Head-to-head comparison of poxvirus NYVAC and ALVAC vectors expressing identical HIV-1 clade C immunogens in prime/boost combination with Env protein 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

indicate that NYVAC may represent an alternative candidate to ALVAC in the development of a future HIV-1 vaccine.

IMPORTANCE

The finding of a safe and effective HIV/AIDS vaccine immunogen is one of the main research priorities. Here, we have generated two poxvirus-based HIV vaccine candidates (NYVAC and ALVAC vectors) expressing the same clade C HIV-1 antigens in separate vectors and tested in non-human primates their immunogenicity profile. The results showed that immunization with NYVAC-C induced a trend toward higher HIV-1-specific cellular and humoral immune responses than those elicited by ALVAC-C, indicating that this new NYVAC vector could be considered a novel optimized HIV/AIDS vaccine candidate for human clinical trials.

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

immune responses and a consistent trend toward higher antibody responses against HIV-1 gp140, gp120 or MuLV gp70-scaffolded V1/V2. These results support the further clinical development of NYVAC-C as a component HIV/AIDS vaccine candidate.

MATERIALS AND METHODS

Recombinant NYVAC and ALVAC vectors expressing HIV-1 antigens

The recombinant NYVAC-C consists of two NYVAC vectors that express different clade C HIV-1 antigens under the same synthetic early/late poxvirus promoter (28): one (NYVAC-gp140) expressing Env gp140 from strain 96ZM651 and one (NYVAC-Gag-Pol-Nef) expressing Gag from strain 96ZM651 and Pol/Nef from strain CN54, and their generation and virological characteristics have been previously described (26). For head-to-head comparison purposes, the recombinant ALVAC-C was generated and consists of a combination of two ALVAC vectors expressing the same clade C HIV-1 antigens present in NYVAC-C (ALVAC-gp140 and ALVAC-Gag-Pol-Nef), and was generated by Sanofi Pasteur. Briefly, Env gp140 or Gag-Pol-Nef HIV-1 genes were inserted into the ALVAC C6 locus under the control of the synthetic early/late poxvirus promoter (28). The ALVAC backbone of ALVAC–C vectors is the same that was used to generate the recombinant ALVAC-vCP1521 vector and the HIV-1 antigens were inserted in the same locus (C6). ALVAC product is licensed for veterinary use under the name KANAPOX®. For the isolation of viral recombinants, 3 x 10^6 primary chicken embryo fibroblast (CEF) cells were first infected with ALVAC parental virus at a multiplicity of infection (MOI) of 10 plaque forming units (PFU)/cell and transfected 1 h later with 8 µg of linearized DNA (containing Env gp140 or Gag-Pol-Nef) using lipofectamine 2000CD (Life Technologies), according to the manufacturer´s

recommendations. After 24 h of incubation, the cells were harvested in 1 ml of 2% FBS-DMEM, sonicated and used for recombinant virus screening. Recombinant ALVAC viruses containing gp140 or Gag-Pol-Nef genes were screened and purified by plaque purification on primary CEF cells. After 4 consecutive rounds of plaque purification, positive plaques were isolated and confirmed to be positive to the gp140 or GPN DNA probe and negative to the ALVAC C6 open reading frame probe. The resulting ALVAC-gp140 and ALVAC-Gag-Pol-Nef recombinant viruses were expanded in primary CEF cells and the crude preparations obtained were used for the propagation of both viruses in large cultures of CEF cells followed by virus purification through two 36% (wt/vol) sucrose cushions and virus titrated. For simplicity of terminology, we subsequently refer to the combined mixed inoculation of NYVAC-gp140 + NYVAC-Gag-Pol-Nef as NYVAC-C and ALVAC-gp140 + ALVAC-Gag-Pol-Nef is labeled as ALVAC-C. The recombinant ALVAC-vCP1521 expresses HIV-1 gp120 from clade E, transmembrane gp41 from clade B and Gag/Pro from clade B, and was used previously in the RV144 phase III clinical trial (1).

HIV-1 proteins

In the immunizations performed in this study, two different HIV-1 gp120 proteins from clades C or B/E were used. Bivalent gp120 protein contains a mixture of TV1 gp120 and 1086 gp120, both from clade C. These proteins were expressed from stably transfected Chinese hamster ovary (CHO) cell lines, purified and characterized as previously described (29). Bivalent AIDSVAX gp120 protein contains a mixture of gp120 from clades B and CRF01_AE was used previously in the RV144 phase III clinical trial (1), and was provided by Global Solutions for Infectious Diseases.

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

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 x 10^8 PFU of each recombinant poxvirus vector (NYVAC-C, ALVAC-C or ALVAC-vCP1521; 2 x 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).

229 **Intracellular cytokine staining (ICS) assay**

230 The HIV-1-specific $CD4^+$ and $CD8^+$ T-cell immune responses induced at weeks 6, 14
231 and 26 were analyzed by polychromatic ICS from PBMCs obtained from each
232 immunized rhesus monkey, as previously described (30). In short, cryopreserved
233 PBMCs were thawed and rested overnight in R10 [RPMI 1640 (BioWhittaker,
234 Walkersville, MD), 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml
235 streptomycin] with 50 U/ml Benzonase (Novagen, Madison, WI) in a 37ºC/5% CO_2
236 incubator. The following morning, cells were stimulated with the corresponding HIV-1
237 Env, Gag, Pol and Nef peptide pools (2 µg/ml) in the presence of GolgiPlug (10 µg/ml;
238 BD Biosciences, San Jose, California) for 6 h. Negative controls received an equal
239 concentration of DMSO instead of peptides. Subsequently, ICS was performed as
240 described (30). The following monoclonal antibodies were used: CD4-BV421 (clone
241 OKT4; BioLegend), CD8-BV570 (clone RPA-T8; BioLegend), CD69-ECD (clone
242 TP1.55.3; Beckman Coulter), CD3-Cy7APC (clone SP34.2; BD Biosciences), IFN-γ-
243 APC (clone B27; BD Biosciences), IL-2-PE (clone MQ1-17H12; BD Biosciences) and
244 TNF-α-FITC (clone Mab11; BD Biosciences). Aqua LIVE/DEAD kit (Invitrogen,
245 Carlsbad, CA) was used to exclude dead cells. All antibodies were previously titrated to
246 determine the optimal concentration. Samples were acquired on an LSR II flow
247 cytometer and analyzed using FlowJo version 9.8 (Treestar, Inc., Ashland, OR).
248
249 **Peptides**

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

pools (Env-1, Env-2, Env-3, Pol-1, Pol-2, Gag-1, Gag/Pol, Gag-2/Pol and Nef), with about 60 peptides per pool.

HIV-1-specific binding antibody assay

HIV-1-specific binding antibodies were measured by Binding Antibody Multiplex Assay (BAMA) for total IgG and IgA antibodies in sera from each immunized rhesus monkey at weeks 0, 6, 14 and 26, as previously described (31, 32). Antigens used to analyze the total IgG or IgA binding antibodies included multiple HIV-1 clades: clade C gp120 TV1 and clade C gp120 1086 (provided by Novartis Vaccines), recombinant gp140 consensus from various subtypes [clade A 00MSA4076 gp140 (gp140. a1Con), clade B JRFL gp140 (gp140. bCon), clade C gp140 (gp140. cCon) and group M consensus (gp140. sCon)], murine leukemia virus (MuLV) gp70-scaffolded V1V2 (from clade C) and 1086 V1/V2 tags [all provided by Drs. H.-X. Liao and B. F. Haynes, Duke University, as previously described (33)]. Different plasma serial dilutions were made and results are expressed as mean fluorescent intensity (MFI) and titer [The area under curve (AUC)]. Furthermore, rectal mucosal IgG binding responses were measured and the specific activity was calculated by dividing the antibody titer by the total IgG concentration, as previously described (34). Positivity criteria were values 3-fold over the baseline visit and the cutoff was established using serum-negative samples. All assays were run under Good Clinical Laboratory Practices (GCLP)-compliant conditions.

