1 **Anti-V2 Antibody Deficiency in Individuals Infected With HIV-1**

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25   **ABSTRACT**

26   The positive correlation of high levels of plasma anti-V2 antibodies (Abs) with protective

27   immunity in the Phase III anti-HIV RV144 vaccine trial generated interest in the induction of

28   these Abs for HIV vaccine development. We analyzed plasma samples from 79 chronically

29   infected Cameroonian individuals for Ab reactivity against three V1V2 fusion proteins and five

30   cyclic V2 peptides and found that HIV-1 infection induces different levels of anti-V2 Abs. While

31   the majority of plasma samples reacted strongly with one or more V2 antigens, 10% (8) of the

32   samples were nonreactive. Deficiency of anti-V2 Abs was consistently found in longitudinal

33   plasma samples tested over 8 to 54 months of HIV infection. There was a strong correlation

34   between binding activities of plasma anti-V2 Abs and anti-gp120 and anti-gp41 Abs, suggesting

35   that deficiency of V2 Abs could be related, in part, to a limited ability to elicit strong Ab

36   responses. Analysis of gp120 sequences revealed that the V2 region of viruses from donors with

37   V2-deficient versus V2-reactive Abs displayed a tendency toward longer length, more glycans,

38   and lower isoelectric point and charge. No differences between these two patient groups were

39   noted in the same parameters measured in the V1 region. These data suggest that immunogens

40   containing a shorter V2 region with fewer glycosylation sites and higher electrostatic charges

41   would be beneficial for induction of anti-V2 Abs, but the ability to mount a strong general Ab

42   response to HIV-1 appears to be a dominant factor.

43   **IMPORTANCE**   The results of the RV144 vaccine clinical trial showed a correlation between

44   plasma Abs against a V1V2 fusion protein and a decreased risk of acquiring HIV-1 infection.

45   This turned the focus of some HIV vaccine design to the induction of elevated levels of anti-V2

46   Abs to increase vaccine efficacy. In plasma samples from Cameroonian individuals infected with

47   HIV-1, we observed broad variations in levels of anti-V2 Abs, and 8 of the 79 plasma samples

48   tested displayed substantial deficiency of V2 Abs. Sequence analysis of the V2 region from

49   plasma viruses and multivariate analyses of V2 characteristics showed a significant difference in

50   several features between V2-deficient and V2-reactive plasma Abs. These results suggest that

51   HIV vaccine immunogens containing a V2 region with shorter length, fewer glycosylation sites,

52   and higher electrostatic charges may be beneficial for induction of a higher level of anti-V2 Abs

53   and thus contribute to HIV vaccine efficacy.

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55   **KEYWORDS**   HIV-1, V1V2 region, V2 conformational antibodies, V2 linear antibodies, V2

56   antibody deficiency

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58   Anti-V2 antibodies (Abs) have been the subject of studies to determine their role in

59   protection against HIV-1 infection due to the results of the Phase III RV144 vaccine trial, which

60   showed an inverse correlation with risk of infection (1). The RV144 data revealed that the V2

61   Abs in vaccinees were highly cross-clade reactive and that high levels, (but not medium or low

62   levels) of plasma Abs against a V1V2 fusion protein correlated with reduced acquisition of HIV-

63   1 infection (1, 2). The V2 region contains at least 3 types of epitopes defined by anti-V2

64   monoclonal Abs (mAbs): i) V2 integrin (V2i), which is a conformation-dependent motif and

65   includes the α4β7 integrin binding site; ii) the V2 peptide (V2p) linear epitope; and iii) the V2

66   quaternary (V2q) epitope, present preferentially on the trimer (3). The majority of anti-V2 Abs

67   are specific for conformation-dependent V2i epitopes; a smaller amount to the linear V2p

68   epitopes, and some are specific for quaternary V2q epitopes and only present in a small

69   proportion of HIV-infected individuals.

70   The frequency of anti-V2 Abs in the plasma of individuals infected with HIV-1 varies

71   among studies, depending on the methods used and the population studied. Screening of immune

72   plasma using V2 consensus peptides with the HxB2 sequence detected 12% and 21% positive

73   sera from HIV-1-infected individuals (4, 5). Using V1V2 proteins expressing both conformation-

74   dependent V2i and linear V2p epitopes, the frequency of plasma anti-V2 Abs is higher, yielding

75   30% and 48% in two studies (6, 7). The prevalence of anti-V2 Abs in clade B-infected

76   individuals was determined to be 45% when tested against a C1-V1V2 BH10 protein and 30%

77   when screened in a competition assay with the 697 mAb specific for the conformational V2i

78   epitope (8).

79   The frequency of anti-V2 Abs induced by the RV144 vaccine was relatively high, given

80   that 84% of vaccinees' sera was reactive with the V1V2-gp70 fusion protein carrying the

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81   sequence of subtype B strain CaseA2 (9). A high percentage of plasma samples was also reactive

82   with the cyclic V2 peptide with the sequence of clade AE 92TH023 (1). Given that the high level

83   of anti-V2 Abs is relevant for protection (2) and that not all immune plasma samples from

84   infected and vaccinated individuals contain V2 Abs, we studied a panel of plasma samples to

85   determine the frequency of anti-V2 Abs against different V1V2 fusion proteins and cyclic V2

86   peptides. Lack of anti-V2 Abs in some plasma samples was in part dependent on a weak general

87   Ab response to HIV envelope (Env) antigens, manifested by significantly lower titers of Abs

88   against V3, gp120, and gp41 proteins compared with plasma with reactive anti-V2 Abs. In

89   addition, differences in the V2 region, including extended length, glycosylation sites, lower

90   isoelectric point, and charge may also contribute to lower immunogenicity and deficiency of

91   anti-V2 Abs in some individuals infected with HIV-1.

92   **RESULTS**

93   **Frequency of plasma Abs against V2 and control antigens.** A panel of 79 plasma

94   samples was obtained at the Medical Diagnostic Center in Yaoundé, Cameroon from a cohort of

95   individuals chronically infected with HIV-1. The plasma samples were screened at a 1:100

96   dilution by ELISA against three V1V2 fusion proteins and five biotinylated cyclic V2 peptides.

