1 **Head-to-head comparison of poxvirus NYVAC and ALVAC vectors expressing**

2 **identical HIV-1 clade C immunogens in prime/boost combination with Env protein**

3 **in non-human primates**

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ABSTRACT

We have compared the HIV-1-specific cellular and humoral immune responses elicited in rhesus macaques immunized with two poxvirus vectors (NYVAC and ALVAC) expressing the same HIV-1 antigens from clade C, Env gp140 as a trimeric cell released protein and Gag-Pol-Nef as Gag-induced virus-like particles (VLPs) (referred as NYVAC-C and ALVAC-C). The immunization protocol consisted of two doses of the corresponding poxvirus vector plus two doses of a combination of the poxvirus vector and a purified HIV-1 gp120 protein from clade C. This immunogenicity profile was also compared to that elicited by vaccine regimens consisting of two doses of the ALVAC vector expressing HIV-1 antigens from clades B/E (ALVAC-vCP1521) plus two doses of a combination of ALVAC-vCP1521 and HIV-1 gp120 protein from clades B/E (similar to the RV144 trial regimen) or clade C. The results showed that immunization of macaques with NYVAC-C stimulated at different times more potent HIV-1-specific CD4$^+$ T-cell responses and induced a trend toward higher magnitude of HIV-1-specific CD8$^+$ T-cell immune responses than ALVAC-C. Furthermore, NYVAC-C induced a trend toward higher levels of binding IgG antibodies against clade C HIV-1 gp140, gp120 or MuLV gp70-scaffolded V1/V2 and toward best cross-clade binding IgG responses against HIV-1 gp140 from clades A, B and group M consensus, compared to ALVAC-C. Of the linear binding IgG responses most were directed against the V3 loop in all immunization groups. Additionally, NYVAC-C and ALVAC-C also induced similar levels of HIV-1 neutralizing antibodies and antibody-dependent cellular cytotoxicity (ADCC) responses. Interestingly, binding IgA antibodies against HIV-1 gp120 or MuLV gp70-scaffolded V1/V2 were absent or very low in all immunization groups. Overall, these results provide a comprehensive survey of the immunogenicity of NYVAC versus ALVAC expressing HIV-1 antigens in non-human primates and

66 indicate that NYVAC may represent an alternative candidate to ALVAC in the

67 development of a future HIV-1 vaccine.

68

69 **IMPORTANCE**

70 The finding of a safe and effective HIV/AIDS vaccine immunogen is one of the main

71 research priorities. Here, we have generated two poxvirus-based HIV vaccine

72 candidates (NYVAC and ALVAC vectors) expressing the same clade C HIV-1 antigens

73 in separate vectors and tested in non-human primates their immunogenicity profile. The

74 results showed that immunization with NYVAC-C induced a trend toward higher HIV-

75 1-specific cellular and humoral immune responses than those elicited by ALVAC-C,

76 indicating that this new NYVAC vector could be considered a novel optimized

77 HIV/AIDS vaccine candidate for human clinical trials.

78

INTRODUCTION

The development of a safe and effective HIV/AIDS vaccine that could prevent HIV-1 infection by inducing effective cellular and humoral immune responses is a key research priority. The Thai phase III HIV-1 vaccine clinical trial (RV144) tested a prime/boost combination of a recombinant poxvirus vector, ALVAC vCP1521 expressing HIV-1 antigens from clades B and E, combined with bivalent HIV-1 gp120 proteins from clades B and CRF01_AE, showing a 31.2% protection against HIV-1 infection in humans (1). This modest efficacy highlighted the poxvirus vector as an important player in these responses, promoting the generation and characterization of new optimized attenuated poxvirus vectors with improved immunogenicity as future HIV-1 vaccine candidates (2-5).

Among poxviruses, the highly attenuated vaccinia virus strain NYVAC (6) is a promising vector that has been broadly used in preclinical and clinical trials as a prototype vaccine against HIV-1, inducing a good immunogenicity profile in different animal models (mice and non-human primates) and in humans (2, 7). In particular, recombinant NYVAC vectors expressing HIV-1 Env, Gag, Pol and Nef antigens from clades B or C elicited strong, broad and polyfunctional T-cell immune responses in mice, non-human primates and humans, together with some levels of humoral responses against HIV-1 gp120 (8-23). An additional feature is that the current NYVAC vectors preferentially triggered CD4$^+$ T-cell responses (13, 14, 24, 25) in both humans and macaques, inferring immunologically the recruitment of stronger B-cell responses than ALVAC-based vectors. In an effort to enhance the magnitude and scope of T- and B-cell responses to HIV-1 antigens delivered by a poxvirus vector, we have recently reported the characterization of two novel attenuated NYVAC vectors expressing HIV-1 clade C trimeric soluble gp140 or Gag-Pol-Nef as a polyprotein processed into Gag-

104   derived VLPs, which triggered specific innate responses in human cells and elicited in

105   mice polyfunctional Env-specific CD4$^+$ and Gag-specific CD8$^+$ T-cell responses,

106   together with antibody responses against HIV-1 gp140 and p17/p24 (26). Furthermore,

107   DNA plasmids producing these improved immunogens lead to higher expression levels

108   and enhanced immunogenicity after DNA vaccination in mice (27) and after DNA

109   prime/NYVAC boost in non-human primates (Asbach B et al, submitted).

110   A comparison of the immunogenicity elicited by different poxvirus vectors expressing

111   the same HIV-1 antigens is of particular importance, as it may provide details of the

112   best-in-class vector to be advanced for future phase III human trials. To this end, in a

113   pre-clinical study in rhesus macaques, we have evaluated head to head, the HIV-1-

114   specific cellular and humoral immune responses elicited by NYVAC and ALVAC pox-

115   vectors, expressing identical clade C HIV-1 inserts, Env gp140 as a trimeric soluble

116   protein and Gag-Pol-Nef as a polyprotein processed into Gag-derived VLPs (referred as

117   NYVAC-C and ALVAC-C). NYVAC-C and ALVAC-C were administered using an

118   immunization protocol consisting of two priming doses of the corresponding

119   recombinant poxvirus vectors boosted with two doses of a combination of the poxvirus

120   vector and HIV-1 gp120 protein from clade C. Moreover, we also compared the

121   immunogenicity elicited by these two vaccine candidates with the one induced by the

122   same ALVAC vector used in the RV144 phase III clinical trial (ALVAC-vCP1251,

123   expressing HIV-1 antigens from clades B and E), and administered following two

124   priming doses of ALVAC-vCP1251 plus two boosts combining ALVAC-vCP1251 and

125   HIV-1 gp120 protein from clades C or B/E. The results showed that while the two

126   vectors triggered both T- and B-cell immune responses, NYVAC-C was more

127   immunogenic than ALVAC-C, inducing at different times higher HIV-1-specific CD4$^+$

128   T-cell responses, with a trend toward higher magnitude of HIV-1-specific CD8$^+$ T-cell

129 immune responses and a consistent trend toward higher antibody responses against

130 HIV-1 gp140, gp120 or MuLV gp70-scaffolded V1/V2. These results support the

131 further clinical development of NYVAC-C as a component HIV/AIDS vaccine

132 candidate.

133

134 **MATERIALS AND METHODS**

135 **Recombinant NYVAC and ALVAC vectors expressing HIV-1 antigens**

136 The recombinant NYVAC-C consists of two NYVAC vectors that express different

137 clade C HIV-1 antigens under the same synthetic early/late poxvirus promoter (28): one

138 (NYVAC-gp140) expressing Env gp140 from strain 96ZM651 and one (NYVAC-Gag-

139 Pol-Nef) expressing Gag from strain 96ZM651 and Pol/Nef from strain CN54, and their

140 generation and virological characteristics have been previously described (26). For

141 head-to-head comparison purposes, the recombinant ALVAC-C was generated and

142 consists of a combination of two ALVAC vectors expressing the same clade C HIV-1

143 antigens present in NYVAC-C (ALVAC-gp140 and ALVAC-Gag-Pol-Nef), and was

144 generated by Sanofi Pasteur. Briefly, Env gp140 or Gag-Pol-Nef HIV-1 genes were

145 inserted into the ALVAC C6 locus under the control of the synthetic early/late poxvirus

146 promoter (28). The ALVAC backbone of ALVAC–C vectors is the same that was used

147 to generate the recombinant ALVAC-vCP1521 vector and the HIV-1 antigens were

148 inserted in the same locus (C6). ALVAC product is licensed for veterinary use under the

149 name KANAPOX®. For the isolation of viral recombinants, 3 x 10^6 primary chicken

150 embryo fibroblast (CEF) cells were first infected with ALVAC parental virus at a

151 multiplicity of infection (MOI) of 10 plaque forming units (PFU)/cell and transfected 1

152 h later with 8 μg of linearized DNA (containing Env gp140 or Gag-Pol-Nef) using

153 lipofectamine 2000CD (Life Technologies), according to the manufacturer´s

154 recommendations. After 24 h of incubation, the cells were harvested in 1 ml of 2%

155 FBS-DMEM, sonicated and used for recombinant virus screening. Recombinant

156 ALVAC viruses containing gp140 or Gag-Pol-Nef genes were screened and purified by

157 plaque purification on primary CEF cells. After 4 consecutive rounds of plaque

158 purification, positive plaques were isolated and confirmed to be positive to the gp140 or

159 GPN DNA probe and negative to the ALVAC C6 open reading frame probe. The

160 resulting ALVAC-gp140 and ALVAC-Gag-Pol-Nef recombinant viruses were

161 expanded in primary CEF cells and the crude preparations obtained were used for the

162 propagation of both viruses in large cultures of CEF cells followed by virus purification

163 through two 36% (wt/vol) sucrose cushions and virus titrated. For simplicity of

164 terminology, we subsequently refer to the combined mixed inoculation of NYVAC-

165 gp140 + NYVAC-Gag-Pol-Nef as NYVAC-C and ALVAC-gp140 + ALVAC-Gag-Pol-

166 Nef is labeled as ALVAC-C. The recombinant ALVAC-vCP1521 expresses HIV-1

167 gp120 from clade E, transmembrane gp41 from clade B and Gag/Pro from clade B, and

168 was used previously in the RV144 phase III clinical trial (1).

