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 Nonhuman Primates

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ABSTRACT

We 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 a Gag-Pol-Nef polyprotein as Gag-induced virus-like particles (VLPs) (referred to 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 HIV-1-specific CD8⁺ T-cell immune responses than did ALVAC-C. Furthermore, NYVAC-C induced a trend toward higher levels of binding IgG antibodies against clade C HIV-1 gp140, gp120, or murine leukemia virus (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, than did 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 antibody levels 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 nonhuman 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 generated two poxvirus-based HIV vaccine candidates (NYVAC and ALVAC vectors) expressing the same clade C HIV-1 antigens in separate vectors, and we analyzed in nonhuman primates their immunogenicity profiles. The results showed that immunization with NYVAC-C induced a trend toward higher HIV-1-specific cellular and humoral immune responses than did ALVAC-C, indicating that this new NYVAC vector could be a novel optimized HIV/AIDS vaccine candidate for human clinical trials.

The development of a safe and effective HIV/AIDS vaccine that can 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; 31.2% protection against HIV-1 infection in humans was reported (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 nonhuman primates) and in humans (2, 7). In particular, recombinant NYVAC vectors expressing HIV-1 Env, Gag, Pol, and Nef antigens from clade B or C elicited strong, broad, and polyfunctional T-cell immune responses in mice, nonhuman primates, and humans, together with varied levels of humoral re-

sponses against HIV-1 gp120 (8–23). An additional feature is that the current NYVAC vectors preferentially trigger 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 recently characterized two novel attenuated NYVAC vectors expressing HIV-1 clade C trimeric soluble gp140 or Gag-Pol-Nef as a polyprotein processed into Gag-derived virus-like particles (VLPs) which triggered specific innate responses in human cells and elicited in mice polyfunctional Env-specific CD4⁺ and Gag-specific CD8⁺ T-cell responses, together with antibody responses against HIV-1 gp140 and p17/p24 (26). Furthermore, DNA plasmids producing these improved immunogens led to higher expression levels and enhanced immunogenicity after DNA vaccination in mice (27) and after DNA prime-NYVAC boost in nonhuman primates (B. Asbach et al., submitted for publication).

A comparison of the immunogenicity elicited by different poxvirus vectors expressing the same HIV-1 antigens is of particular importance, as it may provide details of the best-in-class vector that should be advanced for future phase III human trials. To this end, in a preclinical study in rhesus macaques, we evaluated head to head the HIV-1-specific cellular and humoral immune responses elicited by NYVAC and ALVAC poxvirus vectors expressing identical clade C HIV-1 inserts: Env gp140 as a trimeric soluble protein, and Gag-Pol-Nef as a polyprotein processed into Gag-derived VLPs (referred to here as NYVAC-C and ALVAC-C). NYVAC-C and ALVAC-C were administered using an immunization protocol consisting of two priming doses of the corresponding recombinant poxvirus vector boosted with two doses of a combination of the poxvirus vector and HIV-1 gp120 protein from clade C. Moreover, we also compared the immunogenicity elicited by these two vaccine candidates with the one induced by the same ALVAC vector used in the RV144 phase III clinical trial (ALVAC-vCP1251, expressing HIV-1 antigens from clades B and E) and administered following two priming doses of ALVAC-vCP1251 plus two boosts with combined ALVAC-vCP1251 and HIV-1 gp120 protein from clade C or B/E. The results showed that while the two vectors triggered both T- and B-cell immune responses, NYVAC-C was more immunogenic than ALVAC-C, inducing at different times higher HIV-1-specific CD4⁺ T-cell responses with a trend toward higher-magnitude HIV-1-specific CD8⁺ T-cell immune responses and a consistent trend toward

