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Replication-Competent NYVAC-KC Yields Improved Immunogenicity to HIV-1 Antigens in Rhesus Macaques Compared to Nonreplicating NYVAC

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ABSTRACT As part of the continuing effort to develop an effective HIV vaccine, we generated a poxviral vaccine vector (previously described) designed to improve on the results of the RV144 phase III clinical trial. The construct, NYVAC-KC, is a replication-competent, attenuated recombinant of the vaccinia virus strain NYVAC. NYVAC is a vector that has been used in many previous clinical studies but is replication deficient. Here, we report a side-by-side comparison of replication-restricted NYVAC and replication-competent NYVAC-KC in a nonhuman primate study, which utilized a prime-boost regimen similar to that of RV144. NYVAC-C and NYVAC-C-KC express the HIV-1 antigens gp140, and Gag/Gag-Pol-Nef-derived virus-like particles (VLPs) from clade C and were used as the prime, with recombinant virus plus envelope protein used as the boost. In nearly every T and B cell immune assay against HIV-1, including neutralization and antibody binding, NYVAC-C-KC induced a greater immune response than NYVAC-C, indicating that replication competence in a poxvirus may improve upon the modestly successful regimen used in the RV144 clinical trial.

IMPORTANCE Though the RV144 phase III clinical trial showed promise that an effective vaccine against HIV-1 is possible, a successful vaccine will require improvement over the vaccine candidate (ALVAC) used in the RV144 study. With that goal in mind, we have tested in nonhuman primates an attenuated but replication-competent vector, NYVAC-KC, in direct comparison to its parental vector, NYVAC, which is replication restricted in human cells, similar to the ALVAC vector used in

RV144. We have utilized a prime-boost regimen for administration of the vaccine candidate that is similar to the one used in the RV144 study. The results of this study indicate that a replication-competent poxvirus vector may improve upon the effectiveness of the RV144 clinical trial vaccine candidate.

Though recombinant poxviruses have long been used to express foreign genes in the development of vaccines against multiple disease-causing organisms (1, 2), most of the candidates have been based on the modified and highly attenuated poxviruses: NYVAC (3), MVA (4), and ALVAC (3, 5). Replication of these viruses is restricted in human cells, thus offering the advantage of greater safety than use of replication-competent poxviruses. The safety of attenuation, however, has frequently been achieved at the expense of decreased immunogenicity, driving the field of human immunodeficiency virus (HIV) vaccine development to explore the use of attenuated, replication-competent vectors (reviewed in reference 6).

The first HIV-1 vaccine candidate to demonstrate partial success was the ALVAC-based regimen used in the phase III RV144 clinical trial conducted in Thailand (7). ALVAC is a canary poxvirus and does not replicate in human cells. The results of RV144 were released in 2009, and since then the HIV vaccine field has been working to improve the modest 31% efficacy achieved by the ALVAC vector combined with administration of HIV-1 gp120 protein in a prime-boost model. With this goal in mind, we based our HIV vaccine vector on NYVAC rather than ALVAC and made it replication competent in human cells. This was accomplished by reinsertion of the *K1L* and *C7L* genes, which were two host range viral genes of the 18 open reading frames originally deleted from the Copenhagen strain to create NYVAC (3). The resulting construct, NYVAC-KC, has been previously described (8). In the newborn-mouse model of pathogenesis, NYVAC-KC constructs clustered near those of the replication-deficient NYVAC and MVA vectors, and the 50% lethal dose (LD_{50}) was about 4 logs higher than that of wild-type vaccinia virus. This model is the most sensitive measure we have of poxvirus pathogenicity and demonstrates that NYVAC-KC is highly attenuated even though it is replication competent in human cells. Enhanced expression of the antigen in human cells from this replication-competent vector has been verified (8). Here, we report immunogenicity study results in nonhuman primates (NHPs), comparing immunization with NYVAC versus that with NYVAC-KC. Both viruses expressed novel HIV-1 clade C proteins (9) and therefore are identified as NYVAC-C and NYVAC-C-KC in this NHP study. The study design has combined the advantages of a prime-boost regimen, which were demonstrated in the RV144 trial, with the enhanced antigen expression of a replication-competent vector. The replication-competent vector demonstrated greater HIV-1-specific T cell responses, greater IgG binding to both autologous and heterologous envelope glycoproteins (Envs) and to the V1-V2 loop, and greater neutralization of a panel of pseudotyped tier 1 virus-like particles (VLPs) in TZM-bl assays. Combined, these results provide evidence that the NYVAC-KC construct holds promise as an improved HIV vaccine vector.