Linear peptide microarray assay

Serum from a subset of immunized animals with strong binding IgG antibodies were selected to further evaluate linear epitope specificities by linear peptide microarray,

using an Env peptide library containing 15-mer peptides, overlapping by 12 amino acids, against HIV-1 Env gp160 of consensus clades A, B, C, D, group M, CRF01 and CRF02, as previously described (35, 36).

Antibody-dependent cellular cytotoxicity (ADCC) assay

ADCC activity was detected according to the ADCC-GranToxiLux (GTL) procedure, as previously described (37, 38). The results of the GTL assay were considered positive if % Granzyme B activity after background subtraction was ≥8% for the infected target cells as determined during the standardization of our assay (39). The $\log_{10}$ titer of the ADCC antibodies present in the plasma was calculated by interpolating the $\log_{10}$ reciprocal of the last plasma dilution that yielded positive % Granzyme B activity (≥8%). The GTL-ADCC assay was performed under GCLP-compliant guidelines.

Neutralizing antibodies against HIV-1

Neutralizing antibodies against HIV-1 were measured in TZM-bl cells, as previously described (40). Briefly, a pre-titrated dose of different HIV-1 virus (clade B tier 1 HIV-1 strain MN.3, clade C tier 1 HIV-1 strain MW965.26 and clade CRF01_AE tier 1 HIV-1 strain TH023.6) was incubated with serial 3-fold dilutions of test sample in duplicate in a total volume of 150 µl for 1 h at 37°C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 µl of growth medium containing 75 µg/ml DEAE dextran) were added to each well. One set of control wells received cells plus virus (virus control) and another set received cells only (background control). After 48 h of incubation, 100 µl of cells was transferred to a 96-well black solid plate (Costar) for measurements of luminescence using the Britelite Luminescence Reporter Gene Assay System (PerkinElmer Life Sciences). Neutralization titers are the serum dilution at

which relative luminescence units (RLU) were reduced by 50% compared to virus control wells after subtraction of background RLUs in cell control wells. Assay stocks of molecularly cloned Env-pseudotyped viruses were prepared by transfection in 293T/17 cells (American Type Culture Collection) and titrated in TZM-bl cells as previously described (40). Additional information on the assay and all supporting protocols may be found at: http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm. The assay was done under GCLP-compliant conditions.

Statistical procedures

The Wilcoxon Rank Sum test (when comparing two groups) and the Kruskal Wallis test (when comparing more than two groups) were used at each time point to test the null hypothesis that the groups have the same median response. All values used for analyzing proportionate representation of responses are background-subtracted. Box plots were used to summarize the distribution of various immune responses, where the mid-line of the box indicates the median, and the ends of the box denote the 25th and 75th percentiles, with whiskers extended to the extreme data points that are no more than 1.5 times the interquartile range (IQR) or, if no values meet this criterion, to the data extremes. When there are both positive and negative responses, values showed in the box plots refer to the positive responses.

RESULTS

Immunogenicity in non-human primates immunized with NYVAC and ALVAC vectors

The recombinant poxvirus vector ALVAC expressing HIV-1 antigens provided a modest level of efficacy in a phase III clinical trial in humans (1), highlighting that new

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-

α and/or IL-2) (Fig. 3A) or only IFN-γ (Fig. 3B), TNF-α (Fig. 3C) or IL-2 (Fig. 3D) ($p<0.05$).

In summary, these results showed that immunization with NYVAC-C elicited higher HIV-1-specific $CD4^+$ T-cell immune responses than ALVAC-C, and a trend toward higher $CD8^+$ T-cell immune responses, particularly after a single booster immunization.

NYVAC-C induced a trend toward increased levels of binding IgG antibodies against clade C HIV-1 gp140, gp120 and MuLV gp70-scaffolded V1/V2 proteins compared to ALVAC-C

The RV144 phase III clinical trial showed that IgG antibodies against V1/V2 and V3 regions of HIV-1 gp120 correlated with decreased risk of HIV-1 infection (31, 33, 42-44). Thus, we analyzed the HIV-1-specific humoral immune responses elicited after immunization with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) or A2AP2 (B/E, AIDSVAX), quantifying in individual serum samples obtained from each immunized rhesus monkey at weeks -1, 6, 14 and 26 the total binding IgG antibody levels against clade C HIV-1 gp140, gp120 and MuLV gp70-scaffolded V1/V2 proteins (Fig. 4).

At week 6, immunization with N2NP2 (C) significantly enhanced the levels of binding IgG antibodies against clade C HIV-1 gp140 consensus (Fig. 4A), gp120 from isolate 1086 (Fig. 4B) and gp120 from isolate TV1 (Fig. 4C), compared to immunization with A2AP2 (C), A2AP2 (B/E, C) or with A2AP2 (B/E, AIDSVAX) from which binding IgG antibodies were either rarely present or of lower magnitude. Furthermore, at week 6 immunization with N2NP2 (C) elicited a higher rate of responders than any A2AP2 immunization regimen (Fig. 4A to 4D). Moreover, at week 14 immunization with N2NP2 (C) significantly enhanced the levels of binding IgG antibodies against clade C HIV-1 gp140 consensus (Fig. 4A) and gp120 from isolate 1086 (Fig. 4B), compared to

immunization with A2AP2 (C), A2AP2 (B/E, C) or with A2AP2 (B/E, AIDSVAX). Additionally, at late timepoints (week 26) immunization with N2NP2 (C) slightly enhanced the levels of binding IgG antibodies against the HIV-1 gp140 consensus (Fig. 4A), gp120 from isolate 1086 (Fig. 4B), gp120 from isolate TV1 (Fig. 4C) and MuLV gp70-scaffolded V1/V2 proteins (Fig. 4D), compared to immunization with any A2AP2 immunization regimen, but differences were not significant.

Moreover, rectal IgG binding responses against group M HIV-1 gp140 consensus (Fig. 5A), gp120 from clade C isolate 1086 (Fig. 5B), gp120 from clade C isolate TV1 (Fig. 5C), and MuLV gp70-scaffolded V1/V2 proteins (Fig. 5D) were also detected in all immunization groups, only at weeks 14 and 26 (Fig. 5). The rate of responders against group M and clade C HIV-1 gp140 consensus at week 26 was higher in the NYVAC-C immunization group compared to ALVAC-C (80% versus 57% for both antigens) (Fig 5A and data not shown).

In summary, these results showed that immunization with NYVAC-C generated a trend towards higher binding IgG antibodies against clade C HIV-1 gp140, gp120 and MuLV gp70-scaffolded V1/V2 proteins as compared to ALVAC-C.

NYVAC-C induced a trend toward higher levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A, B and group M consensus than ALVAC-C

Next, we analyzed the ability of the immunizations with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX) to induce cross-clade antibodies against HIV-1 gp140, quantifying in individual serum samples obtained from each immunized rhesus monkey at weeks -1, 6, 14 and 26 the total binding IgG antibody levels against HIV-1 gp140 from clades A, B and group M consensus (Fig. 6).