greater antibody responses against HIV-1 gp140, gp120, or murine leukemia virus (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 vector 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 the other (NYVAC-Gag-Pol-Nef) expressing Gag from strain 96ZM651 and Pol and Nef from strain CN54. Their generation and virological characteristics have been previously described (26). For head-to-head comparison purposes, the recombinant ALVAC-C was generated and consisted 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); this vector 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 was the same that was used to generate the recombinant ALVAC-vCP1521 vector, and the HIV-1 antigens were inserted in the same locus (C6). The ALVAC product is licensed for veterinary use under the name Kanapox. For the isolation of viral recombinants, 3×10^6 primary chicken embryo fibroblast (CEF) cells were first infected with ALVAC parental virus at a multiplicity of infection (MOI) of 10 PFU/cell and transfected 1 h later with 8 µg of linearized DNA (encoding Env gp140 or Gag-Pol-Nef) by 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% fetal bovine serum (FBS)–Dulbecco's modified Eagle's medium, sonicated, and used for recombinant virus screening. Recombinant ALVAC viruses containing gp140 or genes for Gag, Pol, and Nef 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 Gag-Pol-Nef 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 titrations. For simplicity of terminology, here we refer to the combined mixed inoculation of NYVAC-gp140 plus NYVAC-Gag-Pol-Nef as NYVAC-C and that of ALVAC-gp140 plus ALVAC-Gag-Pol-Nef 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. For 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 containing 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.

Nonhuman 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 ret-

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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, AIDS VAX)	8	ALVAC-vCP1521 (B/E)	ALVAC-vCP1521 (B/E)	ALVAC-vCP1521 (B/E) + AIDS VAX (B/E)	ALVAC-vCP1521 (B/E) + AIDS VAX (B/E)

B

FIG 1 Immunization schedule for nonhuman primates. (A) Immunization groups included in the AUP513 study were modeled after the RV144 trial vaccine regimen. Eight nonhuman primates (NHP; rhesus macaques) were immunized in each group at weeks 0 and 4 with the corresponding poxvirus vector (NYVAC-C, ALVAC-C, or ALVAC-vCP1521) and at weeks 12 and 24 with a combination of poxvirus vector plus an HIV-1 gp120 protein (from clade C or B/E), as detailed in Materials and Methods. The composition of NYVAC-C, ALVAC-C, ALVAC-vCP1521, and the bivalent HIV-1 gp120 protein from clade C or from clades B/E (AIDS VAX) are described in detail in Materials and Methods. The HIV-1 subtypes included in the corresponding poxvirus vectors or in the HIV-1 gp120 proteins are indicated in parentheses. (B) Chronological diagram showing the immunization schedule and the immunogenicity endpoints used in this study. At weeks 0, 4, 12, and 24, animals were immunized (see panel A). 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) and 50 µg of each HIV-1 gp120 protein (from clade C or B/E; 100 µg of total protein) were used for each immunization. In groups 1, 2, and 3, the gp120 protein boost was composed of a bivalent clade C gp120 protein containing a mixture of 50 µg of TV1 gp120 plus 50 µg of 1086 gp120, both from clade C (total amount, 100 µg). In group 4, the gp120 protein boost was composed of a bivalent AIDS VAX gp120 protein containing a mixture of 50 µg of clade B gp120 plus 50 µg of clade CRF01_AE gp120 (total amount, 100 µg). Bivalent clade C gp120 protein was administered together with MF59 adjuvant, and bivalent AIDS VAX gp120 protein was administered together with alum adjuvant. At weeks 0, 6, 14, and 26 (at the beginning of the study and 2 weeks after the second, third, and fourth immunizations, respectively), PBMCs and serum samples were obtained from each immunized animal, and HIV-1-specific T-cell and humoral immune responses were analyzed.

rovirus (SRV), simian T-cell leukemia virus 1 (STLV-1), herpesvirus B, simian immunodeficiency virus (SIV), measles, and poxvirus immunogens prior to the study and also demonstrated negative fecal culture for *Salmonella*, *Shigella*, *Campylobacter*, and *Yersinia* genera. Furthermore, animals were immunologically naive for the vaccine components.