169

170 **HIV-1 proteins**

171 In the immunizations performed in this study, two different HIV-1 gp120 proteins from

172 clades C or B/E were used. Bivalent gp120 protein contains a mixture of TV1 gp120

173 and 1086 gp120, both from clade C. These proteins were expressed from stably

174 transfected Chinese hamster ovary (CHO) cell lines, purified and characterized as

175 previously described (29). Bivalent AIDSVAX gp120 protein contains a mixture of

176 gp120 from clades B and CRF01_AE was used previously in the RV144 phase III

177 clinical trial (1), and was provided by Global Solutions for Infectious Diseases.

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Non-human primates

Animals used in this study (designated AUP513) were outbred adult male Indian rhesus macaques (Macaca mulatta) which were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). This study protocol was approved by the Institutional Animal Care and Use Committee of Advanced BioScience Laboratories in accordance with international guidelines. The age of the animals ranged between 2.5 and 2.9 years, with a mean of 2.6 years and the weight range was between 3.1 to 5.7 kg, with a mean of 3.8 kg. All rhesus macaques were negative for tuberculosis, simian retrovirus (SRV), simian T-cell leukemia virus (STLV-1), herpesvirus B, simian immunodeficiency virus (SIV), measles and poxvirus immunogens prior to the study, and have also negative fecal culture for salmonella, shigella, campylobacter and yersinia. Furthermore animals were immunologically naïve for the vaccine components.

Immunization schedule

Four immunizations groups of eight rhesus macaques were included in this study protocol (designated AUP513). Group 1 consisted of two immunizations with NYVAC-C (weeks 0 and 4) boosted with two immunizations of NYVAC-C plus bivalent gp120 proteins from clade C (TV1 + 1086 gp120) (weeks 12 and 24). Group 2 consisted of two immunizations with ALVAC-C (weeks 0 and 4) boosted with two immunizations of ALVAC-C plus bivalent gp120 proteins from clade C (TV1 + 1086 gp120) (weeks 12 and 24). Group 3 consisted of two immunizations with ALVAC-vCP1521 (weeks 0 and 4) boosted with two immunizations of ALVAC-vCP1521 plus bivalent gp120 proteins from clade C (TV1 + 1086 gp120) (weeks 12 and 24). Group 4 consisted of

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two immunizations with ALVAC-vCP1521 (weeks 0 and 4) boosted with two immunizations of ALVAC-vCP1521 plus bivalent gp120 from clades B/E (AIDSVAX gp120) (weeks 12 and 24). The pox-vector priming immunization was carried out at 0 and 4 weeks with the corresponding poxvirus vectors (NYVAC-C, ALVAC-C or ALVAC-vCP1251) and boosted at weeks 12 and 24 with the combination of poxvirus vector plus HIV-1 gp120 proteins (from clades C or B/E) (as depicted in Fig. 1A and 1B). All immunizations for the poxvirus vectors and proteins were given intramuscularly (i.m) in the deltoid muscle in the upper right arm for the poxvirus vectors and in the opposite site, upper left arm for the proteins. A dose of 1×10^8 PFU of each recombinant poxvirus vector (NYVAC-C, ALVAC-C or ALVAC-vCP1521; 2×10^8 PFU of total virus in 1.0 ml) and 50 μg of each HIV-1 gp120 protein (from clades C with adjuvant MF59 or B/E with adjuvant Alum; 100 μg of total protein in 1.0 ml) was used in each immunization. It should be pointed out that group 3 received the identical RV144 immunogen prime (ALVAC-vCP1521), but was boosted with ALVAC-vCP1521 and with the same bivalent clade C gp120 proteins as in groups 1 and 2. Moreover, as an immunological benchmark the immunization regimen used in group 4 was essentially homologous to the one used in the RV144 phase III clinical trial (1) differing in the vaccine dose [RV144 used a lower dose of ALVAC-vCP1521 ($>10^6$ cell culture infectious dose 50%) and higher dose of AIDSVAX B/E gp120 (300 μg of each protein)]. At weeks 0, 6, 14 and 26 (at the beginning of the study and two weeks after the second, third and fourth immunizations, respectively), peripheral blood mononuclear cells (PBMCs) and serum samples were obtained from each immunized animal and HIV-1-specific T-cellular and humoral immune responses were analyzed (Fig. 1B). Blood samples were processed following current procedures (30).

Intracellular cytokine staining (ICS) assay

The HIV-1-specific CD4$^+$ and CD8$^+$ T-cell immune responses induced at weeks 6, 14 and 26 were analyzed by polychromatic ICS from PBMCs obtained from each immunized rhesus monkey, as previously described (30). In short, cryopreserved PBMCs were thawed and rested overnight in R10 [RPMI 1640 (BioWhittaker, Walkersville, MD), 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin] with 50 U/ml Benzonase (Novagen, Madison, WI) in a 37ºC/5% CO_2 incubator. The following morning, cells were stimulated with the corresponding HIV-1 Env, Gag, Pol and Nef peptide pools (2 μg/ml) in the presence of GolgiPlug (10 μg/ml; BD Biosciences, San Jose, California) for 6 h. Negative controls received an equal concentration of DMSO instead of peptides. Subsequently, ICS was performed as described (30). The following monoclonal antibodies were used: CD4-BV421 (clone OKT4; BioLegend), CD8-BV570 (clone RPA-T8; BioLegend), CD69-ECD (clone TP1.55.3; Beckman Coulter), CD3-Cy7APC (clone SP34.2; BD Biosciences), IFN-γ-APC (clone B27; BD Biosciences), IL-2-PE (clone MQ1-17H12; BD Biosciences) and TNF-α-FITC (clone Mab11; BD Biosciences). Aqua LIVE/DEAD kit (Invitrogen, Carlsbad, CA) was used to exclude dead cells. All antibodies were previously titrated to determine the optimal concentration. Samples were acquired on an LSR II flow cytometer and analyzed using FlowJo version 9.8 (Treestar, Inc., Ashland, OR).

Peptides

Overlapping peptides (15-mers with 11 amino acids overlapping) spanning the Env, Gag, Pol and Nef HIV-1 clade C regions were matched to the inserts expressed by NYVAC-C and ALVAC-C. Peptides used in the ICS were grouped in nine peptide

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253 pools (Env-1, Env-2, Env-3, Pol-1, Pol-2, Gag-1, Gag/Pol, Gag-2/Pol and Nef), with

254 about 60 peptides per pool.

255

256 **HIV-1-specific binding antibody assay**

257 HIV-1-specific binding antibodies were measured by Binding Antibody Multiplex

258 Assay (BAMA) for total IgG and IgA antibodies in sera from each immunized rhesus

259 monkey at weeks 0, 6, 14 and 26, as previously described (31, 32). Antigens used to

260 analyze the total IgG or IgA binding antibodies included multiple HIV-1 clades: clade C

261 gp120 TV1 and clade C gp120 1086 (provided by Novartis Vaccines), recombinant

262 gp140 consensus from various subtypes [clade A 00MSA4076 gp140 (gp140. a1Con),

263 clade B JRFL gp140 (gp140. bCon), clade C gp140 (gp140. cCon) and group M

264 consensus (gp140. sCon)], murine leukemia virus (MuLV) gp70-scaffolded V1V2

265 (from clade C) and 1086 V1/V2 tags [all provided by Drs. H.-X. Liao and B. F. Haynes,

266 Duke University, as previously described (33)]. Different plasma serial dilutions were

267 made and results are expressed as mean fluorescent intensity (MFI) and titer [The area

268 under curve (AUC)]. Furthermore, rectal mucosal IgG binding responses were measured

269 and the specific activity was calculated by dividing the antibody titer by the total IgG

270 concentration, as previously described (34). Positivity criteria were values 3-fold over

271 the baseline visit and the cutoff was established using serum-negative samples. All

272 assays were run under Good Clinical Laboratory Practices (GCLP)-compliant

273 conditions.

274

275 **Linear peptide microarray assay**

276 Serum from a subset of immunized animals with strong binding IgG antibodies were

277 selected to further evaluate linear epitope specificities by linear peptide microarray,

278 using an Env peptide library containing 15-mer peptides, overlapping by 12 amino

279 acids, against HIV-1 Env gp160 of consensus clades A, B, C, D, group M, CRF01 and

280 CRF02, as previously described (35, 36).

281

282 **Antibody-dependent cellular cytotoxicity (ADCC) assay**

283 ADCC activity was detected according to the ADCC-GranToxiLux (GTL) procedure, as

284 previously described (37, 38). The results of the GTL assay were considered positive if

285 % Granzyme B activity after background subtraction was ≥8% for the infected target

286 cells as determined during the standardization of our assay (39). The $\log_{10}$ titer of the

287 ADCC antibodies present in the plasma was calculated by interpolating the $\log_{10}$

288 reciprocal of the last plasma dilution that yielded positive % Granzyme B activity

289 (≥8%). The GTL-ADCC assay was performed under GCLP-compliant guidelines.

290

291 **Neutralizing antibodies against HIV-1**

292 Neutralizing antibodies against HIV-1 were measured in TZM-bl cells, as previously

293 described (40). Briefly, a pre-titrated dose of different HIV-1 virus (clade B tier 1 HIV-

294 1 strain MN.3, clade C tier 1 HIV-1 strain MW965.26 and clade CRF01_AE tier 1 HIV-

295 1 strain TH023.6) was incubated with serial 3-fold dilutions of test sample in duplicate

296 in a total volume of 150 µl for 1 h at 37°C in 96-well flat-bottom culture plates. Freshly

297 trypsinized cells (10,000 cells in 100 µl of growth medium containing 75 µg/ml DEAE

298 dextran) were added to each well. One set of control wells received cells plus virus

299 (virus control) and another set received cells only (background control). After 48 h of

300 incubation, 100 µl of cells was transferred to a 96-well black solid plate (Costar) for

301 measurements of luminescence using the Britelite Luminescence Reporter Gene Assay

302 System (PerkinElmer Life Sciences). Neutralization titers are the serum dilution at

303 which relative luminescence units (RLU) were reduced by 50% compared to virus

304 control wells after subtraction of background RLUs in cell control wells. Assay stocks

305 of molecularly cloned Env-pseudotyped viruses were prepared by transfection in

306 293T/17 cells (American Type Culture Collection) and titrated in TZM-bl cells as

307 previously described (40). Additional information on the assay and all supporting

308 protocols may be found at: http://www.hiv.lanl.gov/content/nab-reference-

309 strains/html/home.htm. The assay was done under GCLP-compliant conditions.