Similar to the IgG binding antibody responses against clade C HIV-1 Env, at week 6 animals immunized with N2NP2 (C) produced significantly enhanced levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A (Fig. 6A), B (Fig. 6B) and group M consensus (Fig. 6C) compared to immunization with A2AP2 (C) or with A2AP2 (B/E, C), where cross-clade binding IgG antibodies were either non-existent (against clade B) or of lower magnitude (against clade A and group M consensus). Furthermore, at week 6 immunization with N2NP2 (C) elicited a higher rate of responders than A2AP2 (Fig. 6A to 6D). Nonetheless, the results at late time points (week 26) showed that immunization with N2NP2 (C) and A2AP2 (C) induced similar levels of binding IgG antibodies against HIV-1 gp140 from clades A (Fig. 6A), B (Fig. 6B) and group M (Fig. 6C).

In summary, these results showed that during the priming phase immunization NYVAC-C induced higher levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A, B and group M consensus than ALVAC-C, and boosting with either vector plus protein induced similar levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A, B and group M consensus.

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

The induction of plasma IgG antibodies to linear epitopes in the V2 and V3 regions of HIV-1 gp120 correlates with a reduced risk of infection in the RV144 phase III clinical trial (43). Thus, we next selected a subset of animals that developed strong binding IgG antibodies [belonging to the N2NP2 (C), A2AP2 (C) and A2AP2 (B/E, AIDSVAX) immunization groups] to evaluate linear epitope specificities by peptide microarray against Env gp160 of consensus clades A, B, C, D, group M, CRF01 and CRF02. The

452 results showed that V3-response dominated the binding response in most animals,
453 consisting on average 41% of total gp160 binding, followed by C5 (24%) and C1-V1
454 (11%) epitopes (Figure 7A and 7B). Other linear epitope specific responses were
455 detected to C1.1, C1.2, C2 and V2 epitopes, but with lower magnitude binding.
456
457 **NYVAC-C and ALVAC-C induced similar levels of ADCC responses against HIV-**
458 **1 gp120**
459 It has been suggested that ADCC responses are linked with a reduced risk of infection
460 in the RV144 phase III clinical trial (33). Moreover, antibodies with potent ADCC
461 activity have been isolated from some RV144 vaccinees (45). Thus, we analyzed the
462 ability of the immunizations with N2NP2 (C), A2AP2 (C), A2AP2 (B/E,C) and A2AP2
463 (B/E, AIDSVAX) to induce ADCC responses against clade C HIV-1 gp120 from isolate
464 TV1 in individual plasma samples obtained from each immunized rhesus monkey at
465 weeks 0 and 26 (Fig. 8). The results showed that immunization with N2NP2 (C) and
466 A2AP2 (C) induced similar levels of ADCC responses (Fig. 8).
467
468 **NYVAC-C and ALVAC-C induced similar levels of neutralizing antibodies against**
469 **HIV-1**
470 Broad neutralizing antibodies are a highly desired feature of an HIV-1 vaccine response
471 (46). We analyzed the neutralizing antibody responses to HIV-1 induced in macaques
472 immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E,C) and A2AP2 (B/E,
473 AIDSVAX) in individual serum samples obtained from each immunized rhesus monkey
474 at weeks -1, 6, 14 and 26 (Fig. 9).
475 Neutralizing antibody responses were observed predominantly against HIV-1 Tier 1
476 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

gp120 from isolate 1086 (Fig. 10B). Besides the results presented in Figure 10, we have also analyzed the IgA binding antibodies against multiple HIV-1 clades: clade C gp120 TV1, and recombinant gp140 consensus from various subtypes (clades A 00MSA4076 and A1.con.env03 gp140, clades B JRFL and B.con.env03 gp140, clade C.con.env03 gp140 and group M consensus). The results with the different gp120/gp140 isolates showed that no IgA antibodies against the clades A, B, C and group M analysed were induced in the four immunization groups (data not shown). In summary, these results showed that immunization with NYVAC-C and ALVAC-C induced very low levels of binding IgA antibodies against HIV-1 gp120 and specifically MuLV gp70-scaffolded V1/V2 proteins.

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

(ALVAC-C and NYVAC-C) expressing identical and optimized clade C trimeric gp140 and Gag-Pol-Nef as Gag-derived VLPs. Furthermore, this was bench-marked by comparison of the immune responses elicited by both vectors to the same immunogens and vaccination protocol as in the RV144 trial to define cross-clade responses.

In all immunization groups, HIV-1-specific $CD4^+$ and $CD8^+$ T cells were generated, but interestingly, compared to ALVAC-C, NYVAC-C significantly enhanced post immunization the HIV-1-specific $CD4^+$ T-cell immune responses and elicited a trend toward higher $CD8^+$ T-cell immune responses. Furthermore, NYVAC-C significantly enhanced the magnitude of HIV-1-specific $CD4^+$ T cells producing IFN-γ and/or TNF-α and/or IL-2.

With regard to the humoral immune responses, priming with NYVAC-C resulted in increased magnitude and frequency of clade C Env-specific binding IgG antibodies, with a trend toward higher levels after boosting with protein. In addition, peptide mapping to gp120 indicate that the most frequent linear IgG antibody response to specific linear epitopes was directed against the V3 loop in all animal groups, with reactivity also directed against different protein domains including V2, with animals immunized with NYVAC-C having the highest frequency of antibodies with these specificities. Comparison of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A, B and group M consensus showed that NYVAC-C induced higher levels during the priming phase and similar levels after boosting, compared to ALVAC-C. It should be pointed out that after priming, immunization with NYVAC-C and ALVAC-C gave better antibody responses to clade B gp140 than the immunization with clade B immunogens [A2AP2 (B/E, AIDSVAX)]. This could be due to the nature of the adjuvant, even though both adjuvants (MF59 and Alum) potentially augment the immune response through a common mechanism inducing a similar pattern of

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

612 clinical trial (EV01) in healthy volunteers demonstrating safety profile and inducing T-
613 cell immune responses against HIV-1 antigens in 50% of the vaccinees, with most of
614 the responses being Env-specific (24). Furthermore, a DNA-C prime (two plasmid
615 vectors)/NYVAC-C (vP2010; old single component) boost immunization protocol
616 tested in a phase I clinical trial (EV02) significantly enhanced the HIV-1-specific T and
617 B cell immune responses (25), though again with a Env-antigen specific bias that were
618 polyfunctional and long-lasting (13). In another recent human clinical trial (HVTN078),
619 NYVAC was used in combination with an Ad5-based HIV vaccine, where it was shown
620 that NYVAC was a potent boosting component (23). A similar NYVAC-based
621 HIV/AIDS therapeutic vaccine candidate expressing Env and Gag-Pol-Nef HIV-1
622 antigens from clade B (NYVAC-B) has been evaluated in HIV-1-infected patients on
623 antiretroviral therapy in a phase I clinical trial (Theravac-01). In HIV-infected
624 individuals, this NYVAC immunogen induced broad, polyfunctional HIV-1-specific T-
625 cell responses, triggering both an expansion of pre-existing T-cell immune responses
626 and the appearance of newly detected HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses
627 (14).
628 Importantly, the novel poxvirus vectors NYVAC-C and ALVAC-C express HIV-1
629 antigens from clade C, the most broadly distributed HIV-1 subtype, reinforcing the use
630 of these combined vectors as HIV/AIDS vaccine candidates in those geographical
631 regions where HIV-1 clade C is most prevalent.
632 While there are limited markers that might correlate with protection against HIV, it has
633 been inferred from the RV144 trial and other studies that vaccine efficacy might be
634 related to the induction of antibodies against the V1/V2 and V3 loops, production of
635 neutralizing and non-neutralizing antibodies, cross clade responses, ADCC activation
636 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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FIGURE LEGENDS

