG-3'). Round 2 conditions were one cycle of 94°C

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229 for 2 minutes, 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 3
230 minutes and 30 seconds for the Env 5'in/3'in primers (4min and 30s for Env
231 5'in/2.R3.B6R, 5min and 30s for Low2c/VIF1), followed by 1 cycle of 68°C for 10
232 minutes. Round 2 PCR amplicons were visualized using precast 1% agarose E-gels
233 (Invitrogen), purified with the AMPure XP magnetic bead purification system
234 (Agencourt), and sequenced for the HIV *env* gene by Sanger sequencing (Table 1).
235

236 *HIV env gene genetic analysis*

237 Sequences were assembled using the Sequencher program (Gene Codes) and
238 manually edited. Chromatograms were examined for sites of ambiguity, or double peaks
239 per base read, and sequences containing multiple base peaks at a single position were
240 marked as such and not studied further. Envelope sequences were aligned using the
241 Gene Cutter tool available in the HIV Sequence Database of the Los Alamos National
242 Laboratory (LANL) website
243 (http://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html) and manually
244 edited further in Seaview (Version 4) [26]. Phylogenetic trees were constructed using
245 Seaview and highlighter plots were created using the Highlighter tool on the LANL
246 website (https://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html).
247

248 *Pseudovirus production and infectivity analysis*

249 Using a previously described sequence selection algorithm [23], approximately 8-
250 10 maternal Env variants were selected from the pre-immunization time point (Visit 1)
251 and post-immunization time point (Visit 9) for Env pseudovirus production. Variants

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252 representing major clusters of the phylogenetic trees were selected to represent the full
253 range of *env* genetic diversity in maternal plasma. To produce functional pseudoviruses
254 from the HIV-1 *env* sequences, CMV promoter was added to the *env* genes by
255 overlapping PCR as previously described [27] and the products were co-transfected
256 with a backbone plasmid lacking the *env* gene (SG3Δenv) in 293T cells (American
257 Tissue Culture Collection, Manassas, Virginia). 293T cells (approx. 4.5×10^6) were
258 seeded in a T-75 flask (Corning, Corning, NY) containing growth media (GM)
259 (Dulbecco's modified Eagle's medium (DMEM)-10% fetal bovine serum (FBS)-1%
260 penicillin-streptomycin containing HEPES, ThermoFisher, Waltham, MA) and incubated
261 overnight at 37°C and 8% CO_2. 4μg of Env DNA containing CMV promoter was
262 combined with 4μg of SG3Δenv backbone and FuGene 6 transfection reagent (Roche
263 Diagnostics) was added as per manufacturer's instructions. The mixture was then
264 added to the T-75 flask and it was incubated at 37°C for 48 hours. Supernatant
265 containing pseudovirus was harvested and stored at -80°C with a final concentration of
266 20% FBS. To measure the infectivity of the pseudoviruses, 20μl of pseudovirus was
267 added in duplicate to a 96-well flat bottom plate and then 100μl TZM-bl cells (catalog
268 no. 8129; NIH AIDS Reagent Program; from John Kappes and Xiaoyun Wu) were
269 added (10,000 cells/100μl GM with 10μg/ml of DEAE-dextran). After a 48-hour
270 incubation at 37°C and 8% CO_2, 100μl of culture medium was removed and 100μl of
271 Bright-Glo luciferase reagent (Promega) was added. The mixture was incubated for 2
272 minutes at 25°C, 100μl was subsequently transferred to a 96-well black plate, and
273 luminescence was measured immediately on a Victor X3 multilabel plate reader
274 (PerkinElmer).

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275

276 *TZM-bl neutralization assay*

277 Neutralization of autologous pseudoviruses by maternal plasma was measured
278 using a luciferase (Luc) reporter gene assay in TZM-bl cells (catalog no. 8129; NIH
279 AIDS Reagent Program; from John Kappes and Xiaoyun Wu), as previously described
280 [28]. Before performing the assay, plasma was heat inactivated by incubating for 30
281 minutes at 56°C. Plasma samples were added at a starting dilution of 1:20 and diluted
282 threefold serially. Then, the plasma samples were incubated with virus for 1 hour at
283 37°C. TZM-bl cells were added, and the mixture was incubated for 48 hours.
284 Luminesence was then measured using the Bright-Glo luciferase reagent and Victor X3
285 luminometer and luminescence values used to calculate the ID_{50}, or dilution at which
286 relative luminescence units (RLU) were reduced by 50% compared to virus control
287 wells. VRC01 was used as a positive control in each experiment and murine leukemia
288 virus (SVA.MLV) served as a negative control for the assay [29].

289

290 *Binding antibody multiplex assay (BAMA)*

291 HIV-1 Env-specific IgG responses in maternal plasma against a panel of HIV-1
292 antigens were detected using a customized BAMA, as previously described [30]. HIV-1
293 antigens were covalently coupled to carboxylated fluorescent beads (Bio-Rad
294 Laboratories) and IgG binding to the bead-coupled antigens was measured. The
295 antigen panel for IgG BAMA assays included biotinylated linear V3 loop peptide V3.B
296 (Bio V3 B) and the following 4 proteins: MNgp120, Gp70 B.MN V3, Gp70
297 B.CaseA_V1V2, and HIV-1 MN recombinant gp41 (REC MN gp41, ImmunoDiagnostics)

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298 (Table S1). The antigen-coupled beads were incubated with diluted plasma samples
299 (1:100 for MNgp120, Gp70 B.MN V3, Gp70 B.CaseA_V1V2; 1:2000 for V3.B and REC
300 MN gp41) for 30 minutes at room temperature (20-25°C). HIV Env-specific IgG was
301 then detected with phycoerythrin (PE)-conjugated mouse anti-human IgG (Southern
302 Biotech, Birmingham, AL) at 2 µg/ml. Beads were washed, resuspended, and acquired
303 on a Bio-Plex 200 instrument (Bio-Rad Laboratories). Blank beads were used to
304 account for non-specific binding and HIV immunoglobulin (HIVIG) was used as a
305 positive control for all assays. The magnitude of antibody binding to the panel of HIV-1
306 Env antigens was measured as mean fluorescent intensity (MFI). MFI values for
307 conformational antigens containing gp70 were background-adjusted by subtracting the
308 MFI values of gp70 MulV. All MFI values were background-adjusted by subtracting the
309 MFI values of coupled beads without sample. A positive HIV Env-specific antibody
310 response was considered to be an MFI > 100. The criteria for reporting sample MFI
311 values included coefficient of variation ≤20% with a bead count ≥100 for each sample.
312 All assays tracked the 50% effective concentration and maximum MFI of the positive
313 control HIVIG and protein standards CH58, B12, and 7B2 by Levey-Jennings charts to
314 ensure data consistency.
315
316 *Statistics*
317 We tested for differences in antibody binding and neutralization responses
318 between placebo and vaccinees using 2-sided Wilcoxon tests comparing the values at
319 the first visit to those at the last visit. Because time intervals between visits differed