RESULTS

Study plan. Eight rhesus macaques were assigned to each of two groups. The macaques were randomized, including by weight and Mamu allele status (two in each group were A*01-positive, and one in group 1 was also B*17-positive; all were B*08-negative). As shown in Fig. 1, group 1 was immunized at weeks 0 and 4 with NYVAC-C-KC combined viruses [mixture of NYVAC-KC-Envgp140(96ZM651) plus NYVAC-KC-Gag(96ZM651)-Pol-Nef(97CN54)], and group 2 was immunized at weeks 0 and 4 with NYVAC-C combined viruses ([NYVAC-Envgp140(96ZM651) plus NYVAC-Gag(96ZM651)-Pol-Nef(97CN54)]. Both groups were then boosted with the respective virus plus protein (gp120 plus MF59 adjuvant in place of gp120 plus Rehydragel
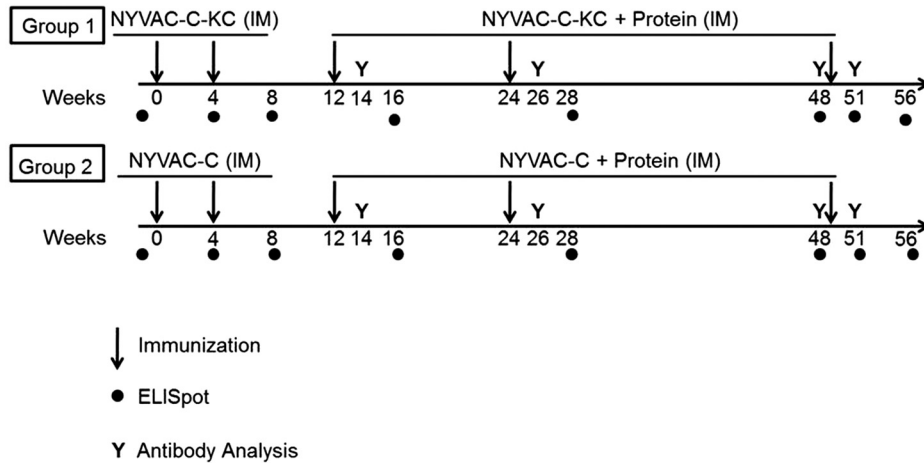
FIG 1 Immunization schedule for the virus prime and virus/protein boost regimen. Two groups of 8 macaques were each immunized twice with virus alone (either NYVAC-C-KC or NYVAC-C), followed by three immunizations with virus plus protein. All immunizations were by the intramuscular (i.m.) route. Blood was collected for ELISpot analysis or antibody analysis at the indicated time points.

as used in the RV144 trial) on weeks 12, 24, and 49. All inoculations were intramuscular (i.m.).

The animals were monitored for any adverse events throughout the study. As in previous studies (10–15), the immunizations were well tolerated. In group 1, one animal following the first virus-only immunization and one following the second virus-only immunization had mild erythema at the inoculation site (both were grade 1). Animals gained weight normally, and there were no noted abnormalities in appetite.

T cell responses. PBMCs were restimulated with peptide sets providing coverage of the HIV-1 antigens, and gamma interferon (IFN-γ)-producing cells were quantified in an enzyme-linked immunosorbent spot (ELISpot) assay. At all time points beginning with week 16, after the third immunization, NYVAC-C-KC induced a statistically significant greater response to the peptides than NYVAC-C (Fig. 2A). In general, the response from both groups was low using this immunization regimen compared to that with the regimen used in the companion paper in this issue (16), and the fold difference between groups, even when significant, ranged from 2 to 8. No animal had a response above 2,000 spot-forming cells (SFC) per 10^6 cells until week 51, with earlier responses clustering in the range of 400 to 1,000 SFC (Fig. 2A). In group 1, which consisted of the animals immunized with NYVAC-C-KC, there was an increase in both the range of SFC counts and total number of SFC at 4 weeks following the immunizations done at weeks 12 and 24, and the greatest increase for both groups came with the sample taken 2 weeks following the week 49 immunization. These results are in contrast to what was observed with the regimen in a parallel study (see companion manuscript of Asbach et al. [16]) in which the prime was DNA, and the response in each of the groups reached above 10,000 SFC after the animals were boosted with NYVAC-C or NYVAC-C-KC. Figure 2B demonstrates the analysis of responders to either immunization at various time points after immunization. While animals immunized with NYVAC-C-KC had T cell responses after the third immunization, appreciable T cell responses were induced with NYVAC-C only after the fourth immunization. While the number of responders in the NYVAC-C-KC arm was stable (7 or 8 responders) from week 16 through the end of the study at week 56, the number of responders in the NYVAC-C arm of the study waned with time after immunization and the number of responders was boosted, even by the fifth immunization at week 48. Thus, animals immunized with NYVAC-C-KC responded earlier than animals immunized with NYVAC-C, and the response was sustained throughout the course of the study.