Figure 1. Immunization schedule in non-human primates. (A) Immunization groups included in the AUP513 study modeled after the RV144 trial vaccine regimen. Eight non-human primates (NHP) (rhesus macaques) were immunized in each group at weeks 0 and 4 with the corresponding poxvirus vectors (NYVAC-C, ALVAC-C or ALVAC-vCP1521) and at weeks 12 and 24 with a combination of poxvirus vector plus a HIV-1 gp120 protein (from clades C or B/E), as detailed in Materials and Methods. 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

line inside the box indicating the median value. *p* values indicate significantly higher responses comparing N2NP2 (C) to A2AP2 (C) at each week (*, $p<0.05$).

Figure 4. Immunization with NYVAC-C induces a trend toward an increase in the levels of binding IgG antibodies against clade C HIV-1 gp140, gp120 and MuLV gp70-scaffolded V1/V2 proteins. Total binding IgG antibody levels against clade C HIV-1 gp140 consensus (cCon) (A), gp120 from isolate 1086 (B), gp120 from isolate TV1 (C) and MuLV gp70-scaffolded V1/V2 proteins (D) induced by the different immunization groups. Individual sera samples were obtained at weeks -1, 6, 14 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 IgG antibodies were measured by BAMA, as indicated in Materials and Methods. The magnitude of the antibody response is expressed as AUC from serial dilutions of plasma. Each dot represents the value from each immunized monkey. *p* values indicate significantly higher levels comparing N2NP2 (C) to A2AP2 (C) at each week (*, $p<0.05$).

Figure 5. Immunization with NYVAC-C and ALVAC-C induces similar levels of rectal IgG binding responses. Rectal binding IgG antibody levels against HIV-1 gp140 group M consensus (sCon) (A), gp120 from isolate 1086 (B), gp120 from isolate TV1 (C) and MuLV gp70-scaffolded V1/V2 proteins (D) induced by the different immunization groups. Individual sera samples were obtained at weeks -1, 6, 14 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). Rectal binding IgG antibodies were measured by analyzing the binding magnitude normalize per total rhesus IgG (specific

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

Figure 6. Immunization with NYVAC-C and ALVAC-C induces a trend toward higher levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A, B and group M consensus. Total cross-clade binding IgG antibody levels against HIV-1 gp140 consensus from clade A (a1Con) (A), clade B (bCon) (B) and group M consensus (sCon) (C) induced by the different immunization groups. Individual sera samples were obtained at weeks -1, 6, 14 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 IgG antibodies were measured by BAMA, as indicated in Materials and Methods. The magnitude of the antibody response is expressed as AUC from serial dilutions of plasma. Each dot represents the value from each immunized monkey. *p* values indicate significantly higher levels comparing N2NP2 (C) to A2AP2 (C) at each week (*, $p<0.05$).

Figure 7. NYVAC-C and ALVAC-C induces IgG antibodies mainly directed against the V3 loop. Plasma linear IgG binding epitope specificity against different linear epitopes covering HIV-1 gp160 of 7 consensus from clades A, B, C, D, group M, CRF01 and CRF02 in a subset of animals that developed strong binding IgG antibodies. (A) Percent of total gp160 binding specific for each epitope. % of total gp160 binding is defined as: maximum binding to the epitope/sum of maximum binding to all epitopes identified. Each slice represents the average percentage values of the 7 animals mapped. (B) Binding magnitude to each linear epitope identified. Binding magnitude is shown as maximum binding to each epitope: highest binding (signal intensity) to a single peptide

in each epitope region. Region of each epitope identified (shown as the range of peptides included in the array library) is listed under the epitope in parentheses.

Figure 8. Immunization with NYVAC-C and ALVAC-C induces similar levels of ADCC responses against HIV-1. ADCC activity induced by the different immunization groups. Individual plasma 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). ADCC activity was measured as indicated in Materials and Methods. 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 9. Immunization with NYVAC-C and ALVAC-C induces similar levels of neutralizing antibodies against HIV-1. Neutralization titers and percentage of responders induced by the different immunization groups. Individual sera samples were obtained at weeks -1, 6, 14, 26 from each rhesus monkey (n=8 per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C) and A2AP2 (B/E, AIDSVAX). Neutralizing antibodies against clade B tier 1 HIV-1 strain MN.3, clade C tier 1 HIV-1 strain MW965.26 and clade CRF01_AE tier 1 HIV-1 strain TH023.6 were measured using the TZM-bl assay, as indicated in Materials and Methods. Each dot represents the value from each immunized monkey. Blue dots indicate non-responders and red dots responders. Box plots represent the distribution of data values, with the line inside the box indicating the median value. Boxes and whiskers represent positive responders only (see Materials and Methods).