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320 across study subjects, we also tested the change per day between visits. All statistical
321 analyses and graphs were produced using R [31].
322
323 **Results**
324
325 ***Env-specific antibody binding responses in vaccinees compared to placebo***
326 ***recipients***
327
328 The magnitude of HIV Env epitope-specific IgG responses prior to and following
329 Env vaccination in HIV-1 infected vaccinated women and placebo recipients was
330 assessed by BAMA. We measured maternal vaccine-elicited responses against clade B
331 MN gp120 protein (Env matched to the vaccine immunogen), gp70 V1V2 protein, and a
332 linear V3 peptide. When comparing the change in clade B MN gp120-specific binding
333 responses between the first and last visit among study participants, all placebo
334 recipients (n=6) did not show increase in binding, whereas 8 out of 11 vaccinees
335 showed increase in binding over time. Statistically, the overall increase in binding to MN
336 gp120 was significantly higher in vaccinees compared to placebo recipients (p=0.027 by
337 Wilcoxon test, Figure 2). To account for differences in the timing of visits for each
338 mother, we calculated the change in MN gp120-specific binding response per day
339 between the first (Visit 1, 4) and last visit (Visit 9). Per day increase in clade B MN
340 gp120-specific binding responses between the first and last visit was statistically
341 significantly higher in vaccinees as compared to placebo recipients (p=0.015 by
342 Wilcoxon test, Figure S1). We observed more than 3-fold increase in antibody binding

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343 responses against MN gp120 in 1/11, 2/11, and 3/11 vaccinees against antigens MN
344 gp120, linear V3.B, and gp70 V1V2, respectively. No change in change in antibody
345 binding responses was observed in placebo recipients except in one against gp70 V1V2
346 (Figure 3, Figure S2).
347

348 ***Autologous virus neutralizing antibody responses in vaccinees compared to***
349 ***placebo recipients***
350

351 To determine if gp120 or gp160 vaccination enhanced functional, virus-specific
352 neutralizing antibody responses, we assessed the ability of maternal plasma to
353 neutralize autologous virus variants isolated from plasma collected in early pregnancy
354 before vaccination and late pregnancy after vaccine boosting. There was no difference
355 between vaccinees and placebo recipients in the ability of maternal plasma collected at
356 delivery to neutralize autologous virus populations isolated from early and late
357 pregnancy visits (Figure 4A-B, Figure S3). Moreover, the difference in the ability of
358 maternal plasma collected at the first visit (Visit 1,4) versus last visit (Visit 9) to
359 neutralize early pregnancy plasma viruses (Visit 1, 4) was comparable between
360 vaccinees and placebo recipients (Figure 4C-D).
361 Additionally, we tested the ability of maternal plasma collected from the pre-
362 vaccination visit (Visit 1), the booster visits (Visits 3, 4, 5, & 6), and the post-vaccination
363 visits (Visits 7 & 9) to neutralize autologous virus populations from the pre-vaccination
364 or early pregnancy visit (Visit 1, 4) (Figure 5). Interestingly, there appears to be a boost
365 over time in neutralization potency against autologous virus populations isolated from

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366 the pre-vaccination visit in the gp160 vaccinee as compared to the corresponding
367 placebo recipient, yet this trend was only observed in the one vaccinee from which
368 samples were available for testing. Taken together, there was no significant change in
369 autologous virus neutralization potency over time for Env vaccinees as compared to
370 placebo recipients.
371
372 ***Plasma HIV env gene sequence diversity in vaccinees compared to placebo***
373 ***recipients***
374
375 Through single genome amplification, we obtained 282 total HIV *env* gene
376 sequences from vaccinees (n=7) and 118 total HIV *env* gene sequences from placebo
377 recipients (n=3) (Table 1). To characterize viral evolution between visits in study
378 participants, we measured viral diversity through the mean pairwise Hamming distance,
379 which was calculated as the number of mutations between all pairs of *env* sequences
380 isolated from one sample divided by the total number of bases in the alignment. The
381 changes in viral diversity between visits observed in the placebo recipients were not
382 significantly different from those observed in the vaccinees (Figure 6). Yet, in a per day
383 analysis of mean Hamming distance since the pre-vaccine visit, we observed a trend in
384 which vaccinees consistently demonstrated lower HIV *env* gene sequence diversity than
385 placebo recipients (Figure S4). However, this trend did not reach significance,
386 potentially due to limited statistical power given the small sample size.
387
388 **Discussion**

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389 Unlike other modes of HIV transmission, MTCT is a unique setting since it occurs
390 in the presence of pre-existing humoral immunity. Maternal IgG antibodies that are
391 passively acquired by the fetus through transplacental transfer during pregnancy and by
392 the infant through breastfeeding are known to provide critical protection against
393 perinatal pathogens for neonates during the first few months of life [32]. Despite the
394 potential source of transmitted virus in a mother-infant pair being confined to the
395 maternal autologous circulating virus pool, our limited understanding of how maternal
396 antibody responses that coevolved with circulating virus populations impact vertical HIV
397 transmission remains a major barrier in developing a maternal immunization strategy for
398 the prevention of MTCT. Yet, there are reasons to believe that enhancing the ability of
399 maternal plasma to neutralize co-circulating viruses may impede vertical virus
400 transmission. Thus, augmentation of maternal autologous virus neutralizing antibody
401 responses through vaccination during pregnancy is a potentially viable strategy to
402 further reduce the rate of MTCT in synergy with ART.