Characteristics of T cell responses. We then examined the polyfunctionality of the CD4$^+$ and CD8$^+$ T cell responses. Peripheral blood mononuclear cells (PBMCs) from
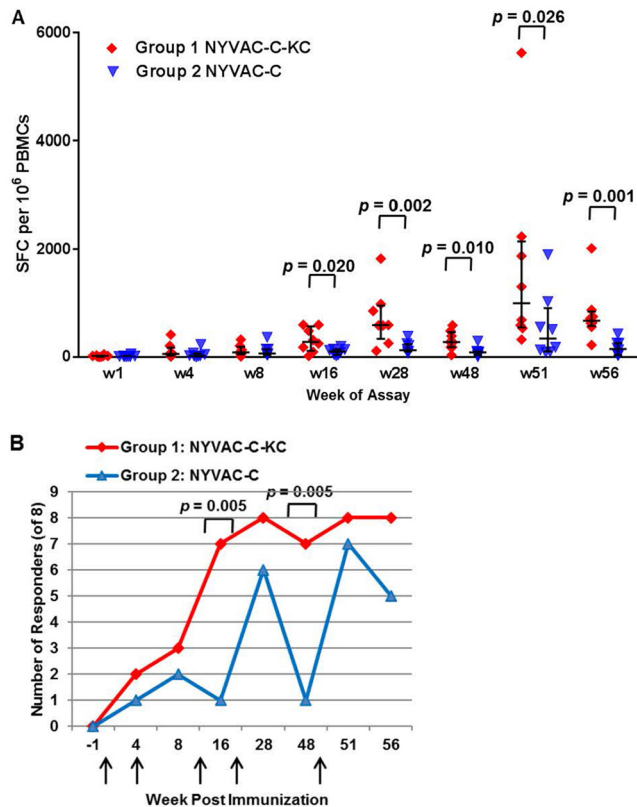
FIG 2 The replication-competent NYVAC-C-KC induces a greater T cell response does replication-deficient NYVAC-C. PBMCs were freshly isolated at the indicated time points and restimulated with a pool of peptides in an ELISpot assay. The number of spot-forming cells (producing IFN-γ) per 10^6 cells is shown. (A) Each value is the sum of the responses from the nine peptides per each of the 8 animals. Statistical significance was determined using the Mann Whitney method, with a confidence interval of 95%. w, week. (B) The number of animals in each group ($n = 8$) that were responders is shown for each time point tested. Criteria for responders were defined as ≤50 SFC/10^6 cells and a 4-fold increase above the baseline level for any pool of peptides. Immunizations were at weeks 0, 4, 12, 24, and 49 (indicated by arrows). Statistical significance was determined using a Wilcoxon rank sum test (A) and by a Fisher exact test (B).

each of the time points indicated in Fig. 3 were stimulated with nine peptide pools. Staining for surface markers and for cytokine production (IFN-γ, interleukin-2 [IL-2], and tumor necrosis factor alpha [TNF-α]) provided a polyfunctionality profile to compare results with the replication-deficient NYVAC-C to those with the replication-competent NYVAC-C-KC. For CD4 T cells, at all three time points (weeks 8, 28, and 48), the percentage of responses that included all three of the assayed cytokines was higher for group 1 animals than for group 2 (Fig. 3A). The polyfunctionality of the CD4$^+$ responses at weeks 8 and 28 was significantly higher for the NYVAC-C-KC-inoculated animals than for those inoculated with NYVAC-C (Fig. 3B), with a 5-fold maximum difference. The CD8$^+$ responses were similar in distribution to those of CD4$^+$ responses, with the triple response greater for group 1 than for group 2 (Fig. 3C) though these were not statistically significant differences (Fig. 3D).

Magnitude of Ab responses. Antibody (Ab) responses to HIV-1 Env and Gag antigens in each group were analyzed in serum samples taken 2 weeks after the first virus/protein immunization done at week 12, 2 and 24 weeks after the second virus/protein immunization done at week 24, and 2 weeks after the third virus/protein immunization done at week 49 (week 51) (Fig. 4). The magnitude of both IgG and IgA binding activities to a set of eight different envelope-derived and two Gag-Pol-derived antigens was measured by a binding antibody multiplex assay (17, 18). Antibody responses were detected at all time points and in similar patterns for both groups.
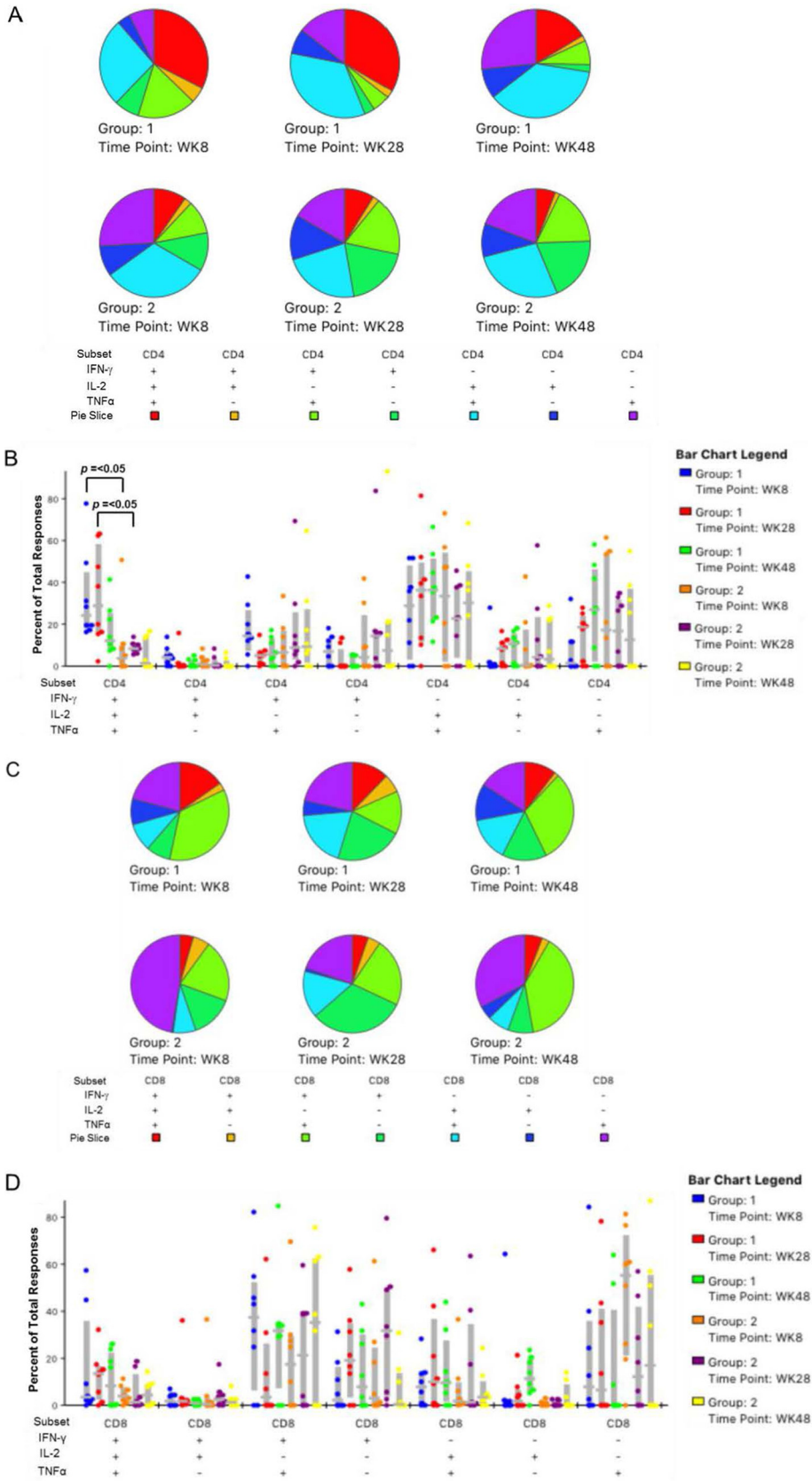
FIG 3 The replication-competent NYVAC-C-KC (group 1) induces more polyfunctional CD4$^+$ and CD8$^+$ T cell responses than replication-deficient NYVAC-C (group 2). PBMCs were obtained at the indicated time points, stimulated with the nine peptide pools, and stained for CD3, CD4, and CD8, as well as intracellular IFN-γ, IL-2,