97   Given that anti-V1 Abs are sequence-specific (10) and don't bind to heterologous V1V2 fusion

98   proteins, plasma samples can be tested against V1V2 fusion proteins with heterologous sequence

99   to detect specific Abs against the V2 region. To confirm that the anti-V1 Abs would not bind to

100   heterologous V1V2 fusion proteins, we screened all plasma samples against two fusion proteins

101   with the major parts of V2 deleted: V1ΔV2$_{A244}$-gp70 and V1ΔV2$_{CaseA2}$-gp70 (Table S1). None of

102   the 79 plasma samples reacted with these two V1ΔV2 proteins, confirming that binding of

103   plasma to heterologous V1V2 fusion proteins usually detects Abs specific for V2.

104   The frequency of plasma with anti-V2 Abs varied depending on the antigen sequence.

105   The highest percentage of plasma samples reacted with $V1V2_{case\ A2}$-gp70 at 85%; 80% reacted

106   with $V1V2_{A244}$-gp70; and 53% of plasma Abs bound to $V1V2_{ZM109}$-1FD6 fusion proteins (Fig.

107   1A). The frequency of anti-V2 peptide Abs was lower, ranging from 72% to 40% for plasma

108   binding to $V2_{A244}$, $V2_{92TH023}$ (clade AE), $V2_{Du422}$ (clade C), $V2_{230}$ and $V2_{200}$ (clade AG) (Fig.

109   1A).

110   All 79 plasma samples reacted by ELISA with $gp120_{A244}$ and $gp41_{MN}$ proteins, while 76

111   (96%) bound to cyclic $V3_{A244}$ peptide, most of the latter reaching the saturation level at OD 3.5

112   to 3.9 (Fig. 1A). The IgG subclasses and IgA of plasma Abs, tested separately against $V1V2_{A244}$-

113   gp70, showed the dominance of IgG1 Abs, whereas IgG3 and IgA were only detected in 3

114   plasma samples each, and IgG2 and IgG4 binding was not detectable (Fig. 1B). The results

115   showed a broad range of ODs for anti-V1V2 fusion proteins and anti-V2 peptides Abs, from

116   close to 0 to 3.9, while the OD range for anti-gp120 was from 1.2 to 3.9 and for anti-gp41 from

117   2.1 to 3.7 OD (all positive). This indicates a weaker immunogenicity of the V2 region compared

118   with gp120 and gp41, which was further confirmed by 50% titers of plasma Abs, as shown

119   below.

120   The CD4$^+$ T cell counts, cells/µL, were comparable in two tested groups (6 samples

121   each): V2 deficient and V2 reactive; mean counts were higher but not significant in the V2-

122   deficient group (558 vs 402, respectively; P=0.1426). Viral loads, HIV RNA copies/mL, were

123   measured in the same plasma samples, and results in the V2-deficient and V2-reactive groups

124   were also comparable: 7,076 vs 16,400, respectively (P=0.2780).

125   Glycoprotein 120 from 12 plasma viruses (6 each from the V2-deficient and V2-reactive

126   groups) was sequenced, and virus subtypes were identified (Table 1). All but one virus (clade C)

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127   were HIV-1 circulating recombinant forms (CRF), which dominate in Cameroon, according to

128   recent analysis with the most prevalent CRF02_AG (64.9%) and CRF22_01A1 (7.1%) (11, 12).

129   The identified subtypes in this study were as follows: CRF02_AG (3 plasma samples),

130   CRF01_AE (2), CRF22_01A1 (2), CRF11_cpx, CRF18_cpx, CRF36_cpx, and CRF37_cpx

131   (Table 1).

132   **Reactivity of plasma anti-V2 Abs.** The majority of 79 plasma samples at 1:100 dilutions

133   (90%) were cross-reactive against eight V2 antigens including three V1V2 fusion proteins and

134   five V2 peptides and representing sequences from HIV-1 subtypes AE, AG, B and C. Eleven

135   plasma samples (14%) reacted with all eight V2 antigens (Fig. 2A), while 60 plasma samples

136   (76%) reacted with several, but not all, V2 antigens (data not shown). Eight plasma samples

137   (10%) did not react with any of the eight V2 antigens (Fig. 2B) with the exception of three

138   plasma samples which each bound weakly to one V2 antigen, but still reacted with cyclic $V3_{A244}$

139   peptide, $gp120_{A244}$ and $gp41_{MN}$ (Fig. 1A).

140   Given that plasma sample reactivity is usually tested against heterologous V2 sequences,

141   we also included cyclic V2 peptides with sequences derived from two blood donors, MDC230

142   and MDC200, to test the reactivity to autologous linear V2 epitopes (Table 1). The results

143   showed strong binding of plasma MDC230-1 (V2-reactive group) to the autologous $V2_{230}$

144   peptide while plasma MDC200-2 (V2-deficient group) bound only weakly to the autologous

145     $V2_{200}$ peptide but not to other heterologous V2 antigens (Table 1). Of interest, the $V2_{200}$ peptide

146   was weakly reactive with 4 of 6 plasma samples from the cross-reactive group (Table 1). One

147   unusual feature of the V2 region represented by the $V2_{200}$ peptide is its exceptionally low pI of

148   4.79, while other V2 regions have pI values between 6.52 and 9.69 (Table S3).

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149   **Longitudinal studies of plasma anti-V2 Abs.** To determine whether the deficiency of

150   V2 Abs is temporary or stable, we analyzed plasma samples from six V2 Ab-deficient donors

151   over 11-54 months of observation and plasma samples from six V2 Ab-reactive donors over 28-

152   60 months of observation (Table 1). These sequential samples were titrated and screened against

153   five Env antigens: V1V2$_{A244}$-gp70, cyclic V3$_{A244}$ peptide, gp120$_{A244}$, gp41$_{MN}$, and p24$_{HXB2}$ (Fig.

154   3). Titers of anti-V2$_{A244}$ Abs in the V2-deficient group were undetectable (<1:100 dilution) (Fig.

155   3A) and designated as a 50% binding titer of 10^0 (Fig. 4). Longitudinal analyses confirmed that

156   lack of V2 Abs in plasma collected from six selected individuals infected with HIV-1 was

157   constant over 11 to 54 months of observation. Similarly, Abs to four other antigens (V3, gp120,

158   gp41, p24) were detected at relatively constant levels, albeit at intermediate to high titers; these

159   results show an exclusive, constant lack of Ab response to V2 (Fig. 3A). The panel of sequential

160   plasma samples collected over 28 to 60 months from six volunteers with reactive V2 Abs

161   displayed relatively constant levels of all five types of Abs, including anti-V2 Abs (Fig. 3B).