403 In this study, we investigated the induction of neutralizing antibodies against
404 autologous circulating viruses in the AVEG 102 and 104 maternal HIV Env vaccine trials
405 (1993-1995), which assessed the safety and immunogenicity of alum-adjuvanted gp120
406 and gp160 subunit vaccines in HIV-infected, pregnant women. Importantly, while the
407 Env vaccines were reported to be safe and immunogenic, the ability of the vaccines to
408 raise neutralizing responses against maternal circulating autologous viruses was not
409 assessed [24]. With the development of novel techniques to isolate and analyze single
410 HIV variants from plasma, as well as more sensitive, bead-based binding antibody
411 detection, we are now better equipped to answer this important question. Another

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412 limitation to the present study is incomplete information from the original study related to
413 which subset of participants may have received antiretroviral therapy, as
414 recommendations for clinical management of HIV-infected pregnant women were
415 updated during the study following the first recognition that zidovudine was effective in
416 decreasing the risk of MTCT of HIV [33]. Additionally, sample quality and restricted
417 availability of time points limited the successful isolation of sufficient number of single
418 genome amplicons from each participant for larger analyses. While the small sample
419 size of 11 vaccinees and 6 placebo is a caveat to this study, these cohorts of HIV-
420 infected pregnant women enrolled in a Phase I HIV Env subunit vaccine clinical trial
421 represented a unique opportunity to understand the ability of HIV Env vaccination to
422 enhance neutralizing antibody responses in the setting of MTCT.

423 Although enhanced autologous virus neutralization was not observed in the
424 AVEG 102 and 104 study cohorts, our work offers key insight in that it suggests more
425 potent B cell stimulation must be achieved for an HIV Env vaccination to be effective in
426 boosting autologous neutralizing antibody responses in pregnancy. There are valuable
427 lessons to be gained and key opportunities for improvement in trial design based on our
428 analysis of this first Phase I trial of HIV Env subunit recombinant gp120 and gp160
429 protein vaccines adjuvanted with alum in HIV-infected, pregnant women. While the first
430 formal recognition that zidovudine (ZDV) was effective in reducing the risk of MTCT
431 occurred during the AVEG 102 and 104 trial periods [33], next-generation maternal HIV
432 Env vaccination strategies should be developed within the contemporary context of
433 widespread availability of ART for pregnant women. Consequently, studies or trials of
434 future maternal vaccine regimens aimed at preventing vertical transmission of HIV

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435 should model the conditions of antiretroviral therapy and viral suppression during
436 pregnancy.
437 Though the majority of vaccines currently licensed for human use in the United
438 States are formulated with an aluminum-based adjuvant, alternative adjuvant selection
439 may play a critical role in the elicitation of protective humoral responses against HIV
440 transmission [34, 35]. In a systematic comparison of the ability of adjuvant formulations,
441 including Toll-like receptor (TLR) agonists, to induce antibody binding, neutralizing, and
442 ADCC responses against transmitted/founder HIV-1 envelope gp140 (B.63521) in
443 rhesus macaques, Moody et al. demonstrated that combination of a TLR7/8 agonist with
444 a TLR9 agonist in a squalene-based oil-in-water emulsion resulted in enhanced HIV
445 Env-specific antibody responses [36]. These potent TLR-based adjuvants may be
446 exploited to synergize with highly antigenic HIV immunogens to induce more potent and
447 durable protective antibody titers. During the 2009 influenza A (H1N1) pandemic, an
448 H1N1 vaccine in combination with a novel squalene-based AS03 adjuvant was safely
449 used among pregnant women in Norway and resulted in reduced risk of both influenza
450 diagnosis and fetal death [37]. Thus, the demonstrated safety profile of AS03 adjuvant
451 among pregnant women has opened the door for implementation of novel adjuvants
452 beyond alum in pregnancy.
453 Moreover, the nature of vertical transmission of HIV necessitates a vaccination
454 strategy that can mount a protective immune response specific to the autologous
455 circulating virus pool of each HIV-infected mother. Thus, the choice of vaccine
456 immunogen must reflect the broad diversity of HIV strains circulating today, specifically
457 clade C and B virus subtypes prevalent in Sub-Saharan Africa and the United States

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458 and Europe, respectively. One potential approach to overcome this barrier is to employ
459 a multi-clade (B/C) HIV Env immunogen that would leverage the immunological
460 phenomenon of original antigenic sin to provoke an anamnestic response against
461 maternal autologous circulating viruses.

462 Additionally, future studies may explore the potentially protective role of antibody-
463 mediated effector functions beyond neutralization in reducing the risk of MTCT of HIV.
464 Notably, a previous study by Overbaugh et al. suggested that HIV Env-specific IgG-
465 mediated antibody dependent cell cytotoxicity (ADCC) activity in breastmilk correlates
466 with reduced risk of postnatal vertical transmission [38]. Moreover, we have previously
467 demonstrated that passive infusion with a cocktail of non-neutralizing antibodies
468 provided partial protection against postnatal SHIV acquisition in an infant non-human
469 primate oral challenge model [39]. Further investigation into the potentially protective
470 roles of other Fc-mediated effector functions including antibody dependent cell
471 phagocytosis (ADCP) and complement activation activity in the setting of MTCT of HIV
472 is warranted. Perhaps elicitation of the full breadth of polyfunctional antiviral activity of
473 the humoral immune response, not only autologous neutralization response, will be the
474 critical target of maternal HIV vaccine design to eliminate MTCT.
475

476 **Conclusion**

477 In this study, we assessed the autologous virus neutralization responses of
478 maternal plasma collected at delivery against circulating viruses isolated from early and
479 late pregnancy in HIV-infected women vaccinated with an HIV Env subunit recombinant
480 gp120/160 adjuvanted with alum from historical AVEG 102 and 104 Phase I trials. We

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481 found that vaccination of HIV-infected pregnant women with recombinant MN gp120 or
482 gp160 adjuvanted with alum boosted HIV Env-specific antibody binding responses
483 between the first and last visit against clade B MN.3 gp120, the original vaccine antigen,
484 compared to placebo recipients. However, vaccination failed to augment the ability of
485 maternal plasma collected at delivery to neutralize clade B MN.3 virus, a tier 1
486 heterologous virus, between the first and last visit. Maternal HIV Env vaccination did not
487 enhance the ability of maternal plasma collected at delivery to neutralize autologous
488 viruses isolated from early pregnancy. Moreover, vaccination had no evident impact on
489 viral diversity. These findings indicate that further optimization in choice of vaccine
490 immunogen and adjuvant will be necessary to effectively augment autologous virus
491 neutralization responses in HIV-infected pregnant women to synergize with ART and
492 reduce MTCT of HIV.
493

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509

510 **Declaration of Competing Interests**

511 The authors declare the following financial interests/personal relationships which
512 may be considered as potential competing interests:
513 S.R.P. is a consultant for Merck, Sanofi, Moderna, and Pfizer CMV vaccine programs
514 and has sponsored programs with Merck and Moderna for CMV vaccine development.
515 All other authors have no competing interests to declare.