(Continued on next page)

However, with few exceptions, the antibody responses from group 1 animals, immunized with NYVAC-C-KC, were significantly greater than those from group 2 animals, immunized with NYVAC-C; for these responses, the fold change between the groups averaged 1.5. The IgG responses to five of the antigens are shown in Fig. 4A: consensus M gp140, consensus C gp140, 1086 gp140, TV1 gp120, and p24. Response patterns were similar following both of the first two virus/protein immunizations (weeks 12 and 24) though the second response was slightly improved. Responses then declined during the period of weeks 26 to 48 and rebounded following the week 49 immunization. In a comparison of the responses of all tested antigens at weeks 14 and 51 (Fig. 4B), the trend in the sera suggested that the reactivity is greatest against autologous antigens: the protein immunizations were derived from TV1, and the 1086 isolate is closely related. The next greatest reactivity was to the consensus sequences (M and C), which would be expected for group C because the immunization antigens are derived from subtype C isolates; however, the response to consensus M gp140 was nearly identical to that of consensus C, indicative of at least some level of cross-reactivity. Other indications of cross-reactivity are the responses to the B and A1 subtype consensus proteins, as well as to other isolates from these subtypes, which were robust although reduced compared to those of the autologous antigens. At all time points postimmunization, NYVAC-C-KC induced greater IgG titers to the HIV-1 envelope than NYVAC-C. Overall, the results demonstrated an improved response with the replication-competent NYVAC-C-KC constructs. Antibodies to Gag, expressed in the vector prime-only regimen, were also elicited. There was no response to reverse transcriptase.

Both groups' responses to IgA were very low (Fig. 4C), and the responses of the two groups were comparable. There were no statistical differences between the responses of either group at any time point and those of the prebleed. Since certain specifications of plasma IgA responses correlated directly with HIV-1 risk in the RV144 study, one hypothesis is that a low plasma Env IgA response, as observed in this study, may be desired as part of a protective immune response (19, 20).

In contrast to IgA responses, the response to the V1-V2 region of Env was strong at a dilution of 1:2430 in an enzyme-linked immunosorbent assay (ELISA), as shown in Fig. 5A, and at the greater dilution of 1:7290 (Fig. 5B) the response was still detectable. At both dilutions, NYVAC-C-KC induced greater reactivity than NYVAC-C.

Functional characteristics of antibody responses. Serum samples were also measured for HIV-1 neutralizing antibody responses in a TZM-bl assay. A profile was created based on results from a panel of viruses (nine tier 1 and three tier 2) with pseudo-Envs (12 pseudo-Envs were measured at week 26, and various combinations of up to 9 were measured at the other time points). Results are shown on a magnitude-breadth (MB) curve in Fig. 6, which indicates that at all time points the NYVAC-C-KC group had an overall broader neutralizing response per animal than the NYVAC-C group. During the span of the time course, the responses among the 8 animals of each group became more consistent, resulting in lower P values over time. By week 48, just prior to the final immunization, and by week 51, 2 weeks following the final immunization on week 49, the difference in the neutralizing antibody responses between the replication-competent NYVAC-C-KC and the replication-deficient NYVAC-C was statistically significant. For both NYVAC-C and NYVAC-C-KC, most of the positive activity was detected against tier 1 viruses (see Data Set S1 in the supplemental material).