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

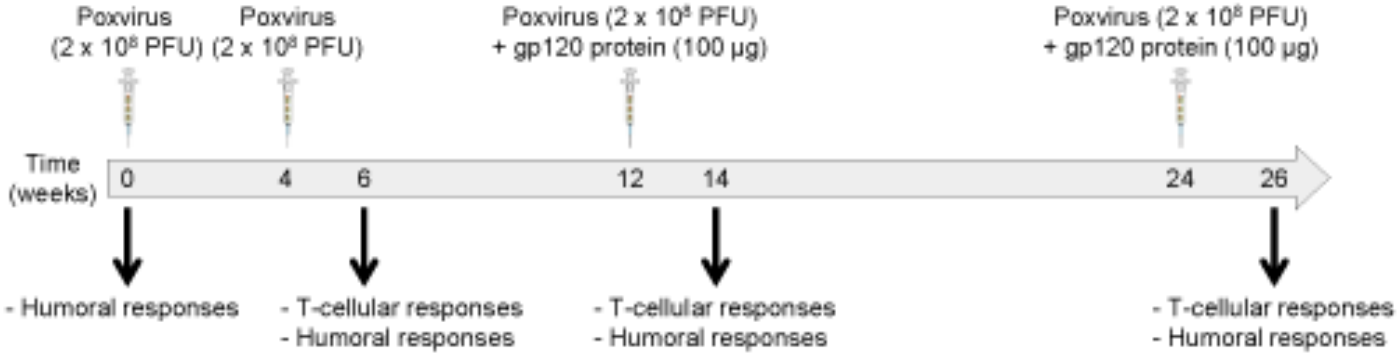


Figure 2

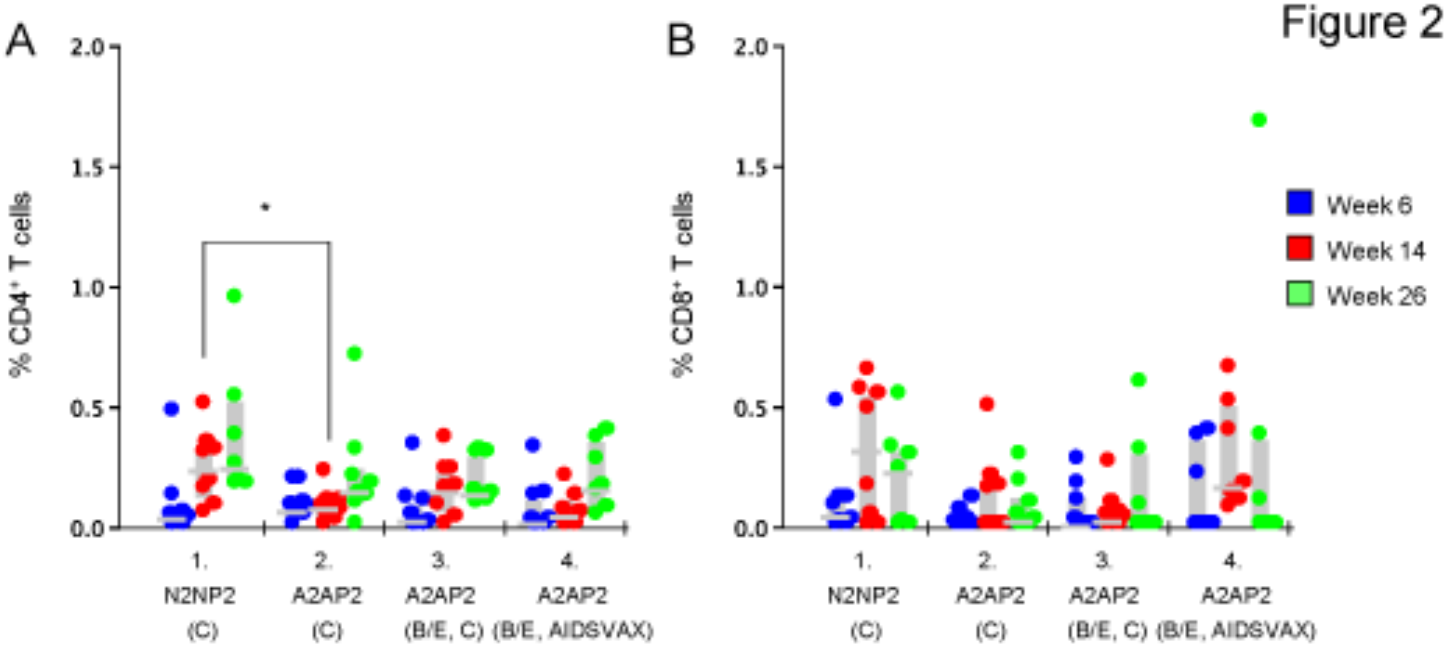

A
B
% CD4+ T cells
% CD8+ T cells
2.0
1.5
1.0
0.5
0.0
*
Week 6
Week 14
Week 26
1.
N2NP2
(C)
2.
A2AP2
(C)
3.
A2AP2
(B/E, C)
4.
A2AP2
(B/E, AIDSVAX)

Figure 3

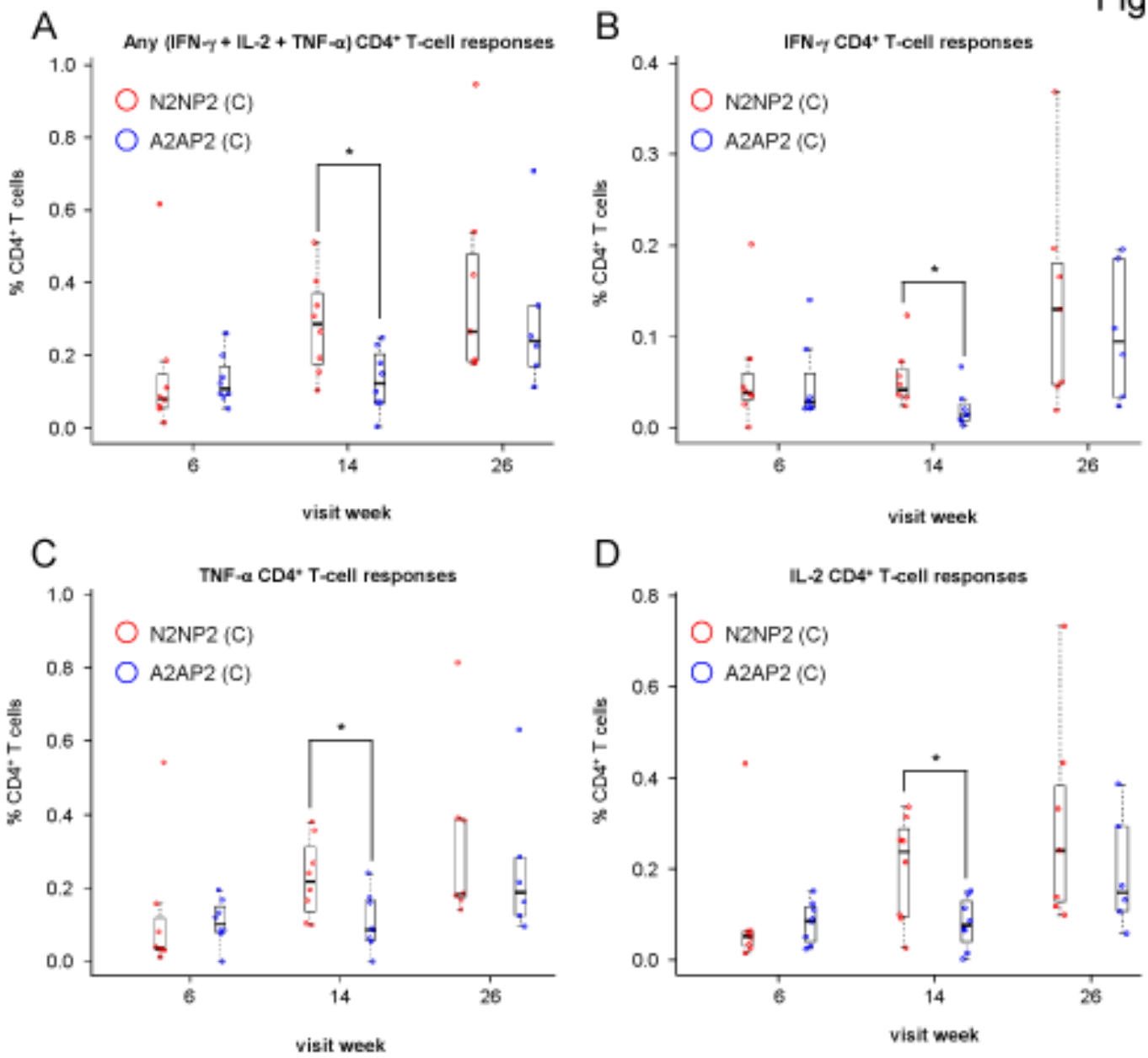

A
Any (IFN-γ + IL-2 + TNF-α) CD4+ T-cell responses
N2NP2 (C)
A2AP2 (C)
% CD4+ T cells
visit week
B
IFN-γ CD4+ T-cell responses
C
TNF-α CD4+ T-cell responses
D
IL-2 CD4+ T-cell responses