516

517 **Author Contributions**

518 **Eliza D. Hompe**: Conceptualization, Data curation, Formal analysis, Funding
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521 original draft. **Joshua A. Eudailey**: Data curation, Formal analysis, Investigation,
522 Writing – review & editing. **Elena E. Giorgi**: Formal analysis, Methodology, Software,
523 Writing – review & editing. **Amit Kumar**: Investigation, Methodology, Writing – review &
524 editing. **Erin McGuire**: Investigation. **Barton F. Haynes**: Writing – review & editing. **M.**
525 **Anthony Moody**: Writing – review & editing. **Peter F. Wright**: Resources, Writing –
526 review & editing. **Genevieve G. Fouda**: Resources, Writing – review & editing,

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527 Supervision. **Feng Gao**: Resources, Writing – review & editing, Supervision. **Sallie R.**
528 **Permar**: Conceptualization, Methodology, Resources, Writing – review & editing,
529 Supervision, Project administration, Funding acquisition. All authors gave final approval
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531 authorship.
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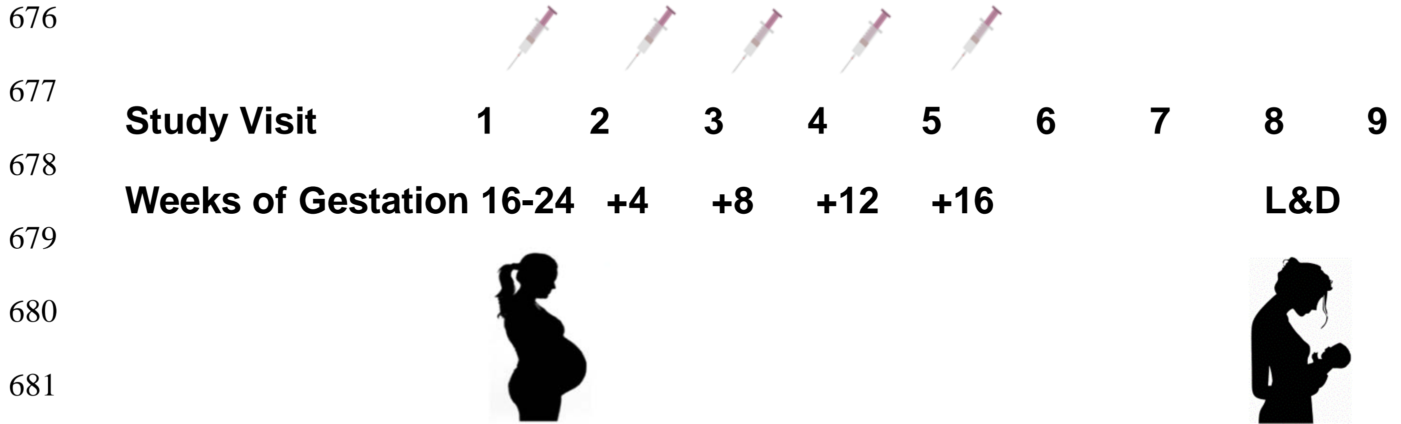

FIGURE 1. Immunization schedule in AVEG102/104 studies. Pregnant, HIV-infected women with $CD4^+$ T cell counts > 400/mm^3 were enrolled in the AVEG 102/104 studies. In the AVEG 102 protocol, women were administered 640 µg of gp160 (n=1) or placebo (n=1). In the AVEG 104 protocol, women received 300 µg of gp120 + alum (n=17) or placebo (alum + diluent) (n=9). The primary immunization was given at Visit 2, between 16-24 weeks of gestation. Monthly booster injections were subsequently given 4 weeks apart for the duration of pregnancy (Visits 3-6) for up to 5 total immunizations (median: 5; range 4-5). Visit 9 was labor and delivery.

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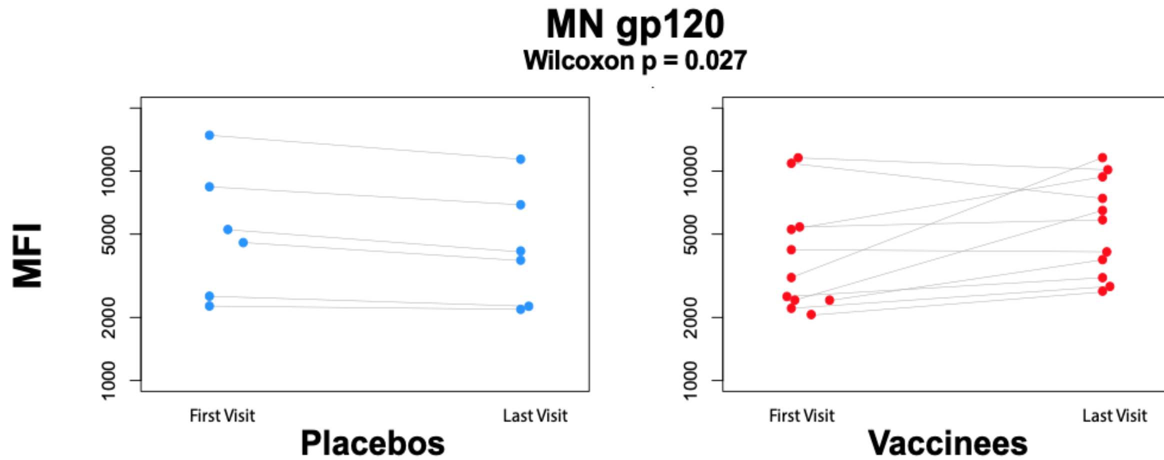

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FIGURE 2. MN gp120-specific binding in vaccinees and placebo recipients at first and last visit. Comparison of changes in gp120-specific binding from the first to the last visit between vaccinees (red) and placebo (blue). The between-visits change in gp120-specific binding was statistically significantly higher in vaccinees (p=0.027 by 2-sided Wilcoxon test). Light gray lines link the same mother.