Immunization schedule. Four immunizations groups of eight rhesus macaques were included in the study protocol (designated AUP513). Group 1 received two immunizations with NYVAC-C (weeks 0 and 4) and was boosted with two immunizations of NYVAC-C plus bivalent gp120 proteins from clade C (TV1 plus 1086 gp120) (weeks 12 and 24). Group 2 received two immunizations with ALVAC-C (weeks 0 and 4) and was boosted with two immunizations of ALVAC-C plus bivalent gp120 proteins from clade C (TV1 plus 1086 gp120) (weeks 12 and 24). Group 3 received two immunizations with ALVAC-vCP1521 (weeks 0 and 4) and was boosted with two immunizations of ALVAC-vCP1521 plus bivalent gp120 proteins from clade C (TV1 plus 1086 gp120) (weeks 12 and 24). Group 4 received two immunizations with ALVAC-vCP1521 (weeks 0 and 4) and was boosted with two immunizations of ALVAC-vCP1521 plus bivalent gp120 from clades B/E (AIDS VAX gp120) (weeks 12 and 24). The poxvirus-priming immunization was carried out at 0 and 4 weeks with the corresponding poxvirus vector (NYVAC-C, ALVAC-C, or ALVAC-vCP1521), and boosting occurred at weeks 12 and 24 with the combination of poxvirus vector plus HIV-1 gp120 proteins (from clades C

or B/E) (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, the 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 clade C with adjuvant MF59 or clades B/E with the adjuvant alum; 100 µg of total protein in 1.0 ml) was used for 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 for group 4 was essentially homologous to the one used in the RV144 phase III clinical trial (1), differing in the vaccine dose (the RV144 trial used a lower dose of ALVAC-vCP1521 [$>10^6$ of the 50% cell culture infectious dose] and a higher dose of AIDS VAX B/E gp120 [300 µg of each protein]). At weeks 0, 6, 14, and 26 (at the beginning of the study and 2 weeks after the second, third, and fourth immunizations, respectively), peripheral blood mononuclear cells (PBMCs) and serum and rectal swab samples were obtained from each immunized animal, and HIV-1-specific T-cell and humoral immune responses were analyzed (Fig. 1B). Blood samples were processed following current procedures (30).

ICS assay. The HIV-1-specific CD4⁺ and CD8⁺ T-cell immune responses induced at weeks 6, 14, and 26 were analyzed by polychromatic intracellular cytokine staining (ICS) from PBMCs obtained from each immunized rhesus macaque, as previously described (30). In short, cryopreserved PBMCs were thawed and rested overnight in R10 medium (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 37°C, 5% CO₂ 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 Golgi-Plug (10 µg/ml; BD Biosciences, San Jose, CA) for 6 h. Negative controls received an equal concentration of dimethyl sulfoxide instead of peptides. Subsequently, ICS was performed as described previously (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-Cy7-allophycocyanin (APC; clone SP34.2; BD Biosciences), gamma interferon (IFN-γ)-APC (clone B27; BD Biosciences), interleukin-2 (IL-2)-phycoerythrin (PE; clone MQ1-17H12; BD Biosciences), and tumor necrosis factor alpha (TNF-α)-fluorescein isothiocyanate (FITC; clone Mab11; BD Biosciences). An 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 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 in a binding antibody multiplex assay (BAMA) for IgG and IgA antibodies in serum and rectal week elutions from each immunized rhesus macaque at weeks 0, 6, 14, and 26, as previously described (31, 32). Antigens used to analyze the 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]), MuLV gp70-scaffolded V1/V2 (from clade C), and 1086 V1/V2 tags (all provided by H.-X. Liao and B. F. Haynes, Duke University [33]). Different plasma serial dilutions were made, and the results are expressed as the mean fluorescent intensity (MFI) and the titer (the area under the 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). Positive criteria were values that were 3-fold above 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 the HIV-1 Env gp160 of consensus clades A, B, C, D, group M, CRF01, and CRF02, as previously described (35, 36).