Figure 4

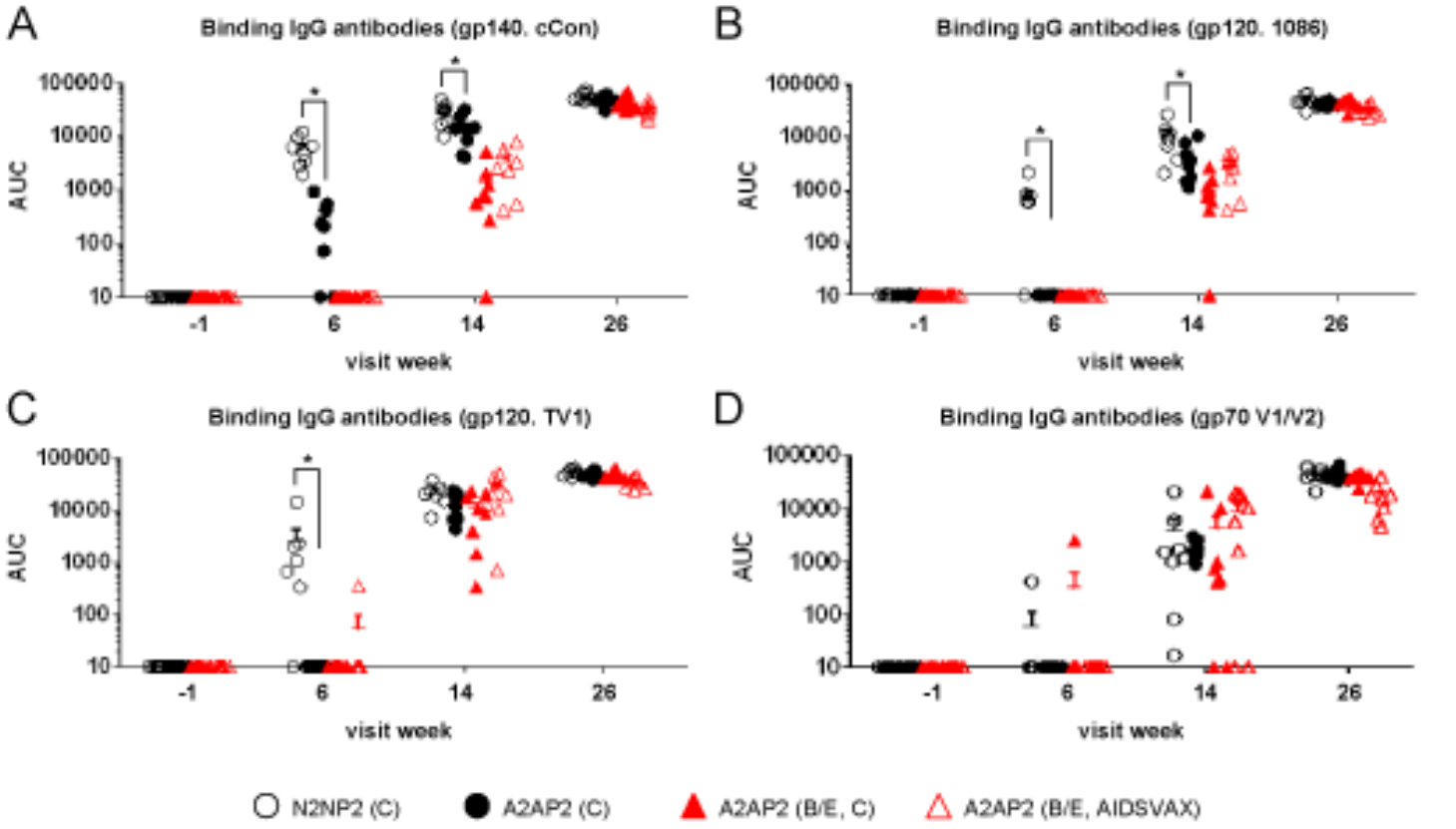

A
Binding IgG antibodies (gp140. cCon)
B
Binding IgG antibodies (gp120. 1086)
C
Binding IgG antibodies (gp120. TV1)
D
Binding IgG antibodies (gp70 V1/V2)
AUC
100000
10000
1000
100
10
-1
6
14
26
visit week
*
N2NP2 (C)
A2AP2 (C)
A2AP2 (B/E, C)
A2AP2 (B/E, AIDSVAX)

Figure 5

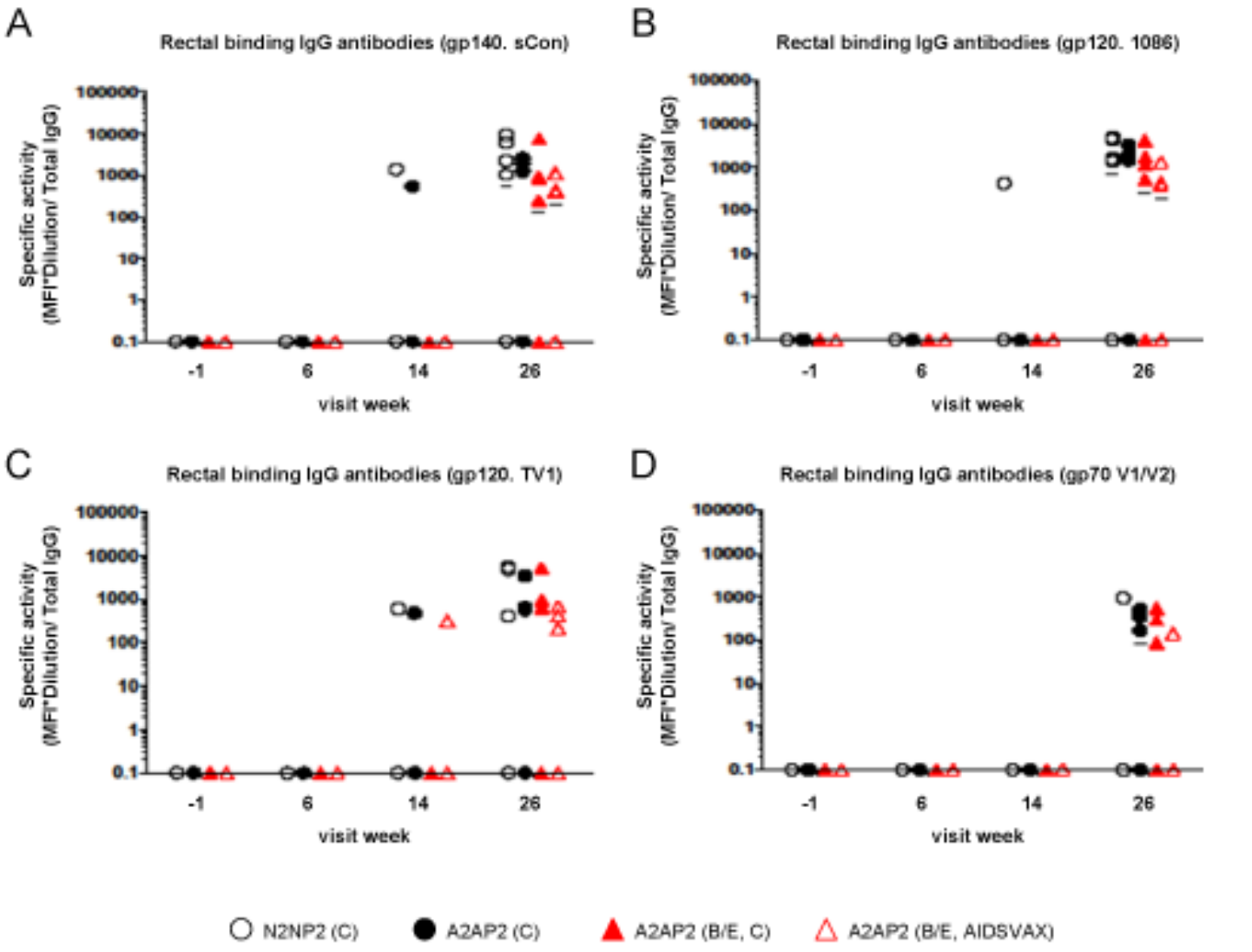

A
Rectal binding IgG antibodies (gp140. sCon)
B
Rectal binding IgG antibodies (gp120. 1086)
C
Rectal binding IgG antibodies (gp120. TV1)
D
Rectal binding IgG antibodies (gp70 V1/V2)
Specific activity (MFI*Dilution/ Total IgG)
100000
10000
1000
100
10
1
0.1
-1
6
14
26
visit week
N2NP2 (C)
A2AP2 (C)
A2AP2 (B/E, C)
A2AP2 (B/E, AIDSVAX)