We also examined individual responses to the specific pseudotyped virus-like particles. Responses to MW965.26 (tier 1, clade C) and MN.3 (tier 1 A, clade B) at all time

FIG 3 Legend (Continued)

and TNF-α. Responding CD4$^+$ (A and B) and CD8$^+$ (C and D) T cells were quantified by flow cytometry. (A) The fraction of triple-positive (red) responses for NYVAC-C-KC vaccinees (group 1) was greater at every time point. (B) Responses at weeks 8 and 28 were significantly higher for NYVAC-C-KC vaccinees than for NYVAC-C vaccinees (group 2). (C) The fraction of triple-positive (red) responses for NYVAC-C-KC vaccinees was greater at every time point. (D) Though not statistically significant, the responses at nearly all time points were higher for those animals vaccinated with NYVAC-C-KC than with NYVAC-C. Statistics calculated by Wilcoxon signed rank test using SPICE software (NIAID).
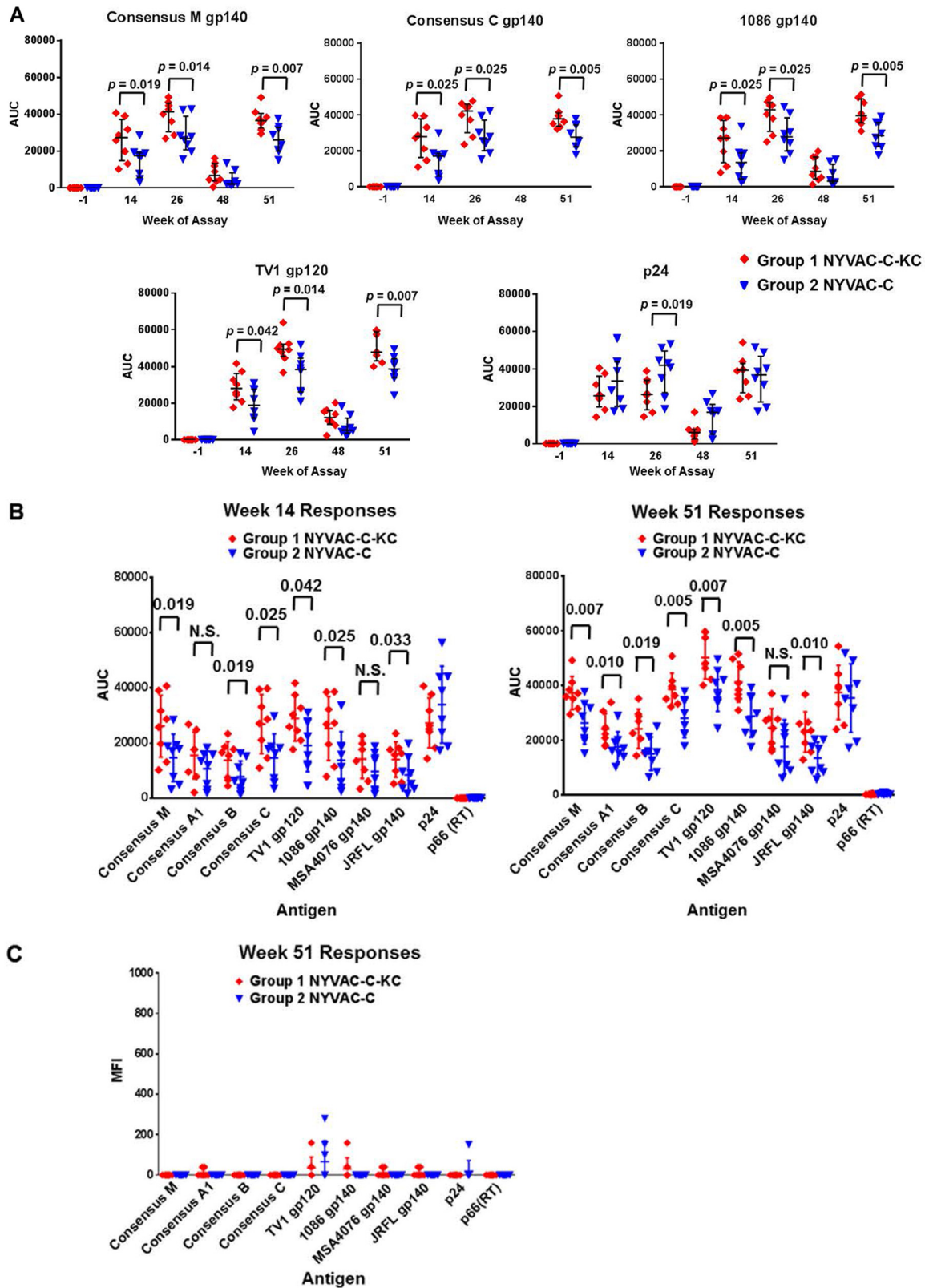
FIG 4 NYVAC-C-KC (group 1) gave improved IgG antibody responses to HIV-1 antigens and both groups had low responses to IgA. (A) A customized binding antibody multiplex assay was used to quantify IgG antibodies that bound to antigen-coated beads. The binding response for each antigen per each animal is shown as the area under the curve (AUC) of the titration curve, and medians and interquartile ranges are shown as horizontal lines. (B) Comparative responses of the week 14 and week 51 sera toward all antigens assessed. Statistical significance was determined using a Wilcoxon rank sum test (for panels A and B). (C) IgA responses of the week 51 sera toward all antigens assessed.
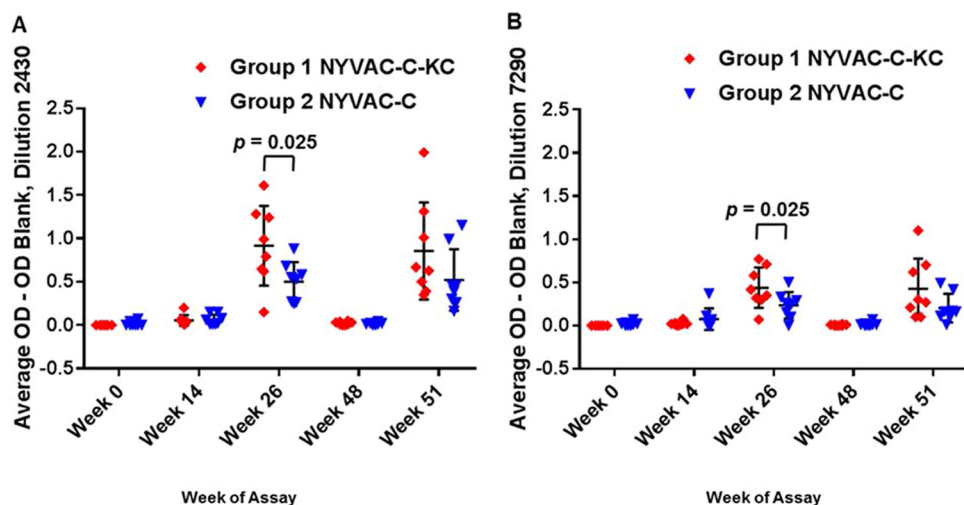
FIG 5 Antibody response to the V1-V2 region was strong for both NYVAC-C-KC and NYVAC-C groups. Response was measured by an ELISA, and the results for dilutions of 1:2430 (A) and 1:7290 (B) are shown. At both dilutions, the antibody levels following the week 24 boost were significantly higher for NYVAC-C-KC (group 1) than for NYVAC-C (group 2). P values were calculated by the Wilcoxon rank-sum test. OD, optical density.