ADCC assay. The antibody-dependent cellular cytotoxicity (ADCC) assay performed according to the ADCC-GranToxiLux (GTL) procedure, as previously described (37, 38). The results of the GTL assay were considered positive if the percentage of Granzyme B activity after background subtraction was ≥8% for the infected target cells, determined during the standardization of our assay (39). The log₁₀ titer of the ADCC antibodies present in the plasma was calculated by interpolating the log₁₀ 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 pretitrated dose of different HIV-1 virus preparations (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 diethylaminoethyl 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 measurement of luminescence using the Britelite luminescence reporter gene assay system (PerkinElmer Life Sciences). Neutralization titers were the serum dilution at which relative luminescence units (RLU) were reduced by 50% compared to virus control wells after subtraction of background RLU 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 (for comparing two groups) and the Kruskal-Wallis test (for 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 were background subtracted. Box plots were used to summarize the distributions of various immune responses, where the midline of the box indicates the median, the ends of the box denote the 25th and 75th percentiles, and 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 shown in the box plots refer to the positive responses.

RESULTS

Immunogenicity in nonhuman 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 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 nonhuman primates divided into four different groups of immunized animals (8 animals/group). The immunization protocols were designed to compare head-to-head the NYVAC and ALVAC poxvirus vectors expressing the same HIV-1 antigens in homologous combination and together with an 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 Materials and Methods for details).

As these protocols aimed to trigger both HIV-1-specific T-cell

FIG 2 Immunization with NYVAC-C enhances the magnitude of HIV-1-specific CD4⁺ T-cell immune responses and induces a trend toward higher HIV-1-specific CD8⁺ T-cell immune responses. The total magnitudes of HIV-1-specific CD4⁺ (A) and CD8⁺ (B) T-cell responses elicited by the different immunization groups are shown. PBMCs were collected at weeks 6, 14, and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C), or A2AP2 (B/E, AIDSVAX). HIV-1-specific CD4⁺ and CD8⁺ T-cell immune responses triggered by the different immunizations were measured in an ICS assay following stimulation of PBMCs with HIV-1 Env, Gag, Pol, and Nef peptide pools. The values represent the sums 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 for one immunized macaque. Box plots represent the distribution of data values, with the line inside the box indicating the median value. P values were determined for significantly higher responses, comparing N2NP2 (C) to A2AP2 (C), at each week (*, $P < 0.05$).

and B-cell responses, with preferential antibody responses to Env, a comprehensive analysis with standardized and validated humoral antibody 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 2 weeks after the second, third, and fourth immunizations, respectively) (Fig. 1B).

NYVAC-C elicited larger HIV-1-specific CD4⁺ T-cell immune responses and a trend toward higher HIV-1-specific CD8⁺ T-cell immune responses than ALVAC-C. We measured the HIV-1-specific CD4⁺ and CD8⁺ T-cell immune responses elicited by the different immunization groups by using multiparameter flow cytometry with ICS, after the stimulation of PBMCs obtained from each immunized rhesus macaque at week 6, 14, and 26 with pools of peptides that spanned the HIV-1 Env, Gag, Pol, and Nef clade C regions present in the inserts expressed by NYVAC-C and ALVAC-C. HIV-1-specific CD4⁺ and CD8⁺ T-cell immune responses were determined based on the frequency of cells expressing IFN- γ and/or TNF- α and/or IL-2 for Env, Gag, Pol, and Nef peptide pools. For each T-cell subset, the response was considered positive if the value in the stimulated samples was greater than previously defined thresholds (41). Moreover, the ICS protocol used was defined previously in ICS qualification experiments (41).

The magnitude of the total HIV-1-specific CD4⁺ T-cell immune responses induced at week 14 by the immunization group N2NP2 (C) was significantly higher than that elicited by the immunization group A2AP2 (clade C) ($P < 0.05$) or by groups A2AP2 (clades B/E, C) and A2AP2 (clades B/E, AIDSVAX), respectively (Fig. 2A). At week 6 (2 weeks after the two priming immunizations), there were no differences between the immunization groups. However, 2 weeks following the booster immunizations (week 26), immunization with N2NP2 (C) induced higher HIV-1-specific total CD4⁺ T-cell immune responses, but this trend was not statistically significant.

On the other hand, at weeks 14 and 26, immunization with N2NP2 (C) elicited a trend toward greater-magnitude HIV-1-spe-

cific CD8⁺ T-cell immune responses than the other immunization groups (Fig. 2B), but the differences were not significant.