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			MN gp120	Linear V3.B	gp70 V1/V2	MN.3 Neut
Vaccinee	AVEG 104	10485S	x	x	x	x
		104ERE	x	x	x	x
		104FHY	x	x	✓	x
		104IR6	✓	✓	✓	✓
		104IRF	x	✓	x	x
		10485T	x	x	✓	x
		104GA1	x	x	x	x
		104IR9	x	x	x	x
		104IRB	x	x	x	x
		104IRC	x	x	x	x
	AVEG 102	102I1G	x	x	x	x
Placebo	AVEG 104	104ERF	x	x	x	x
		104FHX	x	x	x	x
		104HXS	x	x	✓	x
		104IRD	x	x	x	x
		104IRG	x	x	x	x
	AVEG 102	102I1F	x	x	x	x

FIGURE 3. Change in antibody binding response against MN.3 gp120, linear V3.B, gp70 V1V2 and neutralization response against MN.3 before and after vaccination. Vaccinees (red) or placebo recipients (blue). Check marks indicate a three-fold increase.

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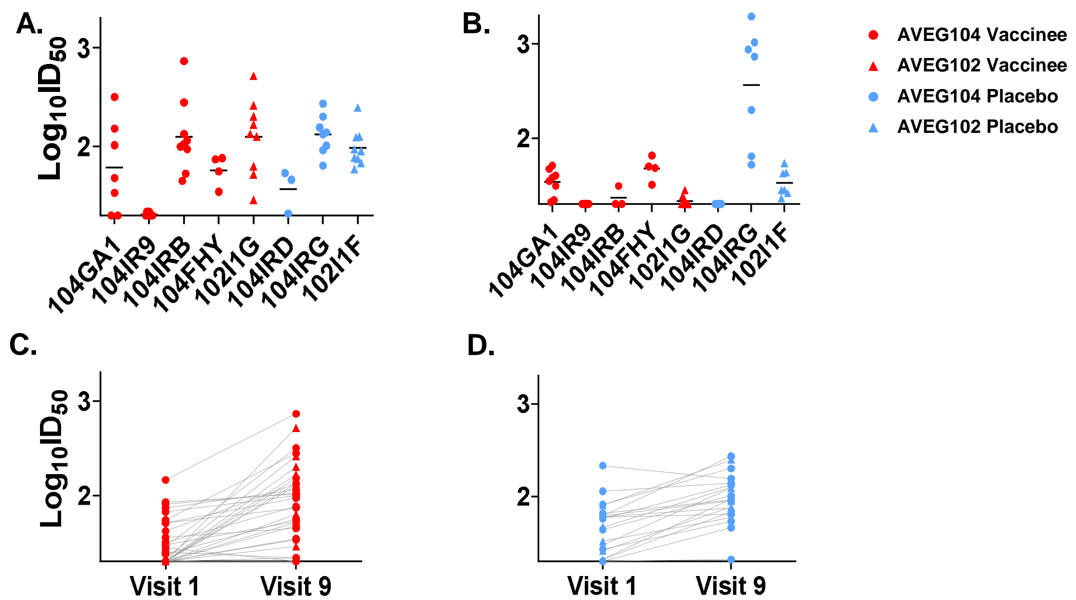

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FIGURE 4. Neutralization of viruses isolated from vaccine and placebo recipient plasma during early and late pregnancy by autologous maternal plasma collected at delivery. For each vaccine and placebo recipient, the neutralization potency of maternal plasma at delivery was assessed against the early pregnancy (Visits 1,4) (A) and late pregnancy (Visits 5-9) (B) autologous virus populations. The left y-axis depicts neutralization potency, in $log_{10}ID_{50}$. Study participants are displayed on the X axis (A,B). AVEG104 study participants are depicted with circles; AVEG 102 study participants with triangles. Vaccine recipients are shown in red; placebos in blue. Bars represent geometric means. Change in neutralization potency of autologous maternal plasma from Visits 1 (pre-immunization) and 9 (delivery) are shown against early pregnancy plasma viruses (C,D), with the exception off autologous viruses isolated from Visit 4 in mother 104FHY.

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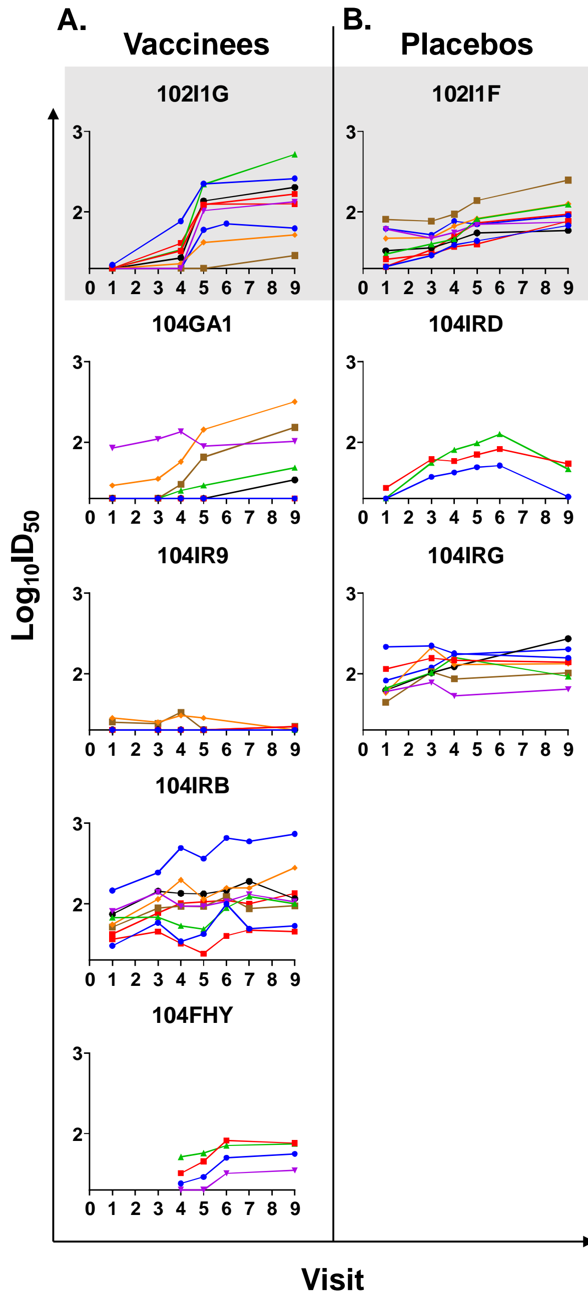