162   The 50% titers of plasma Abs were used to compare the two groups of plasma samples

163   (Fig. 4). There were significantly higher 50% titers of Abs (*P*<0.0001) against V1V2$_{A244}$-gp70,

164   cyclic V3$_{A244}$ peptide, gp120$_{A244}$, and gp41$_{MN}$ in the group of V2-reactive Abs vs those with V2-

165   deficient Abs (Fig. 4, Table 2). Titers of anti-p24 Abs in the two groups were comparable (Fig.

166   4, Table 2).

167   **Correlation between Ab binding to V2 and other Env antigens.** To determine if the

168   deficit of anti-V2 Abs is related to the general Ab response to HIV-1 Env proteins, we analyzed

169   the relationships of the ELISA binding activities (OD) between plasma Abs against V1V2$_{A244}$-

170   gp70 protein and other antigens using linear regression and the Pearson correlation coefficient (r

171   ) (Fig. 5). There was a strong correlation (*P*<0.0001) between a level of Abs against V1V2$_{A244}$-

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172 gp70 versus two other V1V2 fusion proteins (CaseA2 and ZM109) and three cyclic V2 peptides

173 (A244, 92TH023, Du422), as well as gp120$_{A244}$ and gp41$_{MN}$. Thus, correlation of low level of

174 Abs against V2 and gp120/ gp41 suggest that a deficiency of anti-V2 plasma Abs is related to a

175 generally weaker Ab response to HIV-1 Env antigens in a subset of individuals infected with

176 HIV-1.

177   **Characteristics of the V2 and V1 regions.** We sequenced gp120 of the viruses in

178 plasma of 12 selected patients to analyze the V2 and V1 regions, including properties that may

179 have some influence on the immunogenicity of V2. From each plasma virus, 3 to 9 gp120

180 sequences were produced and used to generate consensus sequences for all 12 subjects (Table

181 2S). We analyzed the amino acid (AA) length, number of putative N-linked glycosylation sites,

182 isoelectric point (pI), and charge of the V2 and V1 regions. For each region, we compared these

183 features in the V2-deficient vs V2-reactive groups (Table 2, Table S3). In the V2 region, AA

184 length was significantly longer in the V2-deficient vs V2-reactive group (43.5 vs 39.5 AA;

185 *P*=0.0338), while pI (8.47 vs 9.25; *P*=0.1075) and charge (1 vs. 2.5; *P*=0.1613) were lower, but

186 not significantly different in the V2-deficient vs the V2-reactive group, respectively (Table 3). In

187 contrast to these data, the V1 region displayed statistically comparable AA length of 23.5 vs 24.5

188 (*P*=0.572), pI values of 4.89 vs 4.37 (*P*=0.6991) and charge: 1 vs –1.5 (*P*=0.8068) (Table 3).

189   We also analyzed the β-sheet propensity for the central conserved segment (E153 to

190 I184) and the α-helix propensity (K168 to V172) of the V2 region. The β-sheet propensity

191 correlates with neutralization sensitivity of the virus (13) and may suggest a different

192 immunogenicity profile, i.e., preferential presentation of different epitopes. The α-helix

193 propensity is an estimate of the helical conformation, which is recognized by anti-V2 peptide

194 mAbs such as CH58 (14) and putatively induces anti-V2p Abs; lower propensity suggest a

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195   higher tendency to form a helical structure. The two parameters were comparable between the

196   V2-deficient and V2-reactive groups (Table 3, Table S3). Thus, the V2 consensus sequences

197   from the V2-deficient compared with the V2-reactive group were slightly longer, with one

198   additional N-linked glycosylation site on average and a tendency to a lower pI and electrostatic

199   charge in the V2-deficient group.

200   **Multivariable analysis.** Given that the majority of features characterizing the V2 region,

201   except for length, are not significantly different between the V2-deficient vs V2-reactive groups,

202   we tested whether the combination of multiple features could distinguish these two groups

203   (Table 3). We defined composite indexes by combining 2 or 3 features to determine if the

204   difference between the V2-deficient and V2-reactive groups, in the V2 and V1 regions, is

205   significant, using the nonparametric Wilcoxon test. The difference between "No. AA" and "pI"

206   and the difference between "No. AA" and "Charge" completely separated the V2-deficient from

207   V2-reactive groups. That is, for each of the composite indexes, the maximum value (Max) in the

208   V2-reactive group was lower than the minimum value (Min) in the V2-deficient group. Despite

209   the small sample sizes, the lack of overlap in the values of each composite index between the two

210   groups is statistically significant (P=0.005) (Table 3). Similarly, combining three features ("the

211   sum of No.AA and No. glycosylation sites minus pI," "the sum of No. AA and No. glycosylation

212   sites minus charge," and "the sum of No. AA and α-helix minus pI or charge" also separated the

213   V2-deficient and V2-reactive groups; the non-overlapping index values between the groups are

214   statistically significant (P=0.005 and 0.002, respectively (Table 3). This analysis provides

215   evidence that multivariate predictions are more powerful than any single V2 variable for

216   distinguishing V2-deficient from V2-reactive patient groups (Table 3). The same method was

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217   used for analysis of the V1 region as a control and did not show any significant differences

218   between the two groups (Table 3).

219   **DISCUSSION**

220   The present study analyzed Abs against the V2 region in terms of their frequency and

221   level variation in plasma samples derived from 79 Cameroonian volunteers chronically infected

222   with HIV-1. Given that some samples were negative for anti-V2 Abs, we focused on

223   characterization of the V2 region of these individuals' viruses to look for characteristics that

224   might explain the deficiency of anti-V2 response. This analysis was prompted by the observation

225   that levels of anti-V2 Abs were inversely correlated with the reduced HIV infection rate in

226   recipients of the vaccine used in the RV144 clinical trial (1, 2) although levels of anti-V2 Abs

227   measured by OD were not unusually high (9).

228   In the present study, the frequency of plasma anti-V2 Abs was between 53% and 85% for

229   the three V1V2 fusion proteins and was comparable to results in the RV144 study, which

230   reported 84% frequency, possibly due to using the same V1V2 fusion proteins for screening (9).

231   In previous studies of sera of individuals chronically infected with HIV-1, anti-V2 Abs were

232   detected at a much lower frequency (from 12% to 48%), but either the V2 antigens tested were

233   different or plasma Abs were analyzed by different methods (4-7).