Figure 6

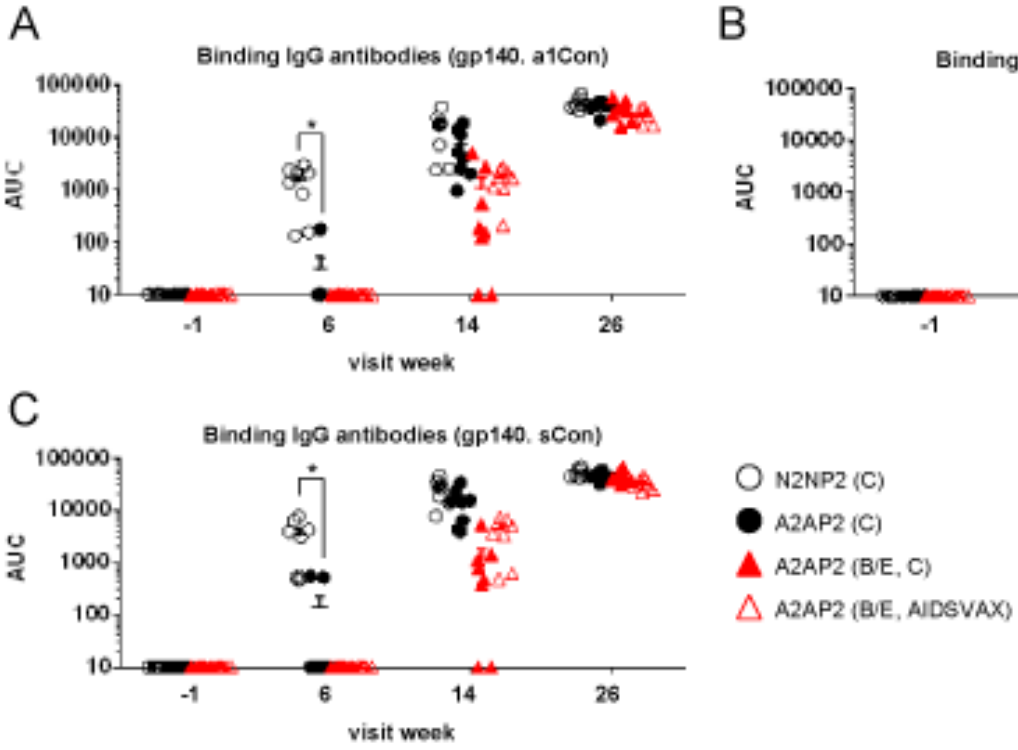
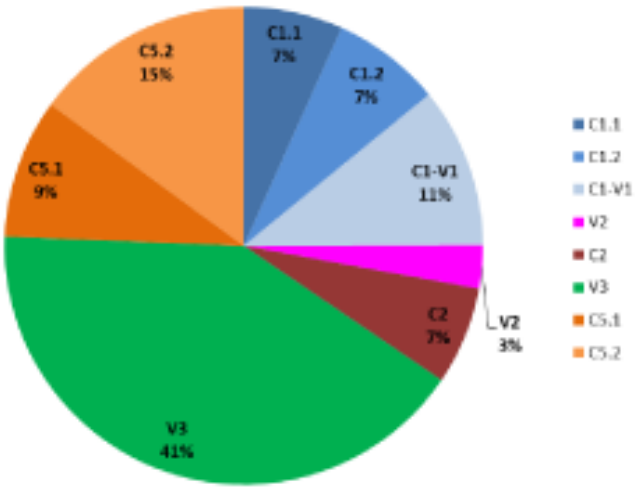

A
Binding IgG antibodies (gp140. a1Con)
AUC
100000
10000
1000
100
10
-1
6
14
26
visit week
*
B
Binding IgG antibodies (gp140. bCon)
AUC
100000
10000
1000
100
10
-1
6
14
26
visit week
*
C
Binding IgG antibodies (gp140. sCon)
AUC
100000
10000
1000
100
10
-1
6
14
26
visit week
*
N2NP2 (C)
A2AP2 (C)
A2AP2 (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

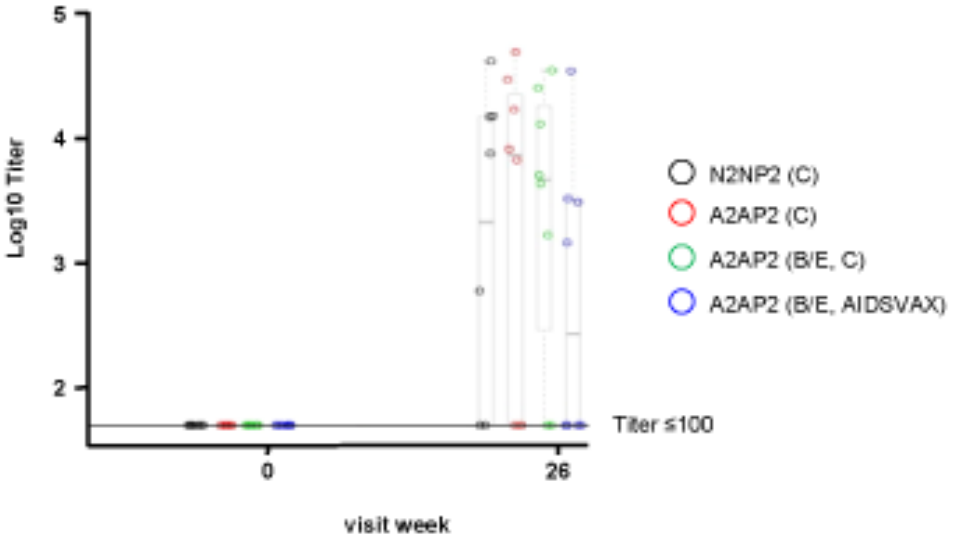

5
4
3
2
Log10 Titer
0
26
visit week
Titer ≤100
N2NP2 (C)
A2AP2 (C)
A2AP2 (B/E, C)
A2AP2 (B/E, AIDSVAX)