Notably, comparison of cytokine responses generated by N2NP2 (C) versus A2AP2 (C) revealed that at week 14, immunization with N2NP2 (C) induced a significantly higher 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 the 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 macaque 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 sequence (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 A2AP2 (B/E, AIDSVAX), from which binding IgG antibodies were either rarely present or of a lower magnitude. Furthermore, at week 6, immunization with N2NP2 (C) elicited a higher rate of responders than any A2AP2 immunization regimen (Fig. 4A to D). Moreover, at week 14 immunization with N2NP2 (C) sig-

FIG 3 Immunization with NYVAC-C enhances the magnitude of HIV-1-specific CD4⁺ T cells producing cytokines. The overall magnitudes 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) are shown. PBMCs were collected at weeks 6, 14, and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C) or 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 sums 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 represents the value for one immunized macaque. 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) each week (*, $P < 0.05$).

nificantly 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 A2AP2 (B/E, AIDSVAX). Additionally, at late time points (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 the 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, but only at weeks 14 and 26 (Fig. 5). The rates of responders against group M and clade C HIV-1 gp140 consensus at week 26 were higher in the NYVAC-C immunization

group than in the ALVAC-C group (80% versus 57% for both antigens) (Fig. 5A and data not shown).

In summary, these results showed that immunization with NYVAC-C resulted in a trend toward higher binding IgG antibodies against clade C HIV-1 gp140, gp120, and MuLV gp70-scaffolded V1/V2 proteins than did ALVAC-C.

NYVAC-C resulted in a trend toward higher levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A and B and the 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, by quantifying in individual serum samples obtained from each immunized rhesus macaque at weeks -1, 6, 14, and 26 the total binding IgG antibody levels against HIV-1 gp140 from clades A and B and the 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) pro-

FIG 4 Immunization with NYVAC-C induced 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 are shown. Individual serum samples were obtained at weeks -1, 6, 14, and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C), or A2AP2 (B/E, AIDS VAX). Binding IgG antibodies were measured by BAMA, as indicated in Materials and Methods. The magnitudes of the antibody responses are expressed as the AUC from serial dilutions of plasma. Each dot represents the value for one immunized macaque. P values indicate significantly higher levels, comparing N2NP2 (C) to A2AP2 (C), at each week (*, $P < 0.05$).

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

In summary, these results showed that during the priming phase, immunization with NYVAC-C induced higher levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A and B and the 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 and B and the 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 correlated 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, AIDS VAX] immunization groups) to evaluate linear epitope specificities in a peptide microarray against Env gp160 of consensus clades A, B, C, and D and group M, CRF01, and CRF02. The results showed that a V3 response dominated the

binding response in most animals, consisting on average of 41% of total gp160 binding, followed by binding to the C5 (24%) and C1-V1 (11%) epitopes (Fig. 7A and B). Other linear epitope-specific responses were detected against 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 were linked to 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, AIDS VAX) to induce ADCC responses against clade C HIV-1 gp120 from isolate TV1 in individual plasma samples obtained from each immunized rhesus macaque 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, AIDS VAX) in individual serum samples obtained from each immunized rhesus macaque 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 the NYVAC-C and ALVAC-C immunization groups, when we used the TZM.BL neutralization assay (Fig. 9). Similar results

FIG 5 Immunization with NYVAC-C or 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), or MuLV gp70-scaffolded V1/V2 proteins (D) induced by the different immunization groups are shown. Individual rectal week samples were obtained at weeks -1, 6, 14, and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C), or A2AP2 (B/E, AIDS VAX). Rectal binding IgG antibodies were measured by analyzing the binding magnitude normalized to total rhesus IgG (specific activity), as described in Materials and Methods. Each dot represents the value for one immunized animal.

were obtained in the A3R5.7 neutralization assay (data not shown). Of note, immunization with N2NP2 (C) and A2AP2 (C) resulted in better neutralization against HIV-1 clade C virus isolates (strain MW965.26), whereas immunization with A2AP2 (B/E, AIDS VAX) elicited 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 we analyzed 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 no 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 an 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, AIDS VAX), quantifying in individual serum samples obtained

from each immunized rhesus macaque 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 Fig. 10, we 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 clades A, B, or C or group M consensus analyzed 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 Thailand, which entailed 16,000 volunteers at risk of infection, 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