234   The level of anti-V2 Abs showed broad variations, in contrast to a relatively high level of

235   Abs against V3, gp120, and gp41, as measured by OD and also by 50% titers (Fig. 1). Eight out

236   of 79 (10%) plasma samples had no reactivity to any of the six V2 antigens in longitudinal

237   studies over 8 to 54 months, while all plasma samples reacted to gp120 and gp41 (Fig. 2C).

238   There was a strong correlation between ODs of plasma Abs binding to V2 antigens and to

239   gp120/gp41 (Fig. 5). For example, plasma samples with high or low OD for gp120 and gp41 Abs

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240   also had high or low levels of anti-V2 Abs, respectively, suggesting that a patient's capacity to

241   mount Ab responses to HIV-1, in general, is associated with the level of anti-V2 Abs.

242   We hypothesized that, in addition to a low Ab response to Env proteins, the lack of anti-

243   V2 Abs in several patients may depend on unusual characteristics of the V2 regions. Analysis of

244   V2 sequences revealed that the V2 region of viruses from donors with V2-deficient versus V2-

245   reactive Abs was significantly longer (P=0.0303), with 1 additional N-linked glycosylation site,

246   on average. Usually, a longer V2 region is accompanied by an increased number of N-linked

247   glycosylation sites (15-17). There is a correlation between the length and number of N-linked

248   glycosylation sites, as has been observed in 41 pseudo-typed viruses in which neutralization-

249   sensitive viruses to anti-V2 mAbs displayed shorter length and fewer glycosylation sites (18).

250   Given that additional glycosylation sites in the V2 region confer resistance to neutralization by

251   V2 mAbs, this may also have an impact on immunogenicity, resulting in weaker or lack of Ab

252   response, particularly in subjects with poor humoral immune response to infecting virus.

253   The charge and pI of the V2 region were also lower in the V2-deficient versus V2-

254   reactive panel of sequences (Table 3). Both these factors play also a role in anti-V3 mAbs; the pI

255   of the VH CDR3 was significantly lower for human anti-V3 mAbs neutralizing Tier 2 and 3

256   pseudo-typed viruses vs non-neutralizing mAbs (19). For example, JR-FL.JB virus (Tier 2) was

257   neutralized by 12 anti-V3 mAbs, and the pI of their VH CDR3 was significantly lower compared

258   with that of 36 non-neutralizing V3 mAbs (data not shown); all these V3 mAbs were developed

259   in our laboratory (19). Although the results do not correspond to the phenomenon of failed Ab

260   response to the V2 region, they underline a functional role of electrostatic interactions between

261   Ab and virus and, possibly, in immunologic recognition as well.

262   All 4 features—length, number of N-glycosylation sites, pI, and charge—were also

263   analyzed in the V1 region for control; there were no significant differences or tendencies in

264   either higher or lower values between the groups with V2-deficient and V2-reactive Abs (Table

265   2).

266   One type of anti-V2 Abs binds to V2 linear epitopes, which are detected using V2

267   peptides. These epitopes were defined by two human mAbs, CH58 and CH59, which were

268   generated from recipients of the vaccine used in the RV144 clinical trial (14). Both mAbs bind to

269   the helical structure of the V2 region, based on crystallographic analysis (14). The higher

270   propensity to helical conformation in the central V2 segment (K168 to V172) in the V2-deficient

271   versus V2-reactive group, which indicates higher flexibility, may be less immunogenic (13).

272   For most features, changes in the V2 region were minimal for the V2-deficient and V2-

273   reactive groups; although length was significantly longer in the V2-deficient group. However,

274   multivariable analysis including combination of 2 or 3 features, for example, differences in

275   length and pI or charge and linear combinations of length, pI, and glycosylation sites, completely

276   separated the two groups and resulted in a highly significant difference between V2-deficient and

277   V2-reactive groups (Table 3). Thus, we conclude that the combination of these small differences

278   in multiple features may be responsible for the lower immunogenicity of the V2 region resulting

279   in lack of anti-V2 Abs in patients with significantly lower Ab response to the Env proteins.

280   In summary, our results show a broad range of binding activity of plasma Abs against 3

281   V1V2 fusion proteins and 5 cyclic V2 peptides, with a frequency of 53% to 85%, whereas in all

282   plasma samples, Abs bound to heterologous gp120 and gp41. A strong correlation between anti-

283   V2 and anti-gp120/ gp41 plasma Abs suggests that lack of V2 Abs depends in part on the general

284   ability of patients to mount Ab responses to HIV-1 proteins. Sequence analysis of the V2 region

285   from plasma viruses in the V2-deficient Ab group indicates that HIV vaccine immunogens

286   containing V2 segments with fewer glycosylation sites and higher electrostatic charge may be

287   advantageous in inducing higher titers of anti-V2 Abs.

288   **MATERIALS AND METHODS**

289   **Ethics Statement.** Blood samples from men and women infected with HIV-1 were

290   received from the Medical Diagnostic Center (MDC), Yaoundé, Cameroon. Written informed

291   consent forms were signed by all participants in the study and approved by the National Ethical

292   Review Board in Cameroon. The study has been reviewed and approved by the Institutional

293   Review Board of New York University School of Medicine, New York, USA.

294   **Specimens.** The panel of 79 plasma samples from Cameroonian patients chronically

295   infected with HIV-1 was randomly selected and received from the collaborative cohort shared

296   between New York University School of Medicine, New York, NY, and the MDC, Yaoundé,

297   Cameroon. Plasma samples were collected longitudinally over a period of 8 to 60 months and

298   analyzed serologically. The donors were presumably infected with non-clade-B viruses, which

299   were confirmed in 12 study subjects by sequencing their plasma viruses (Table 1). Participants

300   enrolled in this study had been referred to the MDC in Cameroon for medical consultation due

301   receiving an HIV-positive diagnosis. Acquisition of HIV-1 infection was self-reported as caused

302   by heterosexual transmission in more than 95% of cases.

303   **Recombinant proteins and peptides.** Seven recombinant proteins—$V1V2_{A244}$-gp70

304   (clade AE), $V1V2_{case\ A2}$-gp70 (clade B), $V1\Delta V2_{A244}$-gp70 (V2 deleted), $V1\Delta V2_{case\ A2}$-gp70 (V2

305   deleted), $gp120_{A244}$, $gp41_{MN}$ (clade B), and $p24_{HXB2}$ (clade B) —were purchased from Immune

306   Technology Corp., New York, NY. Biotinylated cyclic peptides —$V2_{A244}$, $V2_{92TH023}$

307   (CRF01_AE), $V2_{Du422}$ (clade C), $V2_{230}$ (CRF02_AG, plasma virus from donor MDC230, Table

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308   S2) and V3$_{A244}$ —were purchased from Biopeptide Co., Inc., San Diego, CA. One biotinylated

309   cyclic peptide, V2$_{200}$ (CRF02_AG), representing the V2 sequence of plasma viruses from donor

310   MDC200 (Table S2), was purchased from Science Exchange, Palo Alto, CA. V1V2$_{ZM109}$-1FD6

311   (clade C) was produced as previously described (20).