points are shown for neutralization in TZM-bl cells (Fig. 7A). For MW965.26 and MN.3, at all responsive time points postimmunization, the NYVAC-C-KC group demonstrated greater responses than the NYVAC-C group; the differences were statistically significant for MN.3, with fold differences of about 1.5. The values of the 50% infective doses (ID_{50}) for all isolates at weeks 26 and 51 are shown in Fig. 7B. There was neutralizing activity against tier 1 A viruses (MW965.26, MN.3, SF162, and SHIV-SF162P4 SHIV is simian-human immunodeficiency virus), and in each case, animals immunized with NYVAC-C-KC demonstrated a trend toward greater neutralization.

Serum from immunized animals was also tested for ADCC and ADCVI activity. The animals immunized with the replication-competent NYVAC-C-KC (group 1) responded to a greater extent (about 4-fold higher) in an antibody-dependent cell-mediated cytotoxicity (ADCC) assay (Fig. 8A) at week 26 than those receiving the replication-deficient NYVAC-C (group 2). This assay measures the release of granzyme B from natural killer cells (NK cells); such an Ab response has been associated with protective responses and with delayed onset of AIDS in HIV-infected individual patients and infected macaques (21–25). In the antibody-dependent cell-mediated virus inhibition (ADCVI) assay, the two groups had very similar, and strong, responses (Fig. 8B). In this assay, PBMCs were used as effector cells, with serum added to CEM.NKR$_{CCR5}$ cells that had been infected with infectious HIV-1 strain DU156 or DU422. The strong responses indicate that both vaccine candidates induced antibodies capable of binding to HIV-1 peptides, thus making the cells coated with viral proteins a target of NK cells.