310

311 **Statistical procedures**

312 The Wilcoxon Rank Sum test (when comparing two groups) and the Kruskal Wallis test

313 (when comparing more than two groups) were used at each time point to test the null

314 hypothesis that the groups have the same median response. All values used for

315 analyzing proportionate representation of responses are background-subtracted. Box

316 plots were used to summarize the distribution of various immune responses, where the

317 mid-line of the box indicates the median, and the ends of the box denote the 25th and

318 75th percentiles, with whiskers extended to the extreme data points that are no more

319 than 1.5 times the interquartile range (IQR) or, if no values meet this criterion, to the

320 data extremes. When there are both positive and negative responses, values showed in

321 the box plots refer to the positive responses.

322

323 **RESULTS**

324 **Immunogenicity in non-human primates immunized with NYVAC and ALVAC**

325 **vectors**

326 The recombinant poxvirus vector ALVAC expressing HIV-1 antigens provided a

327 modest level of efficacy in a phase III clinical trial in humans (1), highlighting that new

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optimized poxvirus vectors are needed for improved efficacy. Thus, to develop

HIV/AIDS vaccine candidates that could enhance the HIV-1-specific immunogenicity

and efficacy, we have generated two new recombinant NYVAC and ALVAC poxvirus

immunogens expressing in separate vectors the same Env or Gag and Pol/Nef HIV-1

antigens from clade C (termed NYVAC-C and ALVAC-C, respectively). The novelty of

these vectors is the expression of codon-optimized HIV-1 clade C gp140 (ZM96) as a

cell released protein trimer and VLPs of Gag(ZM96) together with Pol-Nef(CN54).

Here, we analyzed the HIV-1-specific T-cell and humoral immune responses induced in

non-human primates by four different groups of immunized animals (8 animals/group).

The immunization protocols were designed to compare head-to-head NYVAC and

ALVAC poxvirus vectors expressing the same HIV-1 antigens in homologous

combination and together with a HIV-1 protein component (gp120) as a booster in order

to determine whether they induced distinct HIV-1-specific T-cell and antibody immune

responses (Fig. 1). Figure 1A summarizes the 4 different immunization groups included

in the study (see also Materials and Methods for details).

As these protocols aimed to trigger both HIV-1-specific T-cell and B-cell responses,

with preferential antibody responses to Env, a comprehensive analysis with

standardized and validated humoral and T-cell assays was performed on serum and

PBMC samples collected at weeks 0, 6, 14 and 26 (at the beginning of the study and

two weeks after the second, third and fourth immunizations, respectively) (Fig. 1B).

NYVAC-C elicited higher magnitude of HIV-1-specific CD4$^+$ T-cell immune

responses and a trend toward higher HIV-1-specific CD8$^+$ T-cell immune

responses than ALVAC-C

352 We measured the HIV-1-specific $CD4^+$ and $CD8^+$ T-cell immune responses elicited by

353 the different immunization groups by multiparameter flow cytometry using ICS, after

354 the stimulation of PBMCs obtained from each immunized rhesus monkey at weeks 6,

355 14 and 26 with pools of peptides that spanned the HIV-1 Env, Gag, Pol and Nef clade C

356 regions present in the inserts expressed by NYVAC-C and ALVAC-C. HIV-1-specific

357 $CD4^+$ and $CD8^+$ T-cell immune responses were determined based on the frequency of

358 IFN-γ and/or TNF-α and/or IL-2 producing cells obtained for Env, Gag, Pol and Nef

359 peptide pools. For each T-cell subset, the response was considered positive if the value

360 in the stimulated samples was greater than previously defined thresholds (41).

361 Moreover, the ICS protocol used was defined previously in ICS qualification

362 experiments (41).

363 The magnitude of the total HIV-1-specific $CD4^+$ T-cell immune responses induced at

364 week 14 by the immunization group N2NP2 (C) was significantly higher than that

365 elicited by the immunization group A2AP2 (C) ($p<0.05$) or by groups A2AP2 (B/E, C)

366 and A2AP2 (B/E, AIDSVAX), respectively (Fig. 2A). At week 6 (two weeks after the

367 two priming immunizations) there were no differences between the immunization

368 groups. However, two weeks following the booster immunizations (week 26),

369 immunization with N2NP2 (C) induced higher HIV-1-specific total $CD4^+$ T-cell

370 immune responses, but this trend was not statistically significant.

371 On the other hand, at weeks 14 and 26 immunization with N2NP2 (C) elicited a trend

372 toward greater magnitude of HIV-1-specific $CD8^+$ T-cell immune responses than the

373 other immunization groups (Fig. 2B), but the differences were not significant.

374 Notably, comparison of cytokine responses generated by N2NP2 (C) versus A2AP2 (C)

375 revealed that at week 14 immunization with N2NP2 (C) induced a significantly higher

376 magnitude of HIV-1-specific $CD4^+$ T cells producing any cytokine (IFN-γ and/or TNF-

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377 α and/or IL-2) (Fig. 3A) or only IFN-γ (Fig. 3B), TNF-α (Fig. 3C) or IL-2 (Fig. 3D)

378 ($p < 0.05$).

379 In summary, these results showed that immunization with NYVAC-C elicited higher

380 HIV-1-specific CD4$^+$ T-cell immune responses than ALVAC-C, and a trend toward

381 higher CD8$^+$ T-cell immune responses, particularly after a single booster immunization.

382

383 **NYVAC-C induced a trend toward increased levels of binding IgG antibodies**

384 **against clade C HIV-1 gp140, gp120 and MuLV gp70-scaffolded V1/V2 proteins**

385 **compared to ALVAC-C**

386 The RV144 phase III clinical trial showed that IgG antibodies against V1/V2 and V3

387 regions of HIV-1 gp120 correlated with decreased risk of HIV-1 infection (31, 33, 42-

388 44). Thus, we analyzed the HIV-1-specific humoral immune responses elicited after

389 immunization with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) or A2AP2 (B/E,

390 AIDSVAX), quantifying in individual serum samples obtained from each immunized

391 rhesus monkey at weeks -1, 6, 14 and 26 the total binding IgG antibody levels against

392 clade C HIV-1 gp140, gp120 and MuLV gp70-scaffolded V1/V2 proteins (Fig. 4).

393 At week 6, immunization with N2NP2 (C) significantly enhanced the levels of binding

394 IgG antibodies against clade C HIV-1 gp140 consensus (Fig. 4A), gp120 from isolate

395 1086 (Fig. 4B) and gp120 from isolate TV1 (Fig. 4C), compared to immunization with

396 A2AP2 (C), A2AP2 (B/E, C) or with A2AP2 (B/E, AIDSVAX) from which binding

397 IgG antibodies were either rarely present or of lower magnitude. Furthermore, at week 6

398 immunization with N2NP2 (C) elicited a higher rate of responders than any A2AP2

399 immunization regimen (Fig. 4A to 4D). Moreover, at week 14 immunization with

400 N2NP2 (C) significantly enhanced the levels of binding IgG antibodies against clade C

401 HIV-1 gp140 consensus (Fig. 4A) and gp120 from isolate 1086 (Fig. 4B), compared to

17

402 immunization with A2AP2 (C), A2AP2 (B/E, C) or with A2AP2 (B/E, AIDSVAX).

403 Additionally, at late timepoints (week 26) immunization with N2NP2 (C) slightly

404 enhanced the levels of binding IgG antibodies against the HIV-1 gp140 consensus (Fig.

405 4A), gp120 from isolate 1086 (Fig. 4B), gp120 from isolate TV1 (Fig. 4C) and MuLV

406 gp70-scaffolded V1/V2 proteins (Fig. 4D), compared to immunization with any A2AP2

407 immunization regimen, but differences were not significant.

408 Moreover, rectal IgG binding responses against group M HIV-1 gp140 consensus (Fig.

409 5A), gp120 from clade C isolate 1086 (Fig. 5B), gp120 from clade C isolate TV1 (Fig.

410 5C), and MuLV gp70-scaffolded V1/V2 proteins (Fig. 5D) were also detected in all

411 immunization groups, only at weeks 14 and 26 (Fig. 5). The rate of responders against

412 group M and clade C HIV-1 gp140 consensus at week 26 was higher in the NYVAC-C

413 immunization group compared to ALVAC-C (80% versus 57% for both antigens) (Fig

414 5A and data not shown).

415 In summary, these results showed that immunization with NYVAC-C generated a trend

416 towards higher binding IgG antibodies against clade C HIV-1 gp140, gp120 and MuLV

417 gp70-scaffolded V1/V2 proteins as compared to ALVAC-C.

418

419 **NYVAC-C induced a trend toward higher levels of cross-clade binding IgG**

420 **antibodies against HIV-1 gp140 from clades A, B and group M consensus than**

421 **ALVAC-C**

422 Next, we analyzed the ability of the immunizations with N2NP2 (C), A2AP2 (C),

423 A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX) to induce cross-clade antibodies against

424 HIV-1 gp140, quantifying in individual serum samples obtained from each immunized

425 rhesus monkey at weeks -1, 6, 14 and 26 the total binding IgG antibody levels against

426 HIV-1 gp140 from clades A, B and group M consensus (Fig. 6).

427 Similar to the IgG binding antibody responses against clade C HIV-1 Env, at week 6

428 animals immunized with N2NP2 (C) produced significantly enhanced levels of cross-

429 clade binding IgG antibodies against HIV-1 gp140 from clades A (Fig. 6A), B (Fig. 6B)

430 and group M consensus (Fig. 6C) compared to immunization with A2AP2 (C) or with

431 A2AP2 (B/E, C), where cross-clade binding IgG antibodies were either non-existent

432 (against clade B) or of lower magnitude (against clade A and group M consensus).