312   **Antibody binding assay (ELISA).** The plasma samples were screened by standard

313   ELISA against recombinant V1V2 fusion proteins, gp120, gp41 and p24 and against biotinylated

314   V2 and V3 peptides according to the methods in (21, 22). In short, proteins were coated directly

315   onto Immulon 2Hb plastic plates at a concentration of 1 µg/mL. After overnight incubation at

316   4°C, the plates were washed with 1X phosphate-buffered saline (PBS) containing 0.05% Tween

317   20 and blocked with PBS containing 2.5% bovine serum albumin and 7.5% fetal bovine serum.

318   Plasma at dilution 1:100 was incubated with antigens in the plate for 1.5 h at 37°C. The plates

319   were washed and incubated with alkaline phosphatase-conjugated goat anti-human IgG (γ

320   specific) (Southern Biotech, Birmingham, AL) followed by washing and incubation with

321   substrate to develop color, and read at 405 nm. IgG subclasses and IgA of plasma Abs against

322   V1V2 fusion protein were detected using mouse mAbs against IgG subclasses and goat anti-

323   human IgA (Southern Biotech). For screenings of plasma against biotinylated peptides, the

324   peptides were immobilized at a concentration of 1 µg/mL onto streptavidin-coated plates

325   (StreptaWell plates, Roche). The 50% titers of plasma Abs were determined by measuring the

326   dilutions of plasma required for 50% maximal binding by linear regression (23). A positive

327   reaction by a plasma sample at 1:100 dilution was defined as an OD of the mean + 3 standard

328   deviations from the 6 plasma samples of healthy individuals from Cameroon, and then rounded

329   to 1 tens digit.

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330   **CD4$^+$ T cell counts.** The CD4$^+$ T cell counts were determined using the Guava EasyCD4

331   Test (Guava Technologies Inc, Hayward, CA) with 2-color direct and absolute counting of

332   number cells per µL (24).

333   **Viral load.** Viral load was determined using Abbott m2000 Real Time HIV-1 assays

334   according to the manufacturer's instructions (Abbott Molecular, Des Plaines, IL).

335   **Sequencing of the virus envelope.** Viruses in 500 µL plasma samples were concentrated

336   by centrifugation for 2 h, 14,000 rpm, at 15°C prior to RNA extraction. After removal of 360 µL

337   supernatant, the virus pellet was resuspended in the remaining 140 µL supernatant by vortexing,

338   and viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen Inc., Valencia, CA)

339   as per manufacturer's instructions. Reverse transcription and nested PCR were performed using

340   the SuperScript One-Step RT-PCR system with platinum *Taq* and Platinum PCR high-fidelity

341   SuperMix (Invitrogen, Carlsbad, CA), respectively, as per manufacturer's instructions, to isolate

342   a portion of *env* (gp120 + fragment of gp41), HXB2 region 6225-7817 (~1600 bp). PCR

343   products were cloned into the pCR4 TOPO cloning vector (Life Technologies, Carlsbad, CA)

344   and transformed into One Shot TOP10 competent *E. coli*. Positive clones were cultured in LB$_{Amp}$

345   medium, and plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen Inc).

346   Plasmids were sequenced for the insert portion using universal primers M13F/R (pCR4 TOPO).

347   Sequence analysis and alignment was performed using DNASTAR and Clustal W (25).

348   Consensus sequences were generated using the Los Alamos National Library program

349   ConsensusMaker.

350   **Statistical analysis.** The nonparametric Mann-Whitney-Wilcoxon test was used for

351   comparing OD, titers of plasma Abs, and composite indexes based on linear combinations of

352   individual characteristics or features (Table 3). Each individual feature or characteristic of the V2

16

353   region (e.g., isoelectric point, charge, number of glycosylation sites) has limited power to

354   distinguish the V2-deficient group from the V2-reactive group. However, composite indexes

355   based on simple difference or sum or linear combinations of more than 1 feature (e.g., No_AA-pI

356   in Table 3) can separate these groups. The relationship of plasma Ab binding to different

357   antigens was determined by Pearson correlation coefficient (r) with *P* values and by linear

358   regression. Statistical analysis and graphing of the data were generated using GraphPad Prism

359   version 7 (GraphPad Software, La Jolla, CA).

360

361

FIGURE LEGEND

Figure 1   Reactivity of plasma Abs against V2 antigens and control proteins. (A) All 79 plasma

samples from Cameroonian HIV-1 infected individuals were tested at 1:100 dilutions by ELISA

against proteins coated at 1 µg/mL. Antigens included: two V1V2-gp70 fusion proteins with V2

deleted (V1ΔV2) with sequences from A244 (clade AE) and CaseA2 (clade B); three V1V2

fusion proteins: $V1V2_{A244}$-gp70 (clade AE), $V1V2_{Case\ A2}$-gp70 (clade B) and $V1V2_{ZM109}$-1FD6

(clade C); five biotinylated cyclic V2 peptides with sequences from A244 (clade AE), TH023

(clade AE), Du422 (clade C), 230 and 200 (clade AG); one biotinylated cyclic $V3_{A244}$ peptide,

$gp120_{A244}$ protein and $gp41_{MN}$ protein. Percentage of plasma samples with specific anti-V2 Abs

is shown below each antigen. Statistical significance between Abs against V2 and control

18

385   antigens was determined by nonparametric Mann-Whitney test. The shape of the distribution is

386   shown by a box with the ends of the box representing the 5th and 95th percentile, and the median

387   marked by a horizontal line inside the box. (B) IgG subclasses and IgA of plasma Abs binding to

388     $V1V2_{A244}$-gp70 fusion protein.