FIGURE 5. Neutralization potency of autologous maternal plasma against plasma viruses isolated from early pregnancy. Maternal plasma from pre-immunization (Visit 1), booster visits (Visits 3, 4, 5, & 6) and post-immunization (Visits 7 & 9) was tested against individual virus variants from Visit 1 (except for 104FHY; virus variants are from Visit 4). Each colored line represents a different virus. The left y-axis depicts neutralization potency, in $\log_{10}ID_{50}$. AVEG 102 study participants are shown on the top row, shaded in gray. With additional gp160 doses, there is a greater boost in neutralization potency against autologous viruses in the gp160 vaccine recipient compared to the placebo. There does not appear to be an increase in autologous virus neutralization with gp120 vaccination compared to placebo.

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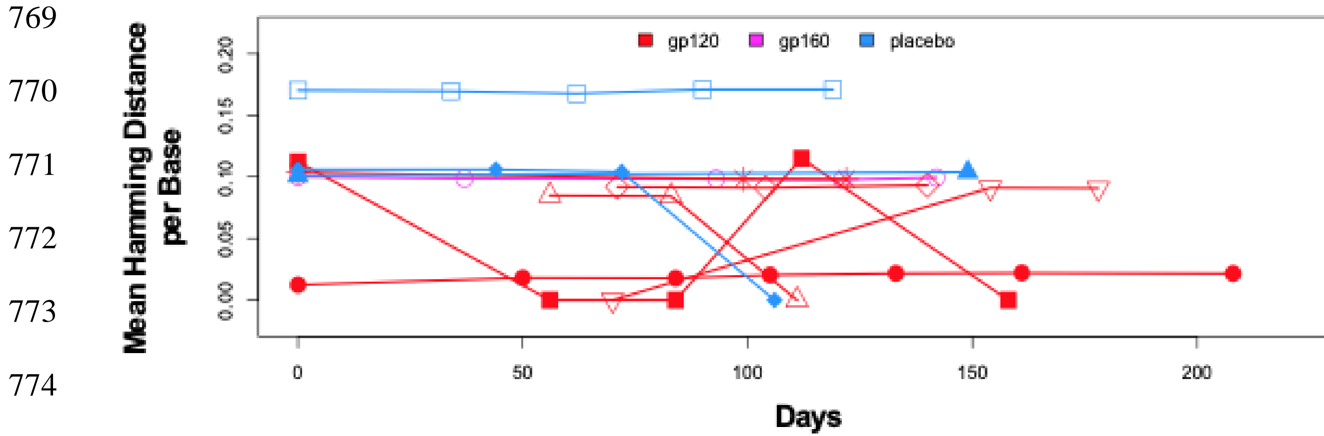

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FIGURE 6. Comparison of change in intersequence Hamming distance per base pair of *env* sequences obtained from vaccinees (n=7) and placebo recipients (n=3) across study visits in days. Each shape is an individual mother. Gp120 (red), gp160 (pink), placebo (blue).

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Study Cohort	Maternal ID	Treatment	Visit	Visit Date	Number of maternal env gene SGAs	Number of functional PSVs
AVEG 102	102I1F	placebo	1	5/21/1993	15	9
			3	6/24/1993	17	
			4	7/22/1993	6	
			5	8/19/1993	9	
			6	9/16/1993		
			9	9/17/1993	21	7
	102I1G	gp160	1	9/14/1993	31	9
			4	10/21/1993	15	
			5	12/16/1993	17	
			6	1/13/1994	6	
			9	2/3/1994	21	12
AVEG 104	104ERE	gp120	2	6/17/1993		
			4	8/12/1993	12	
			5	9/6/1993	11	
			6	10/6/1993	1	
			9	10/18/1993		
	104FHY	gp120	1	10/18/1994		
			4	12/28/1994	7	4
			5	1/30/1995	7	
			6	3/7/1995	6	7
			9	4/5/1995		
	104IR6	gp120	1	6/23/1993		
			3	8/4/1993		
			4	9/1/1993	1	
			5	9/29/1993		
			6	10/27/1993		
			7	11/24/1993	6	
			9	12/16/1993	7	
	104GA1	gp120	1	10/26/1993	6	7
			3	11/30/1993		
			4	1/4/1994		
			5	2/1/1994	22	8
			9	2/24/1994	2	2
	104IR9	gp120	1	1/20/1994	24	6
			3	3/17/1994	1	
			4	4/14/1994	1	
			5	5/12/1994	11	
			9	6/27/1994		
	104IRB	gp120	1	4/20/1994	10	9
			3	6/8/1994	7	
			4	7/13/1994	11	
			5	8/3/1994	4	
			6	8/31/1994	7	
			7	9/28/1994	13	
			9	11/14/1994	9	5
	104IRD	placebo	1	4/16/1994	3	3
			3	6/9/1994		
			4	7/7/1994		
			5	8/4/1994		
			6	9/12/1994		
			9	9/14/1994	4	3
	104IRG	placebo	1	11/1/1994	12	
			3	12/15/1994	17	
			4	1/12/1995	13	
			9	2/16/1995	1	

TABLE 1. Number of maternal *env* gene sequences isolated and functional pseudoviruses produced from each maternal plasma sample.