433 Furthermore, at week 6 immunization with N2NP2 (C) elicited a higher rate of

434 responders than A2AP2 (Fig. 6A to 6D). Nonetheless, the results at late time points

435 (week 26) showed that immunization with N2NP2 (C) and A2AP2 (C) induced similar

436 levels of binding IgG antibodies against HIV-1 gp140 from clades A (Fig. 6A), B (Fig.

437 6B) and group M (Fig. 6C).

438 In summary, these results showed that during the priming phase immunization

439 NYVAC-C induced higher levels of cross-clade binding IgG antibodies against HIV-1

440 gp140 from clades A, B and group M consensus than ALVAC-C, and boosting with

441 either vector plus protein induced similar levels of cross-clade binding IgG antibodies

442 against HIV-1 gp140 from clades A, B and group M consensus.

443

444 **NYVAC-C and ALVAC-C induced IgG antibodies mainly directed against the V3**

445 **loop**

446 The induction of plasma IgG antibodies to linear epitopes in the V2 and V3 regions of

447 HIV-1 gp120 correlates with a reduced risk of infection in the RV144 phase III clinical

448 trial (43). Thus, we next selected a subset of animals that developed strong binding IgG

449 antibodies [belonging to the N2NP2 (C), A2AP2 (C) and A2AP2 (B/E, AIDSVAX)

450 immunization groups] to evaluate linear epitope specificities by peptide microarray

451 against Env gp160 of consensus clades A, B, C, D, group M, CRF01 and CRF02. The

results showed that V3-response dominated the binding response in most animals, consisting on average 41% of total gp160 binding, followed by C5 (24%) and C1-V1 (11%) epitopes (Figure 7A and 7B). Other linear epitope specific responses were detected to C1.1, C1.2, C2 and V2 epitopes, but with lower magnitude binding.

NYVAC-C and ALVAC-C induced similar levels of ADCC responses against HIV-1 gp120

It has been suggested that ADCC responses are linked with a reduced risk of infection in the RV144 phase III clinical trial (33). Moreover, antibodies with potent ADCC activity have been isolated from some RV144 vaccinees (45). Thus, we analyzed the ability of the immunizations with N2NP2 (C), A2AP2 (C), A2AP2 (B/E,C) and A2AP2 (B/E, AIDSVAX) to induce ADCC responses against clade C HIV-1 gp120 from isolate TV1 in individual plasma samples obtained from each immunized rhesus monkey at weeks 0 and 26 (Fig. 8). The results showed that immunization with N2NP2 (C) and A2AP2 (C) induced similar levels of ADCC responses (Fig. 8).

NYVAC-C and ALVAC-C induced similar levels of neutralizing antibodies against HIV-1

Broad neutralizing antibodies are a highly desired feature of an HIV-1 vaccine response (46). We analyzed the neutralizing antibody responses to HIV-1 induced in macaques immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E,C) and A2AP2 (B/E, AIDSVAX) in individual serum samples obtained from each immunized rhesus monkey at weeks -1, 6, 14 and 26 (Fig. 9).

Neutralizing antibody responses were observed predominantly against HIV-1 Tier 1 viruses, with no differences between NYVAC-C and ALVAC-C immunization groups,

using the TZM.BL neutralization assay (Fig. 9). Similar results were obtained using the A3R5.7 neutralization assay (data not shown). Of note, immunization with N2NP2 (C) and A2AP2 (C) performed better neutralization against HIV-1 clade C virus isolates (strain MW965.26), whereas immunization with A2AP2 (B/E, AIDSVAX) elicited a better neutralization against HIV-1 clade B virus isolates (MN-3). Moreover, the kinetics of the neutralization response showed that the higher levels of neutralizing antibodies and the higher rate of responders were elicited at week 26 in all the immunization groups. Interestingly, at week 14 immunization with N2NP2 (C) elicited a higher rate of responders than A2AP2 (C) when analyzing the neutralization against clade C strain MW965.26 and clade AE strain TH023.6.

In summary, these results showed that immunization with NYVAC-C and ALVAC-C induced similar levels of neutralizing antibodies against HIV-1, but NYVAC-C induced a higher rate of responders particularly after a single booster immunization.

NYVAC-C and ALVAC-C induced low or absent binding IgA antibodies against HIV-1 gp120 and MuLV gp70-scaffolded V1/V2 proteins

The RV144 phase III clinical trial showed that high levels of binding plasma IgA antibodies to HIV-1 Env correlated directly with increased risk rate of infection (33, 47). Thus, we next analyzed the binding IgA antibodies elicited after immunization with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX), quantifying in individual serum samples obtained from each immunized rhesus monkey at weeks 0 and 26 the total binding IgA antibody levels against HIV-1 gp120 and MuLV gp70-scaffolded V1/V2 proteins (both from clade C) (Fig. 10). Results revealed that immunization with N2NP2 (C) and A2AP2 (C) induced similar low or absent levels of binding IgA antibodies against MuLV gp70-scaffolded V1/V2 (Fig. 10A) or HIV-1

502   gp120 from isolate 1086 (Fig. 10B). Besides the results presented in Figure 10, we have

503   also analyzed the IgA binding antibodies against multiple HIV-1 clades: clade C gp120

504   TV1, and recombinant gp140 consensus from various subtypes (clades A 00MSA4076

505   and A1.con.env03 gp140, clades B JRFL and B.con.env03 gp140, clade C.con.env03

506   gp140 and group M consensus). The results with the different gp120/gp140 isolates

507   showed that no IgA antibodies against the clades A, B, C and group M analysed were

508   induced in the four immunization groups (data not shown). In summary, these results

509   showed that immunization with NYVAC-C and ALVAC-C induced very low levels of

510   binding IgA antibodies against HIV-1 gp120 and specifically MuLV gp70-scaffolded

511   V1/V2 proteins.

512

DISCUSSION

In 2009, the RV144 phase III clinical trial in 16,000 volunteers at risk of infection in Thailand showed for the first time that an effective HIV/AIDS vaccine could potentially be developed. Immunization with a combination of a recombinant canarypoxvirus vector (ALVAC) expressing HIV-1 antigens from clade E (gp120) and clade B (Gag/Pro) and bivalent HIV-1 gp120 proteins from clades B/E, showed a 31.2% protection against HIV-1 infection in humans (1). There was limited immunogenicity for what was experimentally measured (T-cell and antibody responses) and the efficacy obtained in this clinical trial was considered modest. Nonetheless, this study highlighted the importance of recombinant poxvirus vectors as components of HIV/AIDS vaccine candidates.

Several recombinant poxvirus vectors (including MVA, NYVAC, canarypox and fowlpox) expressing different HIV-1 antigens have been broadly used in several human clinical trials, proving that they are safe and immunogenic, inducing HIV-1-specific cellular and humoral immune responses [reviews in (2-4, 48)]. However, improved immunogens based on optimized poxvirus vectors able to enhance the cellular and humoral immune responses against HIV-1 antigens are needed. Examples include enhancing replication capacity of the vector, co-expression of immunomodulators, heterologous prime/boost approaches and removal of poxviral genes antagonizing host cell-mediated immune responses [reviews in (2, 5)].

Here, we asked whether improved poxvirus vector immunogens can be produced that elicit more broadly reactive T- and B-cell immune responses to HIV-1 antigens. This was examined using a similar prime/boost immunization regimen as in the RV144 trial comparing head-to-head in immunized rhesus macaques the T-cellular and humoral immune responses against HIV-1 antigens triggered by the two poxvirus vectors

23

538 (ALVAC-C and NYVAC-C) expressing identical and optimized clade C trimeric gp140

539 and Gag-Pol-Nef as Gag-derived VLPs. Furthermore, this was bench-marked by

540 comparison of the immune responses elicited by both vectors to the same immunogens

541 and vaccination protocol as in the RV144 trial to define cross-clade responses.

542 In all immunization groups, HIV-1-specific CD4$^+$ and CD8$^+$ T cells were generated, but

543 interestingly, compared to ALVAC-C, NYVAC-C significantly enhanced post

544 immunization the HIV-1-specific CD4$^+$ T-cell immune responses and elicited a trend

545 toward higher CD8$^+$ T-cell immune responses. Furthermore, NYVAC-C significantly

546 enhanced the magnitude of HIV-1-specific CD4$^+$ T cells producing IFN-γ and/or TNF-α

547 and/or IL-2.

548 With regard to the humoral immune responses, priming with NYVAC-C resulted in

549 increased magnitude and frequency of clade C Env-specific binding IgG antibodies,

550 with a trend toward higher levels after boosting with protein. In addition, peptide

551 mapping to gp120 indicate that the most frequent linear IgG antibody response to

552 specific linear epitopes was directed against the V3 loop in all animal groups, with

553 reactivity also directed against different protein domains including V2, with animals

554 immunized with NYVAC-C having the highest frequency of antibodies with these

555 specificities. Comparison of cross-clade binding IgG antibodies against HIV-1 gp140

556 from clades A, B and group M consensus showed that NYVAC-C induced higher levels

557 during the priming phase and similar levels after boosting, compared to ALVAC-C. It

558 should be pointed out that after priming, immunization with NYVAC-C and ALVAC-C

559 gave better antibody responses to clade B gp140 than the immunization with clade B

560 immunogens [A2AP2 (B/E, AIDSVAX)]. This could be due to the nature of the

561 adjuvant, even though both adjuvants (MF59 and Alum) potentially augment the

562 immune response through a common mechanism inducing a similar pattern of

24

563   phenotypical and chemokine responses in monocytes (49). Furthermore, immunization

564   with A2AP2 (B/E, AIDSVAX) also induced good cross-clade antibody responses

565   against clade C HIV-1 gp140, gp120 antigens. Moreover, rectal binding IgG antibody

566   levels against HIV-1 gp140 group M consensus (sCon), gp120 from isolate 1086, gp120

567   from isolate TV1 and MuLV gp70-scaffolded V1/V2 proteins induced by the different

568   immunization groups were comparable. Furthermore, NYVAC-C and ALVAC-C

569   elicited comparable levels of ADCC responses, of neutralizing antibodies and similar

570   low levels of binding IgA antibodies. Although out of 8 animals only one showed high