389   **Figure 2** Cross-reactivity of plasma Abs with eight V2 antigens. (A) Eleven Cameroonian

390   plasma samples (out of 79) at dilution 1:100 tested by ELISA demonstrate binding to all eight

391   V2 antigens: V1V2A244-gp70 (clade AE), $V1V2_{CaseA2}$-gp70 (clade B), $V1V2_{ZM109}$-1FD6 (clade

392   C), and biotinylated cyclic $V2_{A244}$, $V2_{TH023}$, (clade AE), $V2_{Du422}$ (clade C), $V2_{230}$ and $V2_{200}$

393   (clade AG) peptides. (B) Eight plasma samples did not bind to V2 antigens tested with the

394   exception of three plasma samples which each bound weakly to one V2 peptide. All plasma

395   samples displayed reactivity with $gp120_{A244}$ and $gp41_{MN}$ (Fig.1). The dashed lines indicate the

396   cutoff (mean OD + 3xSD of normal human plasma).

397   **Figure 3** Titers of anti-V2 and control Abs in sequential plasma samples. (A) Six donors with

398   lack of anti-V2 Abs over 11 to 54 months of observation, and (B) six donors with cross-reactive

399   anti-V2 Abs over 28 to 60 months. The 50% titers of plasma Abs were determined by ELISA

400   against $V1V2_{A244}$-gp70 (clade AE) protein, biotinylated cyclic $V3_{A244}$ peptide, $gp120_{A244}$,

401     $gp41_{MN}$ (clade B), and $p24_{HXB2}$ (clade B) protein. The antigens were coated or immobilized onto

402   plate at 1 μg/mL; plasma were diluted by 10-fold dilutions starting from 1:100 to 1:1,000,000.

403   **Figure 4** Titers of plasma Abs from HIV-1 infected individuals against HIV-1 antigens, V1V2,

404   V3, gp120, gp41 and p24; two groups of plasma were compared: with non-V2-reactive (crossed

405   circles) and cross-reactive anti-V2 Abs (open circles). The 50% titers of plasma Abs were

406   determined by ELISA at five serial 10-fold dilutions starting from 1:100. Statistical significance

407   was determined by nonparametric Mann-Whitney test.

408   **Figure 5** Correlation between binding activities (OD) of plasma Abs against $V1V2_{A244}$-gp70

409   ($V1V2_{A244}$) and other Env antigens. Plasma binding activity to $V1V2_{A244}$ was correlated with: A)

410    $V1V2_{CaseA2}$-gp70; B) $V1V2_{ZM109}$-1FD6; C) $V2_{A244}$ peptide; D) $V2_{TH023}$ peptide; E) $V2_{Du422}$

411   peptide; F) $gp120_{A244}$; G) $gp41_{MN,}$ but not with H) $V3_{A244}$ peptide.   Correlation was determined

412   by Pearson correlation coefficient (r) and by linear regression with P values.

413

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518

TABLE 1 Infecting HIV clades and ELISA reactivity of plasma antibodies to Env antigens of patients with and without V2 antibodies

MDC donors	HIV-1	V1V2[1] A244 AE	V1V2[1] CaseA2 B	V1V2[1] ZM109 C	V2pept[2] A244 AE	V2pept[2] TH023 AE	V2pept[2] Du422 C	V2pept[2,3] 230 AG	V2pept[2,3] 200 AG	V3pept[2] A244 AE	gp120 A244 AE	gp41 MN B
001-4	CRF18_cpx	**3.2**	**3.1**	**2.0**	**3.5**	**3.5**	**3.5**	**3.5**	0.3	**3.6**	**3.9**	**2.9**
230-1	CRF02_AG	**3.6**	**3.5**	**2.4**	**3.6**	**2.9**	**3.5**	**3.5**	0.8	**3.5**	**3.2**	**3.4**
6501-6	CRF02_AG	**3.5**	**2.2**	**3.6**	**3.5**	**2.2**	**3.0**	**2.1**	1.3	**3.1**	**3.5**	**2.9**
237-2	CRF11_cpx	**2.4**	**3.3**	**2.2**	**3.6**	**1.9**	**1.4**	0.7	**1.6**	**3.6**	**4.0**	**3.2**
060-2	CRF36_cpx	**2.6**	**3.3**	**2.5**	**2.6**	**1.1**	**1.7**	**2.3**	0.8	**3.6**	**3.3**	**3.5**
221-2	CRF01_AE	**2.1**	**2.3**	**2.2**	**3.3**	**1.9**	**3.0**	**3.0**	0.4	**3.7**	**3.8**	**3.3**
014-2	C	0.4	0.5	0.5	0.4	**0.7**	0.3	0.4	0.2	**1.5**	**1.7**	**2.6**
019-3	CRF22_01A1	0.2	0.2	0.4	0.2	0.3	0.3	0.3	0.1	**3.8**	**1.8**	**3.0**
122-3	CRF01_AE	0.5	0.3	0.2	0.3	0.2	0.2	0.3	0.2	**3.6**	**3.9**	**3.5**
195-3	CRF37_cpx	0.4	0.2	0.2	**1.0**	0.2	0.5	0.1	0.1	**3.8**	**3.4**	**3.2**
200-2	CRF02_AG	0.1	0.2	0.3	0.3	0.3	0.5	0.3	**1.4**	**2.9**	**3.2**	**3.4**
211-1	CRF22_01A1	0.3	0.4	0.4	0.3	0.2	0.2	0.3	0.1	**3.0**	**3.0**	**3.3**

[1]V1V2 – V1V2 fusion protein, HIV-1 strain and subtype; [2]V2 and V3pept. – biotinylated cyclic peptides; [3]V2 peptides with sequences from virus of donor's plasma: MDC230-1 and MDC200-2 (this allows testing of two plasma Abs vs. autologous antigens). Plasma samples were screened by standard ELISA at 1:100 dilution against antigens coated at 1 µg/mL; biotinylated cyclic V2 and V3 peptides were immobilized at 1 µg/mL on the streptavidin coated plates. The values bold are above cutoff (mean OD of 6 control healthy plasma samples + 3 standard deviation).

TABLE 2 Titers of plasma Abs, CD4$^+$ T cells, and viral load in two groups of HIV-1 infected subjects with anti-V2 deficient and cross-reactive Abs

Features	V2 deficient[1] n=6	V2 cross-reactive[2] n=6	P value[3]
50% Titer[4]			
Anti-V1V2$_{A244}$	0.0	50,270	**<0.0001**
Anti-V3$_{A244}$	3,252	40,572	**<0.0001**
Anti-gp120$_{A244}$	2,438	70,241	**<0.0001**
Anti-gp41$_{MN}$	36,444	413,169	**<0.0001**
Anti-p24$_{HXB2}$	44,696	72,451	0.0781
CD4$^+$ T cells, VL			
CD4$^+$ T cell count	558	402	0.1426
Viral load (VL)	7,076	16,400	0.2780

[1]Panel of plasma with undetectable anti-V2 Abs; [2]Panel of plasma with cross-reactive anti-V2 Abs; [3]nonparametric Mann-Whitney test; [4]mean of 50% titers.