FIG 6 Immunization with NYVAC-C or ALVAC-C induces a trend toward higher levels of cross-clade binding IgG antibodies against HIV-1 gp140 from clades A or B and the group M consensus. Total cross-clade binding IgG antibody levels against HIV-1 gp140 consensus from clade A (a1Con) (A), clade B (bCon) (B), or the group M consensus (sCon) (C) induced by the different immunization groups are shown. Individual serum samples were obtained at weeks -1, 6, 14, and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C), or A2AP2 (B/E, AIDSVAX). Binding IgG antibodies were measured by BAMA, as described in Materials and Methods. The magnitude of the antibody response is expressed as the AUC from serial dilutions of plasma. Each dot represents the value for one immunized macaque. P values indicate significantly higher levels, comparing N2NP2 (C) to A2AP2 (C) at each week (*, $P < 0.05$).

clade E (gp120) and clade B (Gag/Pro) and bivalent HIV-1 gp120 proteins from clades B/E showed 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 for HIV/AIDS vaccine candidates.

Several recombinant poxvirus vectors (including MVA, NYVAC, canarypox virus, and fowlpox virus) expressing different HIV-1 antigens have been broadly used in several human clinical trials, proving that they are safe and immunogenic and induce HIV-1-specific cellular and humoral immune responses (reviewed in references 2 to 4 and 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 use of vectors with enhanced replication capacity, coexpression of immunomodulators, heterologous prime-boost approaches, and removal of poxviral genes that antagonize host cell-mediated immune responses (for reviews, see references 2 and 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 issue was examined by using a similar prime-boost immunization regimen as in the RV144 trial, comparing head to head in immunized rhesus macaques the T-cell and humoral immune responses against HIV-1 antigens triggered by two poxvirus vectors (ALVAC-C and NYVAC-C) expressing identical and optimized clade C trimeric gp140 and the Gag-Pol-Nef polypeptide as Gag-derived VLPs. Furthermore, this comparison used as a benchmark the immune responses elicited by both vectors to the same immunogens and

vaccination protocol as used 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 postimmunization 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 an 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 indicated 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 and 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 phenotypical and chemokine responses in monocytes (49). Furthermore, immunization with A2AP2 (B/E, AIDSVAX) also induced good cross-clade antibody responses against clade C HIV-1

FIG 7 NYVAC-C and ALVAC-C induce IgG antibodies mainly directed against the V3 loop. Plasma linear IgG binding epitope specificities against different linear epitopes covering HIV-1 gp160 of seven consensus sequences from clades A, B, C, or D or group M, CRF01, and CRF02 in a subset of animals that developed strong binding IgG antibodies are shown. (A) Percentage of total gp160 binding specific for each epitope. The percentage of total gp160 binding was defined as the maximum binding to the epitope divided by the sum of maximum binding to all epitopes identified. Each slice represents the average percentage for the 7 animals mapped. (B) Binding magnitude to each linear epitope identified, shown as the maximum binding level (signal intensity) to a single peptide in each epitope region. The region of each epitope identified (shown as the range of peptides included in the array library) is listed in parentheses in the column labels, under each epitope name.

FIG 8 Immunization with NYVAC-C and ALVAC-C induces a similar level of ADCC responses against HIV-1. ADCC activity induced by the different immunization groups is shown. Individual plasma samples were obtained at weeks 0 and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C), or A2AP2 (B/E, AIDSVAX). ADCC activity was measured as described in Materials and Methods. Each dot represents the value for one immunized macaque. Box plots represent the distribution of data values, with the line inside the box indicating the median value.