571   values of IgA against gp70 V1/V2 and clade C gp120 1086 in the N2NP2(C) group,

572   however this macaque induced low levels of IgA antibodies against clades A, B, C and

573   group M. Also the group of animals that got the vaccine similar to the RV144 trial had

574   low IgA antibody responses. These observations clearly showed that these protocols

575   trigger low IgA responses. The induction by NYVAC-C of a trend toward higher levels

576   of binding IgG antibodies against HIV-1 gp140, gp120 and MuLV gp70-scaffolded

577   V1/V2 and low levels of binding IgA antibodies against Env is particularly important

578   since antibody responses against V1/V2 loops of HIV-1 gp120 correlated with lower

579   infection risk in RV144, whereas higher plasma levels of Env-specific IgA were

580   correlated with a lack of protection (33). These improvements in NYVAC-C were likely

581   attributed to the higher magnitude of HIV-1-specific CD4$^+$ T helper cells induced in the

582   NYVAC-C immunization group. Thus, the immunological profiles elicited by NYVAC-

583   C and ALVAC-C are compatible with possible protective mechanisms against HIV-1,

584   including induction of HIV-1-specific CD4$^+$ and CD8$^+$ T-cell immune responses (50-

585   54) and high levels of IgG antibodies directed against HIV-1 gp120 and MuLV gp70-

586   scaffolded V1/V2, together with low levels of IgA antibodies against HIV-1 gp120 and

MuLV gp70-scaffolded V1/V2 (33). Challenge studies in non-human primates immunized with NYVAC-C and ALVAC-C may help define the best-in-class vector.

The differences in the immune responses between the two poxvirus vectors are likely related to the nature of the pox vector, as the viral genomes of NYVAC and ALVAC differ in content of immunomodulatory genes, use of promoters or insertion sites of the HIV genes. In fact, it has been recently described that ALVAC induces distinct cytokine responses compared to NYVAC in rhesus macaques (55), a difference that can influence the HIV-1-specific T-cell and humoral immune responses elicited by the recombinant ALVAC-C versus NYVAC-C.

A head-to-head comparison of NYVAC and ALVAC vectors expressing Gag-Pol-Env from SIV has been undertaken, but in SIVmac251-infected rhesus macaques treated with antiretroviral therapy (ART). These findings demonstrated that both vectors were immunogenic, inducing similar virus-specific $CD8^{+}$ T-cell responses and comparable lymphoproliferative responses to the SIV p27 Gag and gp120 Env proteins (56). However, no Env protein boost and no antibody responses were investigated in the SIV study.

The NYVAC and ALVAC immunogens used in this investigation are distinct from any other previous poxvirus vector. The advantage of these vectors is that they express independently Env and Gag-Pol-Nef and induced potent innate immune responses (26), reinforcing that a mixture of two vectors could be a better approach over a single virus vector as was used in the RV144 trial vaccine regimen (ALVAC-vCP1521). Notably, previous generation of NYVAC-based HIV-1 immunogens were designed to express both Env and Gag-Pol-Nef from the same viral TK locus, such as NYVAC-C (vP2010), a NYVAC HIV-1 immunogen expressing HIV-1 gp120 and Gag-Pol-Nef proteins from clade C 97CN54. This has been tested as an homologous component in a phase I

clinical trial (EV01) in healthy volunteers demonstrating safety profile and inducing T-cell immune responses against HIV-1 antigens in 50% of the vaccinees, with most of the responses being Env-specific (24). Furthermore, a DNA-C prime (two plasmid vectors)/NYVAC-C (vP2010; old single component) boost immunization protocol tested in a phase I clinical trial (EV02) significantly enhanced the HIV-1-specific T and B cell immune responses (25), though again with a Env-antigen specific bias that were polyfunctional and long-lasting (13). In another recent human clinical trial (HVTN078), NYVAC was used in combination with an Ad5-based HIV vaccine, where it was shown that NYVAC was a potent boosting component (23). A similar NYVAC-based HIV/AIDS therapeutic vaccine candidate expressing Env and Gag-Pol-Nef HIV-1 antigens from clade B (NYVAC-B) has been evaluated in HIV-1-infected patients on antiretroviral therapy in a phase I clinical trial (Theravac-01). In HIV-infected individuals, this NYVAC immunogen induced broad, polyfunctional HIV-1-specific T-cell responses, triggering both an expansion of pre-existing T-cell immune responses and the appearance of newly detected HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses (14).

Importantly, the novel poxvirus vectors NYVAC-C and ALVAC-C express HIV-1 antigens from clade C, the most broadly distributed HIV-1 subtype, reinforcing the use of these combined vectors as HIV/AIDS vaccine candidates in those geographical regions where HIV-1 clade C is most prevalent.

While there are limited markers that might correlate with protection against HIV, it has been inferred from the RV144 trial and other studies that vaccine efficacy might be related to the induction of antibodies against the V1/V2 and V3 loops, production of neutralizing and non-neutralizing antibodies, cross clade responses, ADCC activation and induction of $CD4^+$ and $CD8^+$ T-cell responses (31, 33, 42-44, 46). From the

findings described here it is clear that the poxvirus vectors NYVAC and ALVAC induced responses to all of these vaccine markers. Whether these immune markers correlate with control of HIV infection and which of the two poxvirus vectors is best in eliciting protective efficacy remains to be defined.

Overall, this head-to-head comparison in non-human primates has revealed how NYVAC-C and ALVAC-C elicit a wide spectrum of different T and B cell immune responses that may be relevant in protection from HIV infection. These results support the further clinical development of NYVAC as an HIV vaccine candidate.

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898

899 **FIGURE LEGENDS**

900 **Figure 1. Immunization schedule in non-human primates.** (A) Immunization groups

901 included in the AUP513 study modeled after the RV144 trial vaccine regimen. Eight

902 non-human primates (NHP) (rhesus macaques) were immunized in each group at weeks

903 0 and 4 with the corresponding poxvirus vectors (NYVAC-C, ALVAC-C or ALVAC-

904 vCP1521) and at weeks 12 and 24 with a combination of poxvirus vector plus a HIV-1

905 gp120 protein (from clades C or B/E), as detailed in Materials and Methods.

906 Composition of NYVAC-C, ALVAC-C, ALVAC-vCP1521 and the bivalent HIV-1

907   gp120 protein from clade C or from clades B/E (AIDSVAX) is detailed in Materials and

908   Methods. The HIV-1 subtypes included in the corresponding poxvirus vectors or in the

909   HIV-1 gp120 proteins are indicated between brackets. (B) Chronological diagram

910   showing the immunization schedule and the immunogenicity endpoints used in this

911   study. At weeks 0, 4, 12 and 24 animals were immunized as detailed in Figure 1A. A

912   dose of 1 x 10^8 PFU of each recombinant poxvirus vector (NYVAC-C, ALVAC-C or

913   ALVAC-vCP1521; 2 x 10^8 PFU of total virus) and 50 μg of each HIV-1 gp120 protein

914   (from clades C or B/E; 100 μg of total protein) were used in each immunization. In

915   groups 1, 2 and 3, gp120 protein boost is composed of a bivalent clade C gp120 protein

916   containing a mixture of 50 μg of TV1 gp120 plus 50 μg of 1086 gp120, both from clade

917   C (total amount of 100 μg). In group 4, gp120 protein boost is composed of a bivalent

918   AIDSVAX gp120 protein containing a mixture of 50 μg of clade B gp120 plus 50 μg of

919   clade CRF01_AE gp120 (total amount of 100 μg). Bivalent clade C gp120 protein was

920   administered together with MF59 adjuvant, and bivalent AIDSVAX gp120 protein was

921   administered together with alum adjuvant. At weeks 0, 6, 14 and 26 (at the beginning of

922   the study, two weeks after the second, third and fourth immunizations, respectively),

923   PBMCs and serum samples were obtained from each immunized animal and HIV-1-

924   specific T-cellular and humoral immune responses were analyzed.

925

926   **Figure 2. Immunization with NYVAC-C enhances the magnitude of HIV-1-specific**

927   **CD4$^+$ T-cell immune responses and induced a trend toward higher HIV-1-specific**

928   **CD8$^+$ T-cell immune responses.** Total magnitude of HIV-1-specific CD4$^+$ (A) and

929   CD8$^+$ (B) T-cell responses elicited by the different immunization groups. PBMCs were

930   collected at weeks 6, 14 and 26 from each rhesus monkey (n=8 per group) immunized

931   with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX). HIV-1-

specific CD4$^+$ and CD8$^+$ T-cell immune responses triggered by the different

immunization groups were measured by ICS assay following stimulation of PBMCs

with HIV-1 Env, Gag, Pol and Nef peptide pools. The values represent the sum of the

percentages of T cells producing IFN-γ and/or TNF-α and/or IL-2 against

Env+Gag+Pol+Nef peptide pools. Values from unstimulated controls were subtracted in

all cases. Each dot represents the value from each immunized monkey. Box plots

represent the distribution of data values, with the line inside the box indicating the

median value. p value indicates significantly higher response comparing N2NP2 (C) to

A2AP2 (C) at each week (*, $p < 0.05$).

Figure 3. Immunization with NYVAC-C enhances the magnitude of HIV-1-specific

CD4$^+$ T-cells producing cytokines. Overall magnitude of HIV-1-specific CD4$^+$ T cells

elicited by the different immunization groups and producing any cytokine (IFN-γ and/or

TNF-α and/or IL-2) (A), only IFN-γ (B), only TNF-α (C) or only IL-2 (D). PBMCs

were collected at weeks 6, 14 and 26 from each rhesus monkey (n=8 per group)

immunized with N2NP2 (C) and A2AP2 (C). HIV-1-specific CD4$^+$ T-cell immune

responses triggered by both immunization groups were measured by ICS assay

following stimulation of PBMCs with HIV-1 Env, Gag, Pol and Nef peptide pools. The

values represent the sum of the percentages of T cells producing IFN-γ and/or TNF-α

and/or IL-2 against Env+Gag+Pol+Nef peptide pools (A), or the percentages of T cells

producing IFN-γ (B) or TNF-α (C) or IL-2 (D) against Env+Gag+Pol+Nef peptide

pools. Values from unstimulated controls were subtracted in all cases. Each dot

represented the value from each immunized monkey. Each dot represents the value from

each immunized monkey. Box plots represent the distribution of data values, with the

956 line inside the box indicating the median value. *p* values indicate significantly higher

957 responses comparing N2NP2 (C) to A2AP2 (C) at each week (*, *p*<0.05).