TABLE 3 Characteristics of the V2 and V1 regions in HIV-infected individual who were had V2-deficient antibody responses or cross-reactive antibody response

Features	V2 region		P[1]	V1 region		P[1]
	V2 Deficient Group	V2 Cross Group		V2 Deficient Group	V2 Cross Group	
	Median	(min, max)		Median	(min, max)	
# amino acids (AA)	43.5 (40, 58)	39.5 (37, 41)	**0.034**	23.5 (22, 32)	24.5 (21, 38)	0.572
# glycosylation sites	2 (2, 7)	2 (0, 2)	0.115	3 (2, 4)	2.5 (1, 4)	0.452
pI	8.47 (4.79, 9.66)	9.25 (6.52, 9.69)	0.108	4.89 (3.88, 9.37	4.37 (3.99, 9.58)	0.699
Charge	1 (-1, 3)	2.5 (0, 3)	0.161	-1 (-3, 2)	-1.5 (-4, 2)	0.807
α-helix K168-V172	1.78 (1.6, 1.9)	1.57 (1.13, 2)	0.376	nt	nt	nt
β-sheet E153-I184	-19.82 (-22, -16.3)	-20.32 (-22.97, -18.88)	0.699	nt	nt	nt
Composite Indexes*						
1. AA–pI	35.37 (31.98, 49.5)	30.89 (27.87, 31.75)	**0.005**	18.07 (13.63, 26.4)	18.74 (14.42, 34.0)	1.000
2. AA–Charge	42 (40, 56)	37.5 (35, 39)	**0.005**	24.5 (21, 33)	25.5 (22, 42)	0.748
3. AA–pI+ Glyc.	37.37 (33.98, 56.5)	32.39 (27.87, 33.75)	**0.005**	20.44 (16.63. 30.4)	21.74 (15.42, 38)	1.000
4. AA–pI– Glyc.	33.37 (29.98, 42.5)	29.39 (26.31, 29.75)	**0.005**	16.07 (10.63, 22.4)	15.74 (13.42, 30)	1.000
5. AA–Charge+Glyc.	44 (42, 63)	39 (35, 41)	**0.005**	27 (24, 37)	28.5 (23, 46)	0.936
6. AA–Charge-Glyc.	40 (38, 49)	36 (33, 37)	**0.005**	22.5 (18, 29)	22.5 (20, 38)	0.808
7. AA–pI+helix	37.2 (33.76, 51.4)	32.39 (29.44, 33.7)	**0.002**	nt	nt	nt
8. AA–Charge+helix	43.82 (41.6, 57.9)	38.99 (36.57, 40.13)	**0.002**	nt	nt	nt

[1] P value, V2 deficient versus cross-reactive group (non-paired tests), Wilcoxon test; nt – not tested.

*Composite indexes are based on difference or sum or linear combinations of more than one features; 1. # AA–pI; 2. # AA–Charge; 3. # AA–pI+ # glycosylation sites; 4. # AA–pI - # glycosylation sites; 5. # AA–Charge+# glycosylation sites;