gp140 and gp120 antigens. Moreover, rectal binding IgG antibody levels against HIV-1 gp140 group M consensus (sCon), gp120 from isolate 1086, gp120 from isolate TV1, and MuLV gp70-scaffolded V1/V2 proteins induced by the different immunization groups were comparable. Furthermore, NYVAC-C and ALVAC-C elicited comparable levels of ADCC responses and of neutralizing antibodies and similar low levels of binding IgA antibodies. Of 8 animals only 1 showed high levels of IgA against gp70 V1/V2 and clade C gp120 1086 in the N2NP2 (C) group; however, this macaque had low levels of IgA antibodies against clades A, B, and C and group M. Also, the group of animals that received the vaccine that was similar to that in the RV144 trial had low IgA antibody responses. These observations clearly showed that these protocols trigger low IgA responses. The induction by NYVAC-C of a trend toward higher levels of binding IgG antibodies against HIV-1 gp140, gp120, and MuLV gp70-scaffolded V1/V2 and low levels of binding IgA antibodies against Env is particularly important since antibody responses against V1/V2 loops of HIV-1 gp120 correlated with a lower infection risk in RV144, whereas higher plasma levels of Env-specific IgA correlated with a lack of protection (33). These

FIG 9 Immunization with NYVAC-C or ALVAC-C induces a similar level of neutralizing antibodies against HIV-1. Neutralization titers and the percentage of responders induced by the different immunization groups are shown. Individual serum samples were obtained at weeks -1, 6, 14, and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C), or 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 by using the TZM-bl assay, as described in Materials and Methods. Each dot represents the value for one immunized macaque. Blue dots indicate nonresponders, and red dots are results for 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.

improvements in NYVAC-C were likely attributable to the higher magnitude of HIV-1-specific CD4⁺ T helper cells induced in the NYVAC-C immunization group. Thus, the immunological profiles elicited by NYVAC-C and ALVAC-C are compatible with possible protective mechanisms against HIV-1, including induction of HIV-1-specific CD4⁺ and CD8⁺ T-cell immune responses (50–54) and high levels of IgG antibodies directed against HIV-1

gp120 and MuLV gp70-scaffolded V1/V2, together with low levels of IgA antibodies against HIV-1 gp120 and MuLV gp70-scaffolded V1/V2 (33). Challenge studies in nonhuman 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 poxvirus vector,

FIG 10 Immunization with NYVAC-C or ALVAC-C induces low levels of binding IgA antibodies against HIV-1 gp120 or 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 are shown. Individual serum samples were obtained at weeks 0 and 26 from each rhesus macaque ($n = 8$ per group) immunized with N2NP2 (C), A2AP2 (C), A2AP2 (B/E, C), or A2AP2 (B/E, AIDS-VAX). Binding IgA antibodies against MuLV gp70-scaffolded V1/V2 and gp120 from isolate 1086 were measured by BAMA, as described in Materials and Methods. The magnitude of the antibody response is expressed as the fluorescence intensity, minus the background intensity (FI bg) at a dilution of 1/80. Each dot represents the value for one immunized macaque. Box plots represent the distribution of data values, with the line inside the box indicating the median value.

as the viral genomes of NYVAC and ALVAC differ in their contents of immunomodulatory genes, use of promoters, and 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 could influence the HIV-1-specific T-cell and humoral immune responses elicited by recombinant ALVAC-C versus NYVAC-C.

A head-to-head comparison of NYVAC and ALVAC vectors expressing Gag-Pol-Env from simian immunodeficiency virus (SIV) has been undertaken, but it is being conducted in SIVmac251-infected rhesus macaques treated with antiretroviral therapy (ART) (56). The study's 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 evaluated. The advantage of these vectors is that they express independently Env and Gag-Pol-Nef and induce 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 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 that expresses HIV-1 gp120 and Gag-Pol-Nef proteins from clade C 97CN54. This has been tested as a homologous component in a phase I clinical trial (EV01) in healthy volunteers that demonstrated a safety profile and induced 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) re-

sulted in significantly enhanced HIV-1-specific T- and B-cell immune responses (25), though again with an Env antigen-specific bias that was polyfunctional and long-lasting (13). In another recent human clinical trial (HVTN078), NYVAC was used in combination with an adenovirus 5 (Ad5)-based HIV vaccine, and 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 preexisting 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 nonneutralizing 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 induce 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 remain to be determined.

Overall, this head-to-head comparison in nonhuman 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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