958

959 **Figure 4. Immunization with NYVAC-C induces a trend toward an increase in the**

960 **levels of binding IgG antibodies against clade C HIV-1 gp140, gp120 and MuLV**

961 **gp70-scaffolded V1/V2 proteins.** Total binding IgG antibody levels against clade C

962 HIV-1 gp140 consensus (cCon) (A), gp120 from isolate 1086 (B), gp120 from isolate

963 TV1 (C) and MuLV gp70-scaffolded V1/V2 proteins (D) induced by the different

964 immunization groups. Individual sera samples were obtained at weeks -1, 6, 14 and 26

965 from each rhesus monkey (n=8 per group) immunized with N2NP2 (C), A2AP2 (C),

966 A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX). Binding IgG antibodies were measured

967 by BAMA, as indicated in Materials and Methods. The magnitude of the antibody

968 response is expressed as AUC from serial dilutions of plasma. Each dot represents the

969 value from each immunized monkey. *p* values indicate significantly higher levels

970 comparing N2NP2 (C) to A2AP2 (C) at each week (*, *p*<0.05).

971

972 **Figure 5. Immunization with NYVAC-C and ALVAC-C induces similar levels of**

973 **rectal IgG binding responses.** Rectal binding IgG antibody levels against HIV-1

974 gp140 group M consensus (sCon) (A), gp120 from isolate 1086 (B), gp120 from isolate

975 TV1 (C) and MuLV gp70-scaffolded V1/V2 proteins (D) induced by the different

976 immunization groups. Individual sera samples were obtained at weeks -1, 6, 14 and 26

977 from each rhesus monkey (n=8 per group) immunized with N2NP2 (C), A2AP2 (C),

978 A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX). Rectal binding IgG antibodies were

979 measured by analyzing the binding magnitude normalize per total rhesus IgG (specific

980 activity), as indicated in Materials and Methods. Each dot represents the value from

981 each immunized animal.

982

983 **Figure 6. Immunization with NYVAC-C and ALVAC-C induces a trend toward**

984 **higher levels of cross-clade binding IgG antibodies against HIV-1 gp140 from**

985 **clades A, B and group M consensus.** Total cross-clade binding IgG antibody levels

986 against HIV-1 gp140 consensus from clade A (a1Con) (A), clade B (bCon) (B) and

987 group M consensus (sCon) (C) induced by the different immunization groups.

988 Individual sera samples were obtained at weeks -1, 6, 14 and 26 from each rhesus

989 monkey (n=8 per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) and

990 A2AP2 (B/E, AIDSVAX). Binding IgG antibodies were measured by BAMA, as

991 indicated in Materials and Methods. The magnitude of the antibody response is

992 expressed as AUC from serial dilutions of plasma. Each dot represents the value from

993 each immunized monkey. *p* values indicate significantly higher levels comparing

994 N2NP2 (C) to A2AP2 (C) at each week (*, p<0.05).

995

996 **Figure 7. NYVAC-C and ALVAC-C induces IgG antibodies mainly directed**

997 **against the V3 loop.** Plasma linear IgG binding epitope specificity against different

998 linear epitopes covering HIV-1 gp160 of 7 consensus from clades A, B, C, D, group M,

999 CRF01 and CRF02 in a subset of animals that developed strong binding IgG antibodies.

1000 (A) Percent of total gp160 binding specific for each epitope. % of total gp160 binding is

1001 defined as: maximum binding to the epitope/sum of maximum binding to all epitopes

1002 identified. Each slice represents the average percentage values of the 7 animals mapped.

1003 (B) Binding magnitude to each linear epitope identified. Binding magnitude is shown as

1004 maximum binding to each epitope: highest binding (signal intensity) to a single peptide

1005   in each epitope region. Region of each epitope identified (shown as the range of

1006   peptides included in the array library) is listed under the epitope in parentheses.

1007

1008   **Figure 8. Immunization with NYVAC-C and ALVAC-C induces similar levels of**

1009   **ADCC responses against HIV-1.** ADCC activity induced by the different

1010   immunization groups. Individual plasma samples were obtained at weeks 0 and 26 from

1011   each rhesus monkey (n=8 per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2

1012   (B/E, C) and A2AP2 (B/E, AIDSVAX). ADCC activity was measured as indicated in

1013   Materials and Methods. Each dot represents the value from each immunized monkey.

1014   Box plots represent the distribution of data values, with the line inside the box

1015   indicating the median value.

1016

1017   **Figure 9. Immunization with NYVAC-C and ALVAC-C induces similar levels of**

1018   **neutralizing antibodies against HIV-1.** Neutralization titers and percentage of

1019   responders induced by the different immunization groups. Individual sera samples were

1020   obtained at weeks -1, 6, 14, 26 from each rhesus monkey (n=8 per group) immunized

1021   with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX).

1022   Neutralizing antibodies against clade B tier 1 HIV-1 strain MN.3, clade C tier 1 HIV-1

1023   strain MW965.26 and clade CRF01_AE tier 1 HIV-1 strain TH023.6 were measured

1024   using the TZM-bl assay, as indicated in Materials and Methods. Each dot represents the

1025   value from each immunized monkey. Blue dots indicate non-responders and red dots

1026   responders. Box plots represent the distribution of data values, with the line inside the

1027   box indicating the median value. Boxes and whiskers represent positive responders only

1028   (see Materials and Methods).

1029

38

Figure 10. Immunization with NYVAC-C and ALVAC-C induces low levels of binding IgA antibodies against HIV-1 gp120 and MuLV gp70-scaffolded V1/V2 proteins. Total binding IgA antibody levels against MuLV gp70-scaffolded V1/V2 (A) and gp120 from the clade C primary isolate 1086 (B) induced by the different immunization groups. Individual sera samples were obtained at weeks 0 and 26 from each rhesus monkey (n=8 per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX). Binding IgA antibodies against MuLV gp70-scaffolded V1/V2 and gp120 from isolate 1086 were measured by BAMA, as indicated in Materials and Methods. The magnitude of the antibody response is expressed as the fluorescence intensity background (FI bg)-subtracted at dilution 1/80. Each dot represents the value from each immunized monkey. Box plots represent the distribution of data values, with the line inside the box indicating the median value.