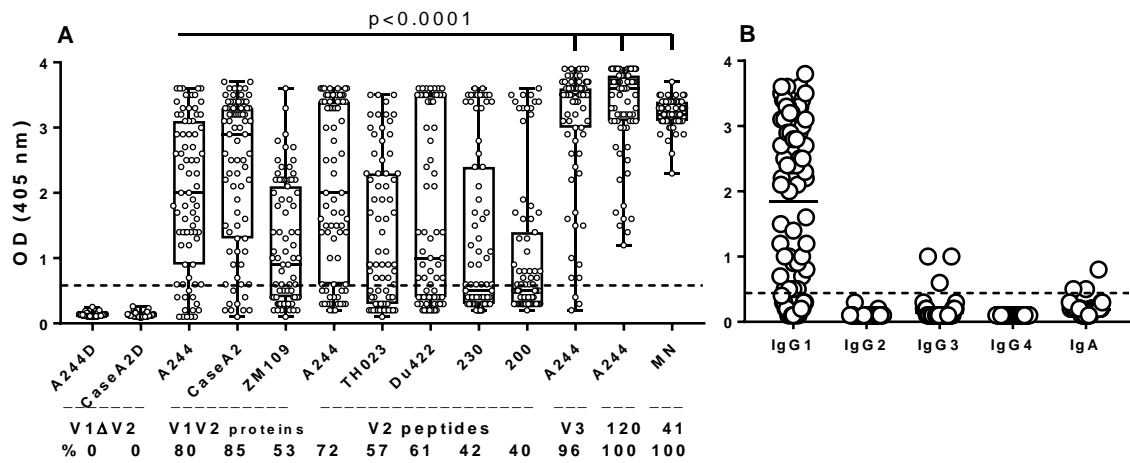6. # AA–Charge-# glycosylation sites; 7. # AA–pI+α-helix propensity; 8. # AA–Charge+α-helix propensity.
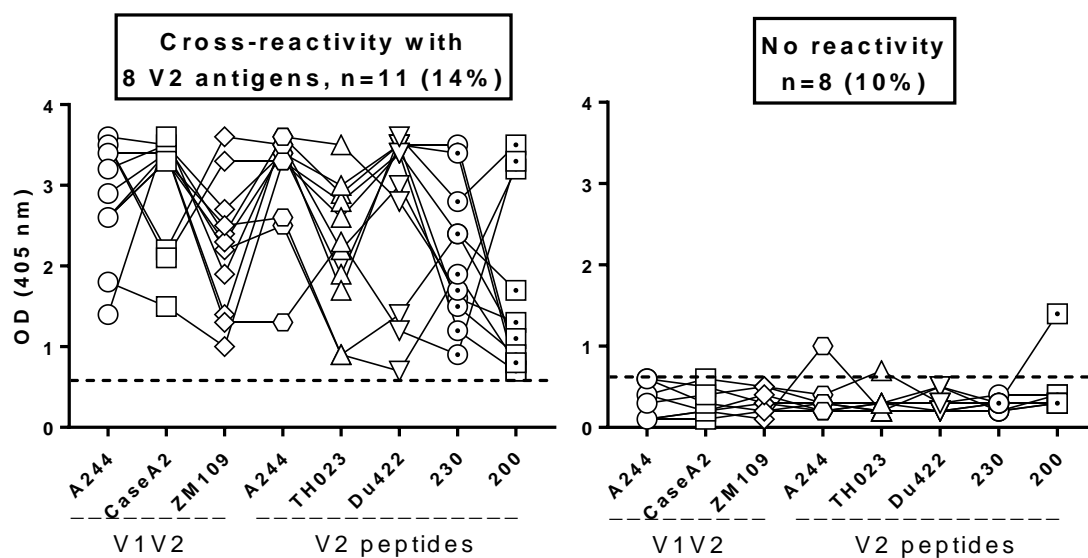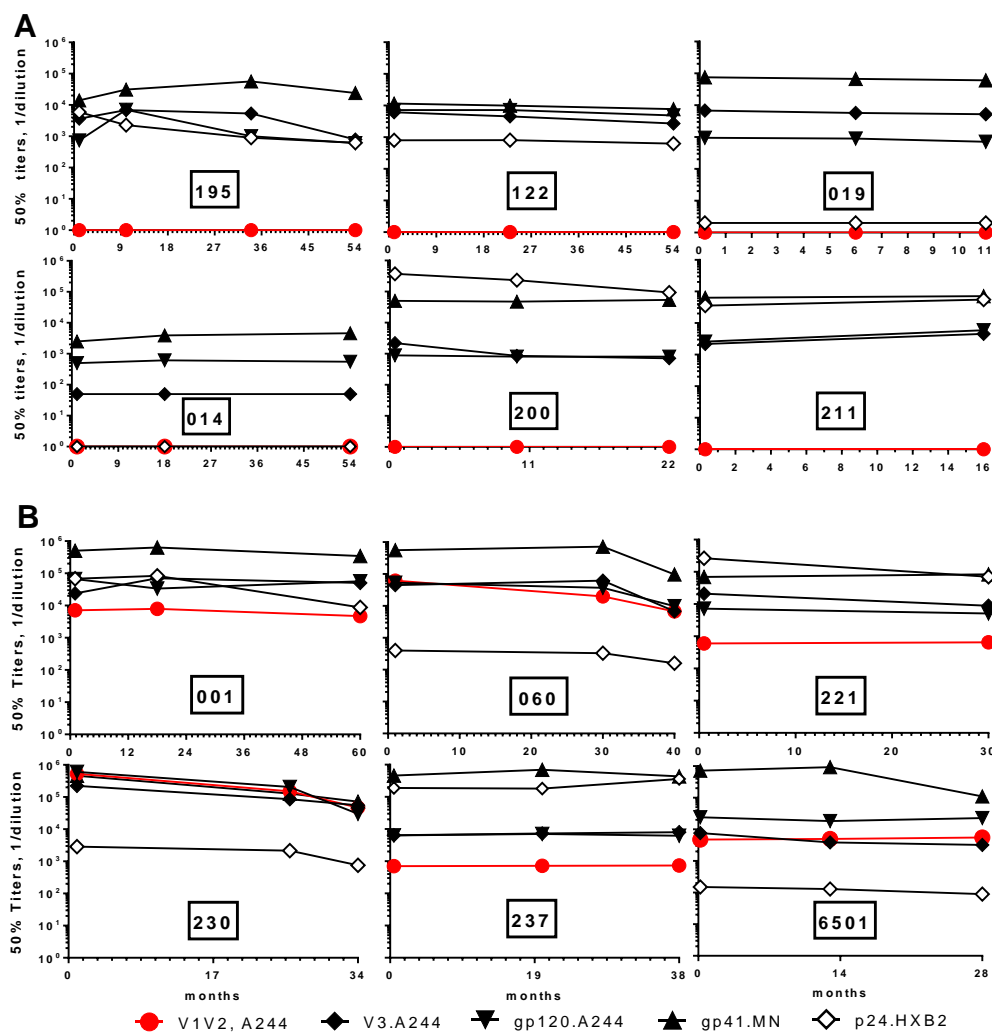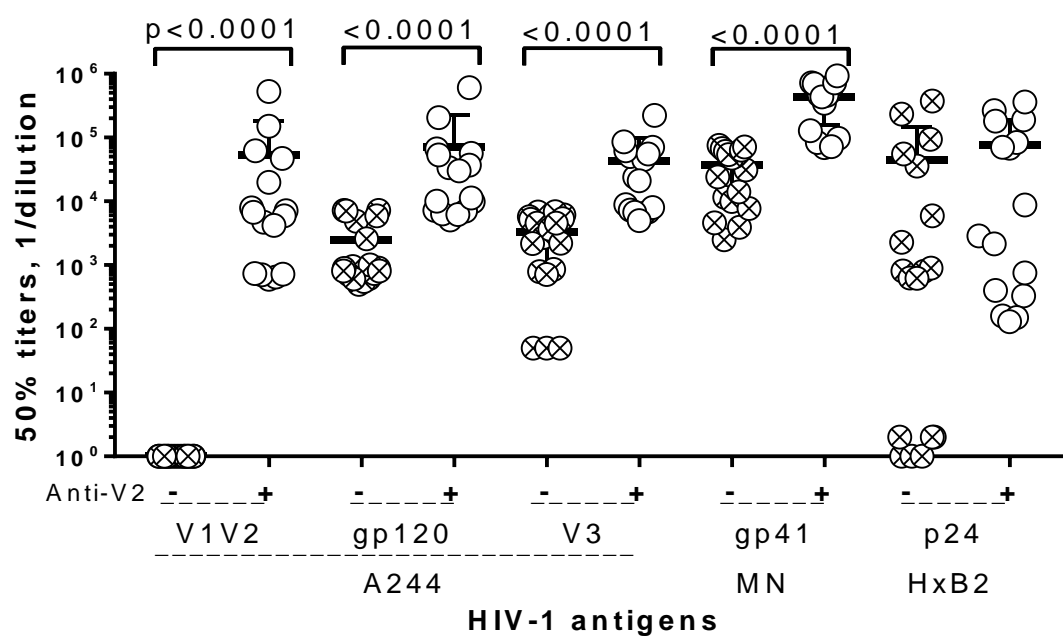
FIG 1

FIG 2

FIG 3

FIG 4

FIG 5

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