Figure 9

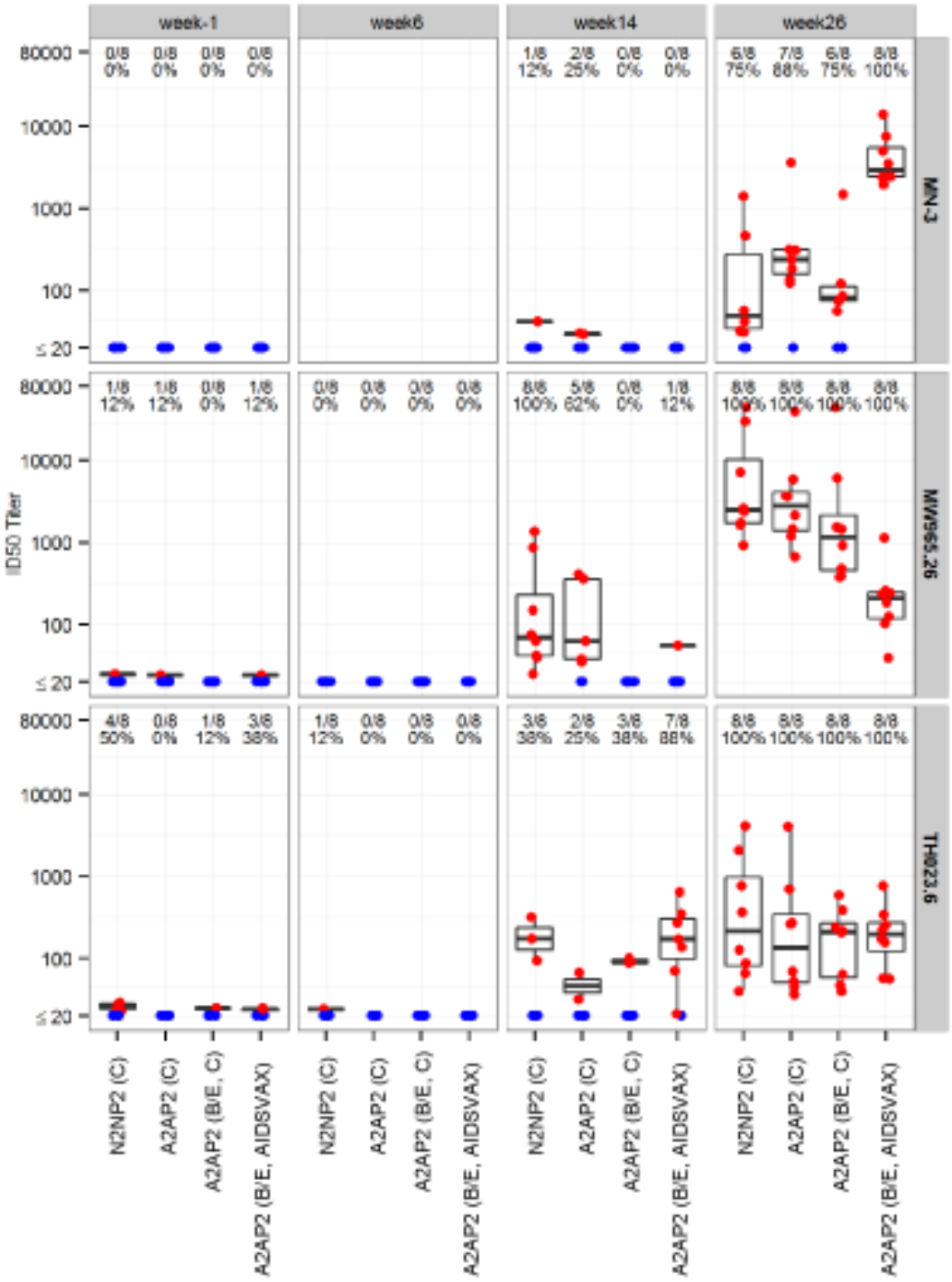
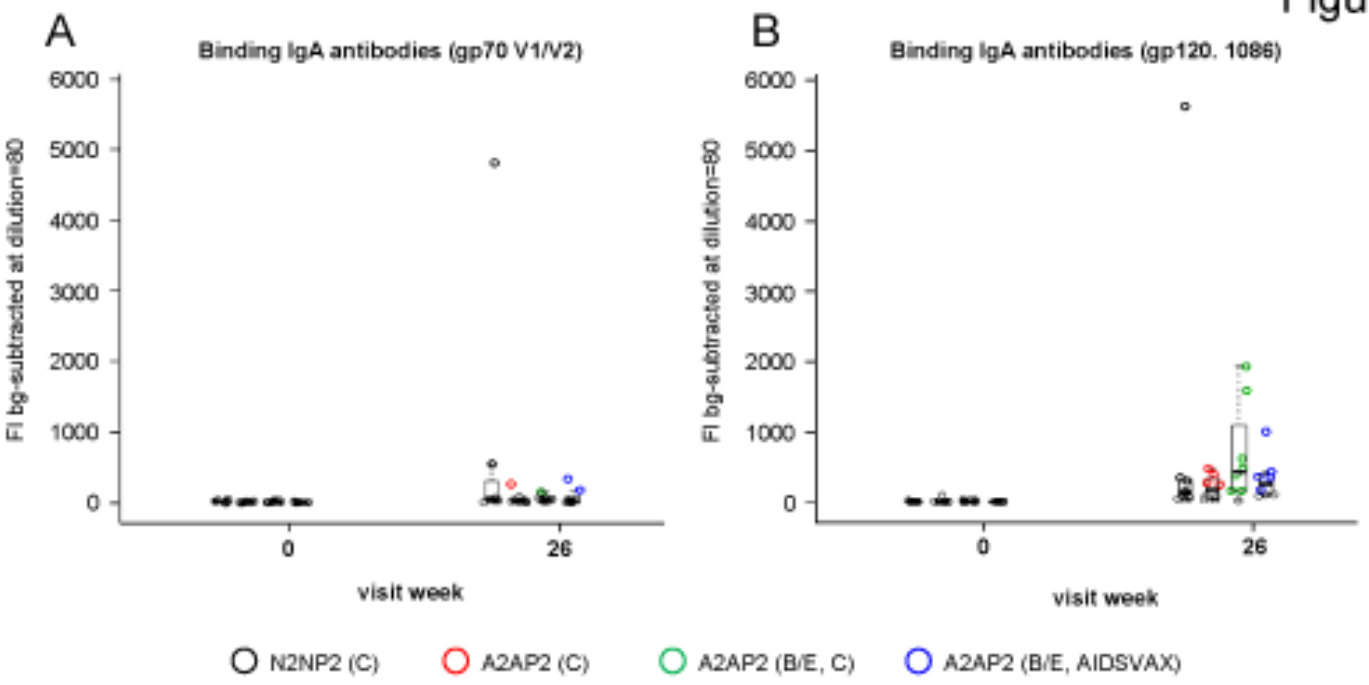

week-1
week6
week14
week26
MN-3
MWRS.26
TH023.6
ID50 Titer
80000
10000
1000
100
≤ 20
0/8 0%
1/8 12%
2/8 25%
5/8 62%
6/8 75%
7/8 88%
8/8 100%
4/8 50%
3/8 38%
N2NP2 (C)
A2AP2 (C)
A2AP2 (B/E, C)
A2AP2 (B/E, AIDSVAX)

Figure 10


A
Binding IgA antibodies (gp70 V1/V2)
FI bg-subtracted at dilution=80
6000
5000
4000
3000
2000
1000
0
0
26
visit week
B
Binding IgA antibodies (gp120. 1086)
FI bg-subtracted at dilution=80
6000
5000
4000
3000
2000
1000
0
0
26
visit week
N2NP2 (C)
A2AP2 (C)
A2AP2 (B/E, C)
A2AP2 (B/E, AIDSVAX)