Figure 1

A

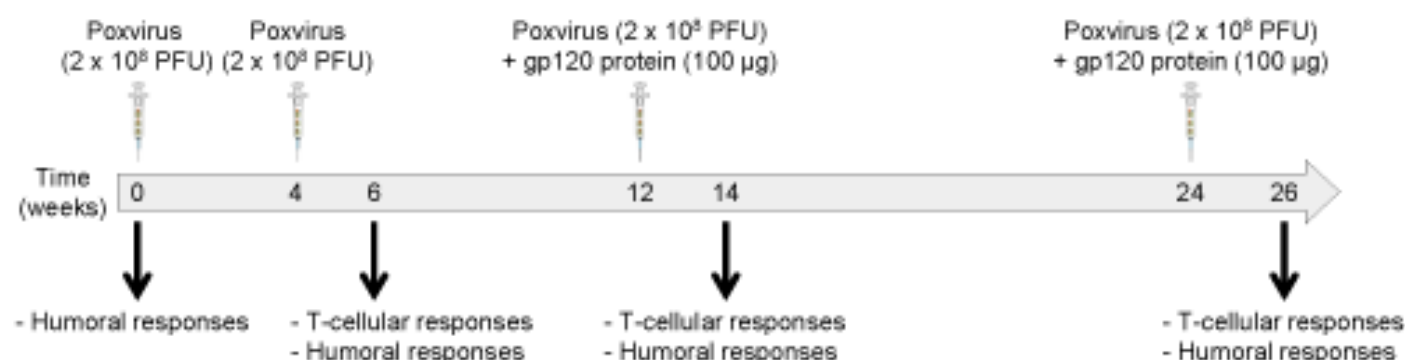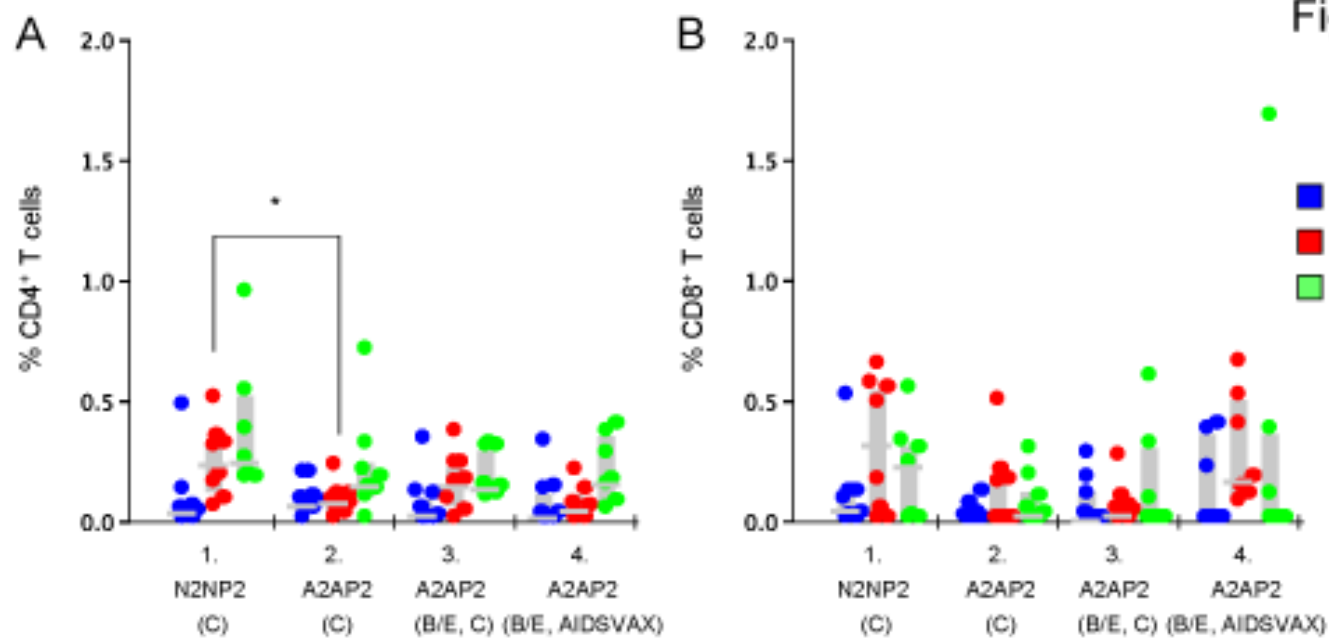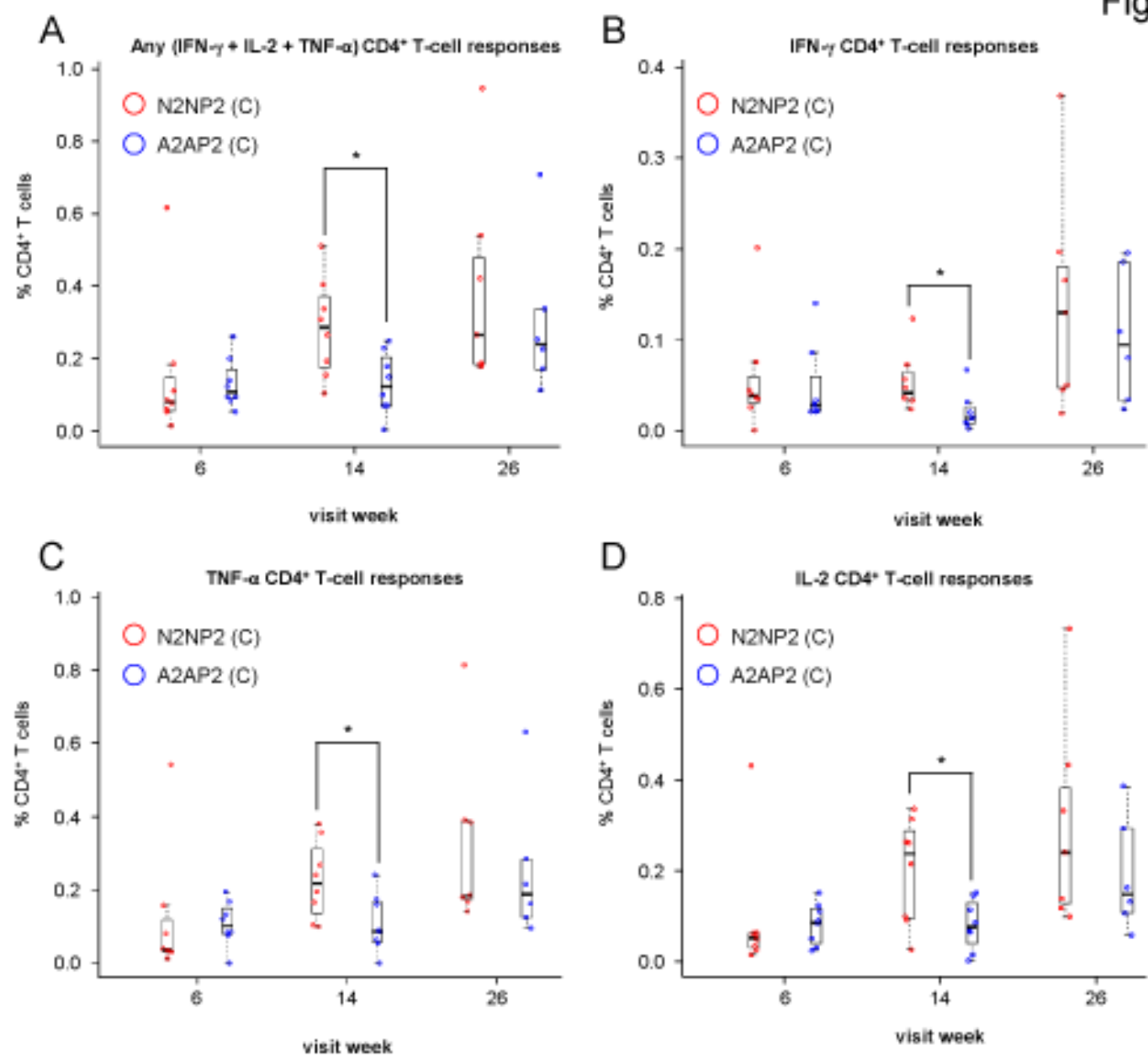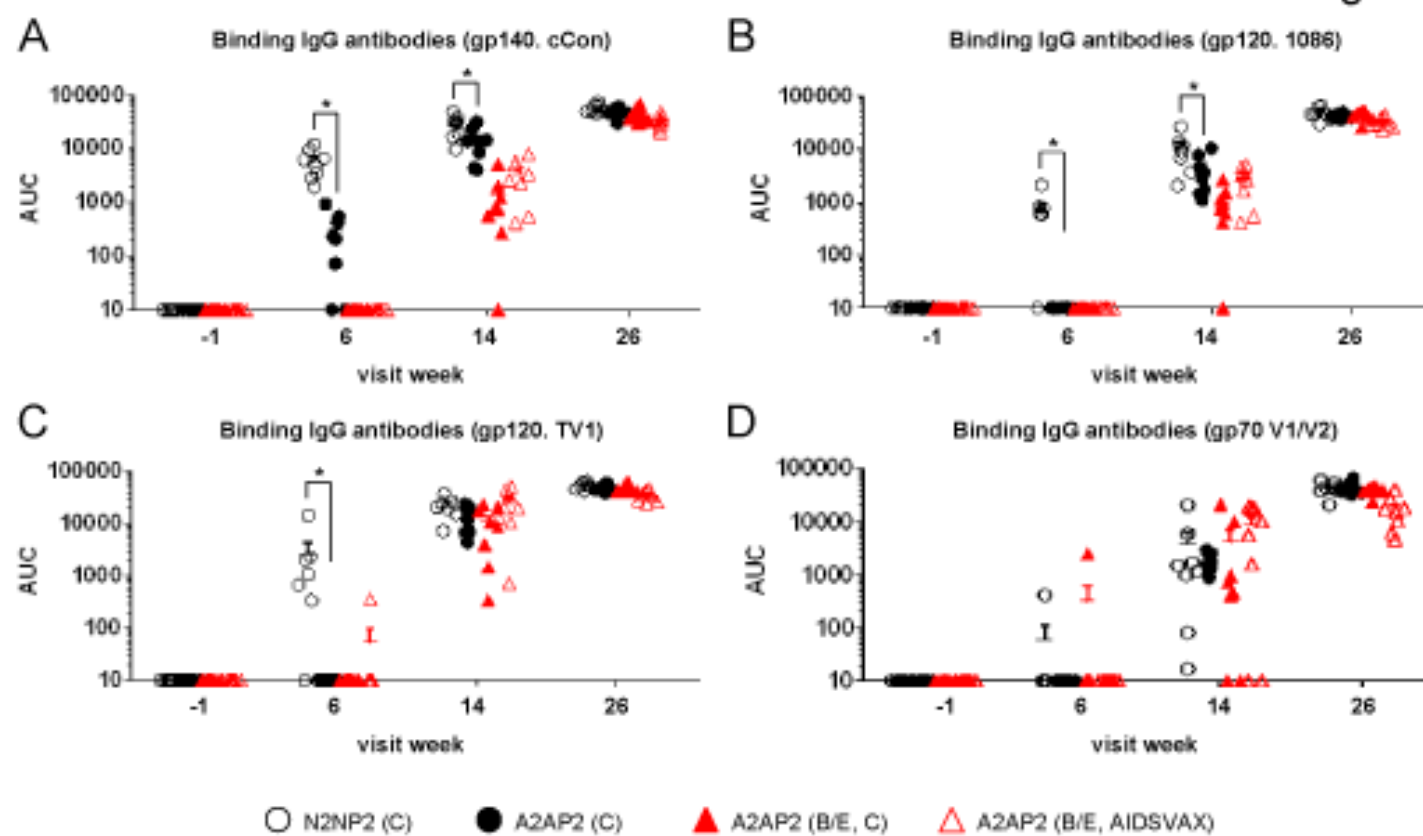| Immunization group	Number of NHP immunized	Immunization schedule			
		week 0	week 4	week 12	week 24
1. N2NP2 (C)	8	NYVAC-C (C)	NYVAC-C (C)	NYVAC-C (C) + gp120 protein (C)	NYVAC-C (C) + gp120 protein (C)
2. A2AP2 (C)	8	ALVAC-C (C)	ALVAC-C (C)	ALVAC-C (C) + gp120 protein (C)	ALVAC-C (C) + gp120 protein (C)
3. A2AP2 (B/E,C)	8	ALVAC-vCP1521 (B/E)	ALVAC-vCP1521 (B/E)	ALVAC-vCP1521 (B/E) + gp120 protein (C)	ALVAC-vCP1521 (B/E) + gp120 protein (C)
4. A2AP2 (B/E, AIDSVAX)	8	ALVAC-vCP521 (B/E)	ALVAC-vCP1521 (B/E)	ALVAC-vCP1521 (B/E) + AIDSVAX (B/E)	ALVAC-vCP1521 (B/E) + AIDSVAX (B/E)

B

Poxvirus (2×10^8 PFU)   Poxvirus (2×10^8 PFU)   Poxvirus (2×10^8 PFU) + gp120 protein (100 µg)   Poxvirus (2×10^8 PFU) + gp120 protein (100 µg)

Time (weeks)   0   4   6   12   14   24   26

- Humoral responses

- T-cellular responses
- Humoral responses

- T-cellular responses
- Humoral responses

- T-cellular responses
- Humoral responses

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

Binding IgG antibodies (gp140. a1Con)

B

Binding IgG antibodies (gp140. bCon)

C

Binding IgG antibodies (gp140. sCon)