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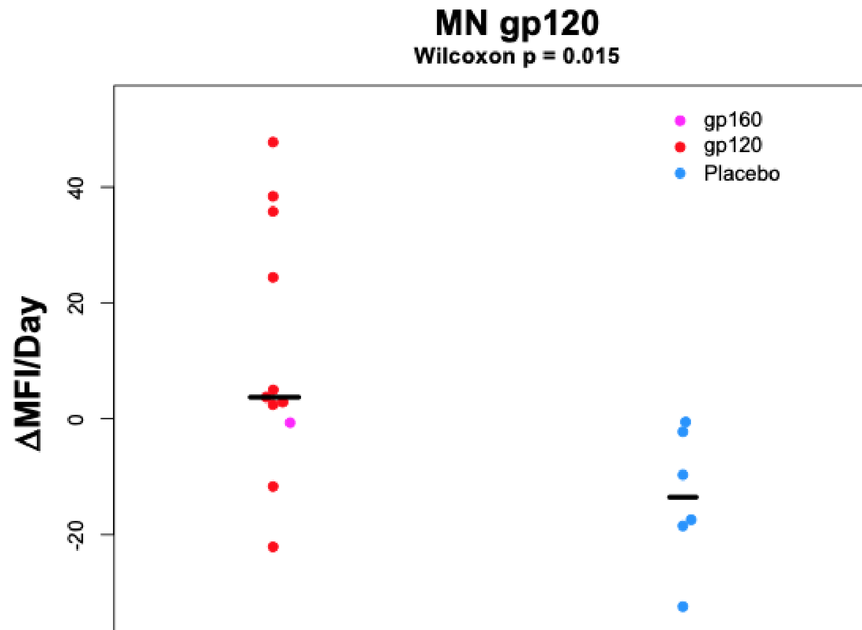

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FIGURE S1. **Change in MN gp120-specific binding per day in vaccinees and placebo recipients between first and last visit.** Comparison of changes in gp120-specific binding per day from the first to the last visit between vaccinees (red or pink) and placebo (blue). The gp120-specific binding increase per day was higher in vaccinees compared to placebo (p=0.015 by 2-sided Wilcoxon test).

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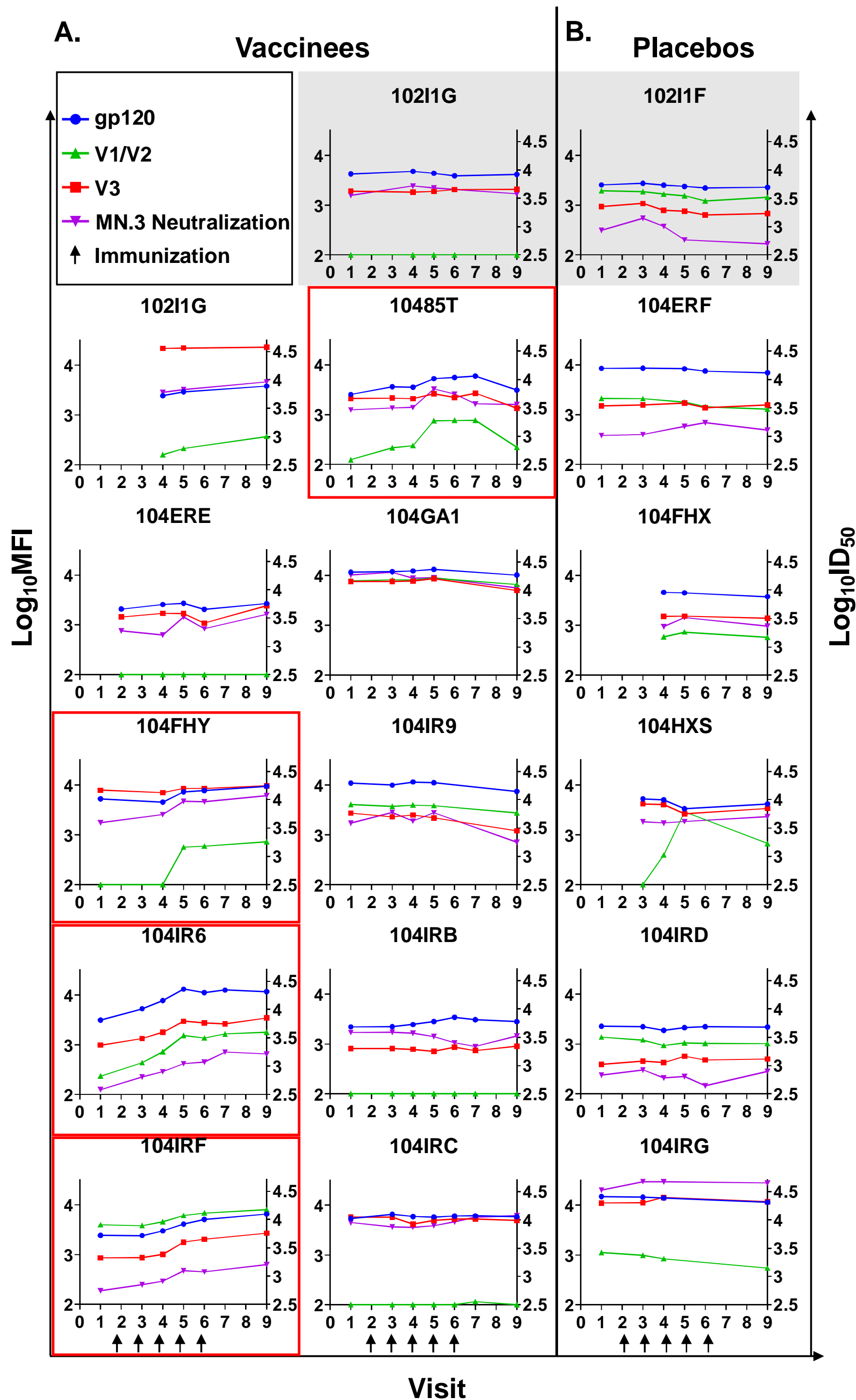

FIGURE S2. HIV Env gp120, V1V2, and V3-specific IgG binding and neutralizing Ab responses in HIV-infected, pregnant women following immunizations with MN gp120 or gp160. The left y-axis depicts binding antibody response, in $\log_{10}$MFI, and gp120 binding responses are indicated by the blue lines, V3 responses by the red lines, and V1V2 responses by the green lines. The right y-axis depicts neutralization potency, in $\log_{10}ID_{50}$, and is indicated by the purple lines. AVEG 102 study participants are shown on the top row, shaded in gray. Red boxing indicates 3-fold increase in gp120, V1V2, V3, or neutralizing antibody responses over time.