DISCUSSION

In this study, we have examined the immune response of the replication-restricted poxvirus vector NYVAC in a head-to-head comparison with the replication-competent NYVAC-KC vector in an NHP model and have shown that the replication-competent NYVAC-C-KC expressing HIV-1 antigens induces greater HIV-1-specific T cell responses in NHPs than NYVAC expressing the same HIV-1 antigens, as well as a greater HIV-1-specific humoral response in the regimen that was utilized. Our hypothesis was that the results of RV144 (19, 26) could be improved upon by using a vector that achieved higher levels of antigen expression than levels attained with a replication-deficient vector and that the higher levels could be reached through use of a vector that is replication competent in human cells (8). Each of the two groups in this study was primed and boosted (once) with virus only and then boosted three times more but with virus plus protein at each of the final three time points. This regimen was based on that
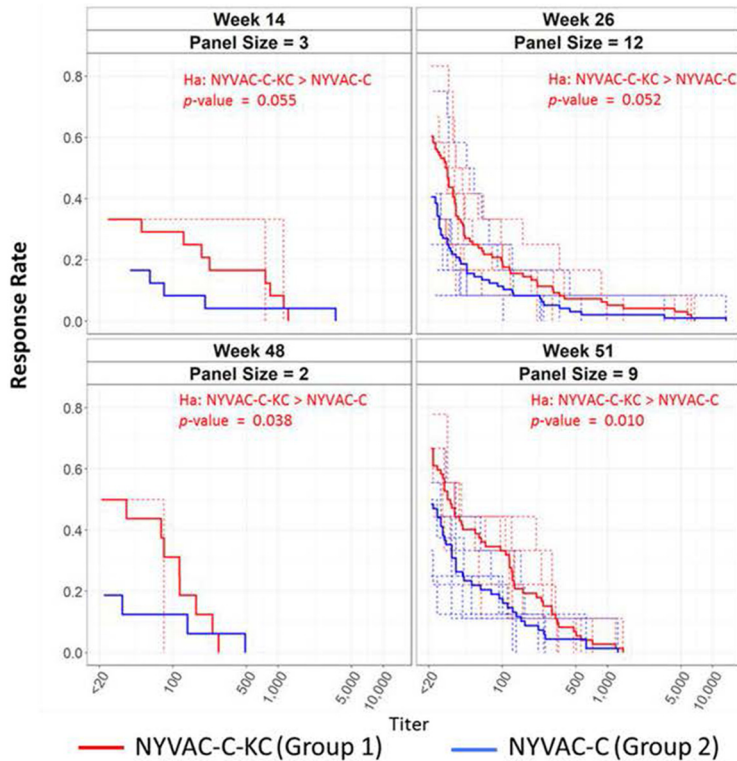
FIG 6 Neutralizing antibody panel potency subject-specific and average magnitude breadth curves for TZM-bl assay. A magnitude-breadth (MB) curve of 50% inhibitory dose (ID$_{50}$) neutralizing antibody titers against pseudoenvelopes shown over time (x axis, neutralization titers; y axis, fraction of viruses neutralized). Isolates were the following: MN.3, MW965.26, and TV1.21 for week 14; TV1.21, BaL.26, Bx08.16, MN.3, MW965.26, SF162.LS, SHIV SF162P4, SHIV-SF162.P3, SS1196.1, SHIV 1157ipEL-p, SHIV 1157ipd3N4, and SHIV BAL-P4 for week 26; MN.3 and MW965.26 for week 48; TV1.21, BaL.26, Bx08.16, MN.3, MW965.26, SF162.LS, SHIV SF162P4, SHIV-SF162.P3, and SS1196.1 for week 51. Values of <20 (limit of detection of the assay) were assigned a value of 10. Dashed lines represent subject-specific responses. Solid lines represent group averages. P values were derived from a Wilcoxon rank sum test.

of RV144, which was the same schedule with the single exception that this study had a fifth (final) inoculation whereas the RV144 trial had only the first four administered.

NYVAC-C has been studied previously in a head-to-head comparison with ALVAC-C, with both vector mixtures expressing the same antigens and used in identical regimens (11); in that study, NYVAC-C was found to induce improved T cell and humoral responses compared to those of ALVAC-C. Therefore, this current report focused on the comparison between NYVAC-C and NYVAC-C-KC in our efforts to improve upon the RV144 immune responses; we started with the premise that if NYVAC has an improved response compared to that of ALVAC, then a response that exceeds that of NYVAC is also an improvement compared to that of RV144. Retrospective analyses of RV144 have shown that the correlates of protection are complex and most likely will be dependent on many variables and combinations of variables (27–38). Therefore, with this study we have tried to gain perspective based on some of the ideas brought forth in retrospective reviews of what was learned from RV144.

The T cell responses with the poxvirus/protein regimen in this report were modest, and while differences between the two groups were statistically significant, they ranged from minimal to 5-fold changes. In contrast, in the companion study in this issue (16), the magnitude of the T cell response was greater, whether measuring IFN-γ responses, HIV-1-specific CD4$^+$ and CD8$^+$ T cell responses, or polyfunctionality of the responses; this is most likely a result of the DNA prime used in the companion study. For a more in-depth comparison of the T cell responses, please see the companion paper by Asbach et al. (16).

IgG antibody responses in the study reported here were more robust than the T cell
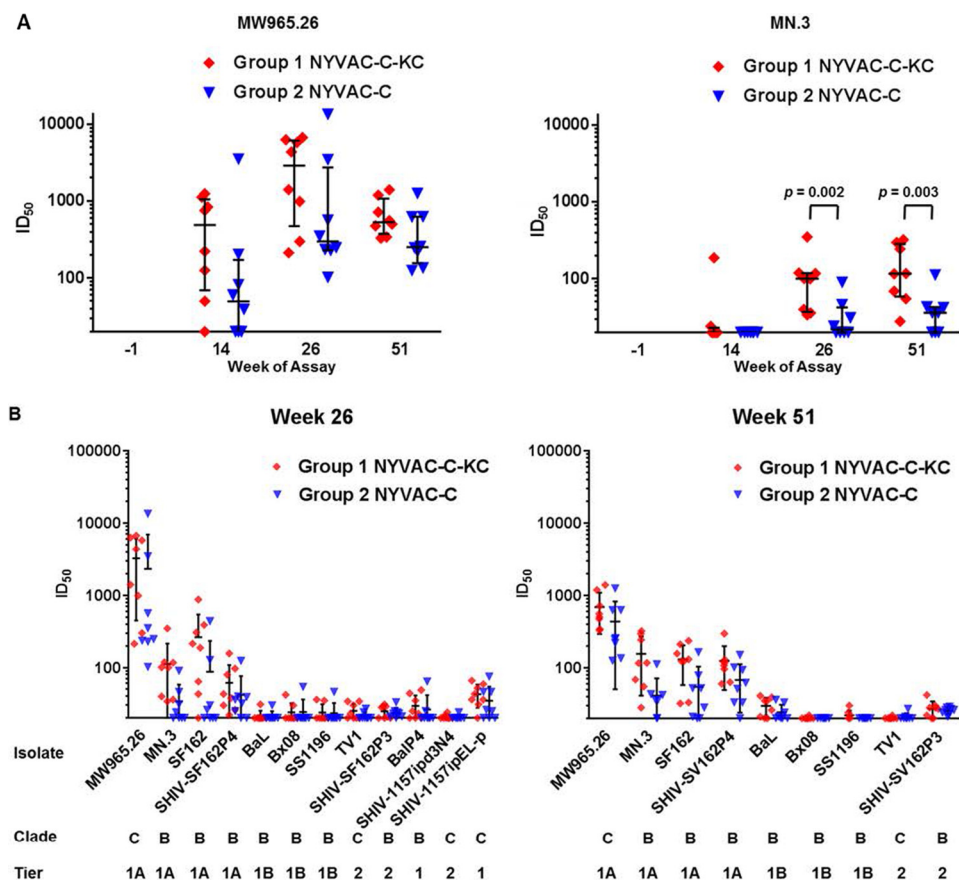
FIG 7 Macaque sera neutralization in TZM-bl assay. (A) Serum samples were diluted and tested for neutralization against the indicated pseudoviruses. The value shown is the reciprocal of dilution at which 50% inhibition (ID_{50}) was observed for each animal; median and interquartile range are shown at the indicated time points of the pseudoviruses carrying MW965.26 or MN.3. Statistical significance was determined using a Mann-Whitney method. (B) ID_{50} values of the serum samples from week 26 and week 51 for pseudoviruses carrying Envs from the indicated isolates.