○ N2NP2 (C)
● A2AP2 (C)
▲ A2AP2 (B/E, C)
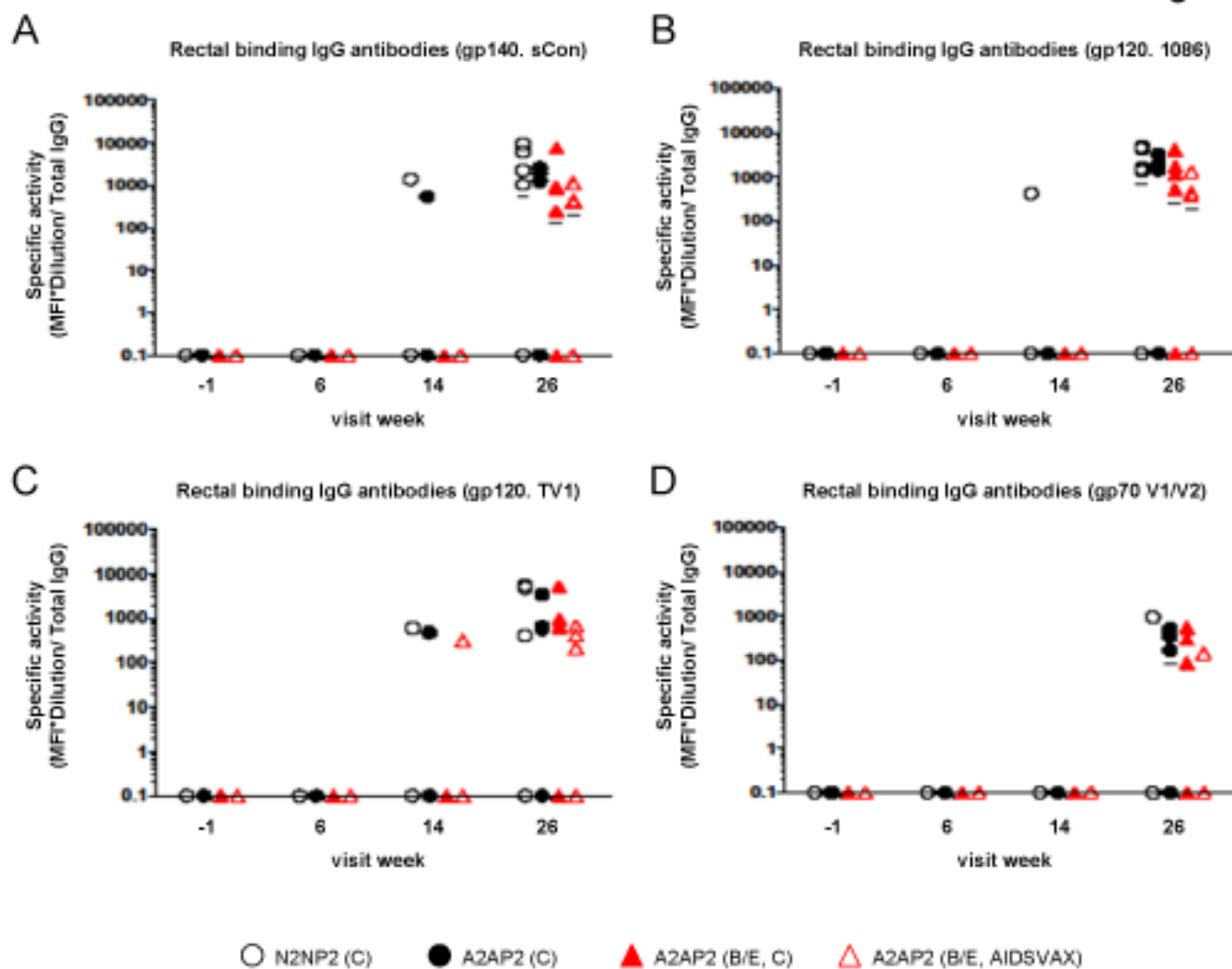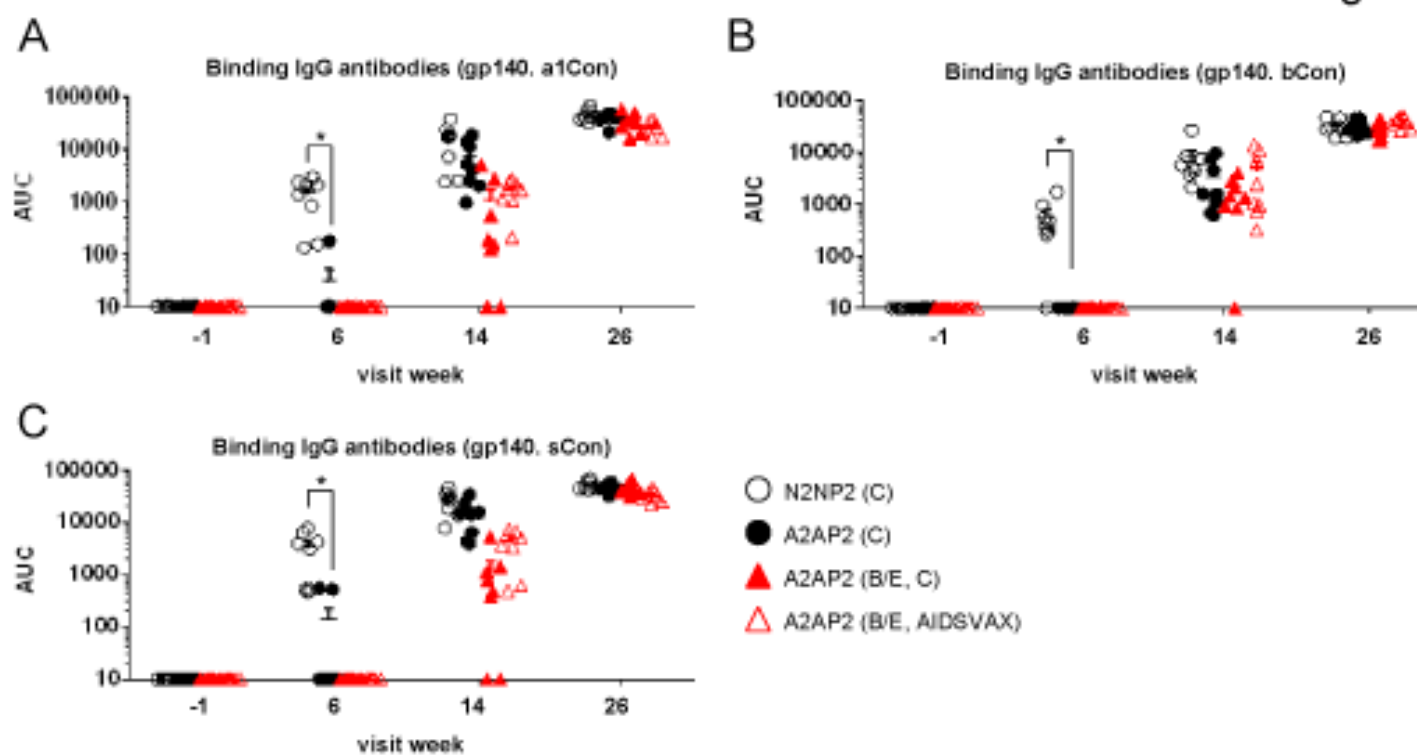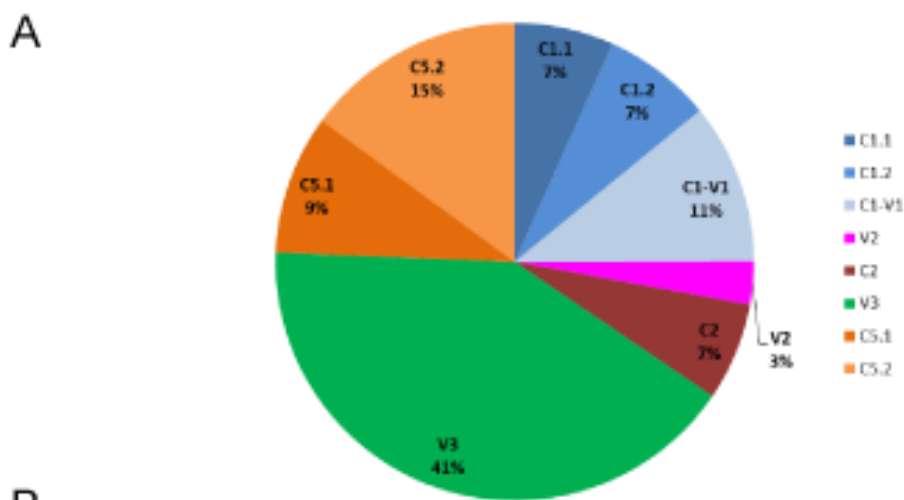△ A2AP2 (B/E, AIDSVAX)

Figure 7

A

B

Immunization group	NHP	C1.1 (#21-30)	C1.2 (#33-36)	C1-V1 (#38-42)	V2 (#53-57)	C2 (#64-68)	V3 (#98-103)	C5.1 (#147-155)	C5.2 (#156-164)
1. N2NP2 (C)	R120	25,721	2,866	4,055	5,794	3,271	65,279	14,586	10,222
1. N2NP2 (C)	R174	19,131	3,006	58,393	4,398	10,612	65,400	46,169	57,769
1. N2NP2 (C)	R185	36,857	37,782	46,759	17,378	22,751	65,280	25,419	25,110
1. N2NP2 (C)	R202	3,486	30,174	17,092	5,451	14,432	59,843	14,326	31,800
1. N2NP2 (C)	R209	846	3,941	4,511	1,987	7,757	33,323	3,316	4,217
2. A2AP2 (C)	R212	647	2,226	13,186	1,126	1,044	65,394	8,791	6,644
4. A2AP2 (B/E, AIDSVAX)	R140	1,915	5,794	2,296	358	6,223	22,581	3,903	22,339

Figure 8

Figure 9

Figure 10

A Binding IgA antibodies (gp70 V1/V2)

B Binding IgA antibodies (gp120, 1086)

○ N2NP2 (C)   ○ A2AP2 (C)   ○ A2AP2 (B/E, C)   ○ A2AP2 (B/E, AIDSVAX)