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Neutralization of Autologous Viruses (ID_{50})

	Virus ID	Vaccinee					Placebo		
		104GA1	104IR9	104IRB	104FHY	102I1G	104IRD	104IRG	102I1F
Early Autologous Plasma Viruses (Visits 1,4)	1	<20	<20	53	56	261	21	156	68
	2	<20	22	45	76	167	54	139	77
	3	48	<20	100	74	519	46	92	124
	4	103	<20	106	35	134		64	75
	5	317	20	279		52		133	126
	6	34	<20	115		202		271	59
	7	152	22	94		29		102	248
	8			733		63		201	90
	9			134		126			94
Late Autologous Plasma Viruses (Visits 5-9)	1	40	<20	31	65	28	<20	52	23
	2	47	<20	<20	48	24	<20	64	54
	3	38	<20	<20	50	<20	<20	730	26
	4	31	<20		32	<20	<20	1939	28
	5	22	<20			<20	<20	198	28
	6	51	<20			<20	<20	864	42
	7	35	<20			23	<20	1030	43
	8	21	<20			<20			
	9					21			
	10					21			
	11					<20			

ID_{50}
<20
20-60
61-180
181-540
>540

FIGURE S3. Neutralization of viruses isolated from vaccine and placebo recipient plasma during early and late pregnancy by autologous maternal plasma collected at delivery. For each vaccine and placebo recipient, the neutralization potency of maternal plasma at delivery was assessed against the early (Visits 1,4) and late pregnancy (Visits 5-9) autologous virus populations. Higher ID_{50} (darker color) represents greater neutralization potency.

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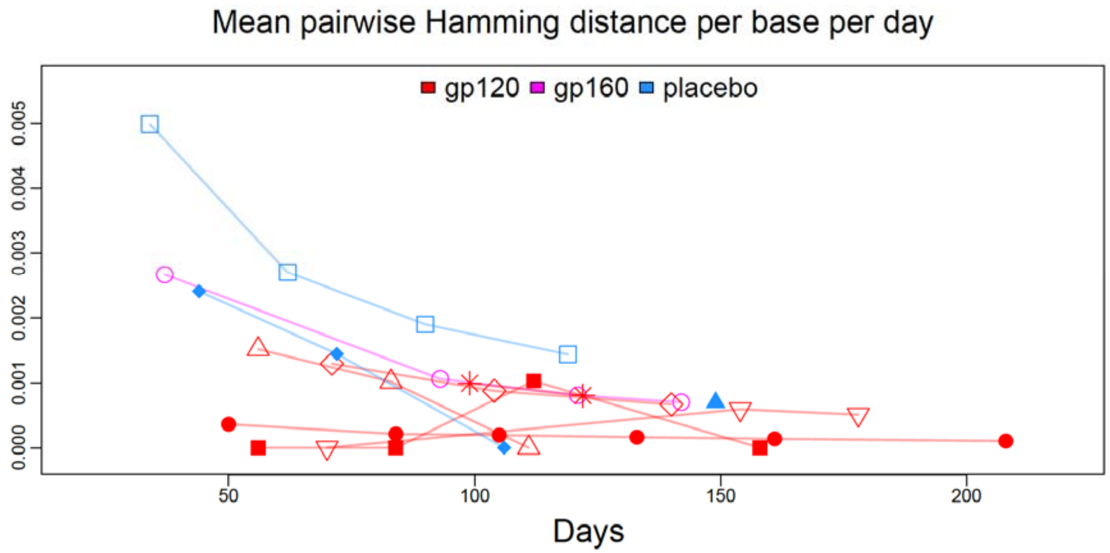

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FIGURE S4. Comparison of change in intersequence Hamming distance per base pair per day of *env* sequences obtained from vaccinees (n=7) and placebo recipients (n=3) across study visits. Each shape is an individual mother. Gp120 (red), gp160 (pink), placebo (blue).

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Assay	Protein	Peptide	Sequence
BAMA	MN gp120		TEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEAHNVWATHACVPTDPNPQEVELVN VTENFNMWKNNMVEQMHEDIISLWDQSLKPCVKLTPLCVTLNCTDLRNTTNTNNSTDN NNSKSEGTIKGGEMKNCSFNITTSIGDKMQKEYALLYKLDIEPIDNDSTSYRLISCNT SVITQACPKISFEPIPIHYCAPAGFAILKCNDKKFSGKGSCKNVSTVQCTHGIRPVVS TQLLLNGSLAEEEVVIRSEDFTDNAKTIIVHLKESVQINCTRPNYNKRKRIHIGPGRA FYTTKNIKGTIRQAHCIISRAKWNDTLRQIVSKLKEQFKNKTIVFNPSSGGDPEIVMH SFNCGGEFFYCNTSPLFNSIWNGNNTWNNTTGSNNNITLQCKIKQIINMWQKVGKAMY APPIEGQIRCSSNITGLLLTRDGGEDTDTNDTEIFRPGGGDMRDNWRSELYKYKVVTI EPLGVAPTKAKRRVVQREKR
	Rec MN gp41		TVQARLLLSGIVQQQNNLLRAIEAQQNMLQLTVWGIKQLQARVQAVERYLKDQQLLGF WGCSGKLICTTTVPWNASWSNKSLDDIWNNMTWMQWEREIDNYTSLIYSLLEKSQTQQ EKNEQELLGLDKWESLWNWFDITNWLENRVRQGYSPLSLQTRPPVPRGPDRPEGIEEE GGERDRDTSGRLVHGFLAIIWVD
		Bio-V3.B	NNTRKSIHIGPGRAFYATGDIIGDIRQAHC
		gp70 B.MN V3	TRPNYNKRKRIHIGPGRAFYTTKNIKGTIRQAH
		gp70 B.CaseA V1V2	CIDLRNATNATSNSNTTNTTSSSGGLMMEQGEIKNCSFNITTSIRDKVQKEYALFYKL DIVPIDNPKNSTNYRLISC

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TABLE S1. Amino acid sequences for antigens used in AVEG102/104 plasma binding antibody multiplex assays (BAMA).