responses, and the replication-competent NYVAC-C-KC responses at most time points were significantly higher than those of the replication-restricted NYVAC-C. In many cases, direct comparison of the results in this report to RV144 humoral response assays is difficult because the assays were not performed identically between the clinical trial and this NHP study. While it is difficult to directly compare responses in humans to responses in macaques, it would appear that NYVAC-C-KC induced a much stronger binding response to V1-V2 than ALVAC did in RV144. We have previously shown that NYVAC was an improved vector over ALVAC in a head-to-head comparison in NHPs (11). In this study, we show that in IgG binding assays, in V1-V2 loop-binding assays, in neutralizing antibody assays, and in the ADCC assay, NYVAC-C-KC induced a greater response than the replication-restricted NYVAC-C. Thus, we believe that NYVAC-KC will likely be superior to ALVAC as a vector for humans.

We also compared the prime-boost regimen used in this study to that of a DNA-primed study, which is described in the companion paper in this issue (16). Though the DNA-primed regimen clearly and predictably produced a greater T cell response than the regimen described here of poxvirus and protein components, the reverse was true for antibody responses, with mostly higher HIV-1 antibody responses in terms of IgG binding, V1-V2 binding, and neutralization in the TZM-bl assay in the virus-primed groups. This was especially clear in a comparison of the response kinetics of the two groups, where there were major differences. Except for ADCVI activity, peak responses were mostly already achieved at week 26 in this study (following the second poxvirus/
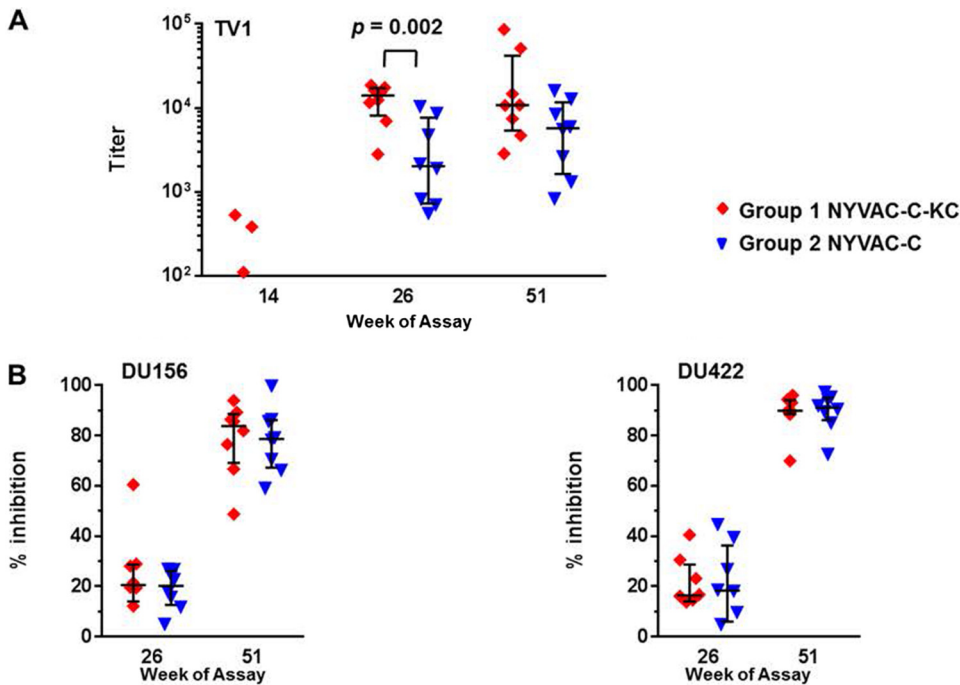
FIG 8 Serum samples from macaques collected at week 51 demonstrated a strong HIV-1 response in both ADCC and ADCVI assays. (A) ADCC assay. CEM.NKRCCR5 target cells coated with TV1 gp120 were coincubated with PBMC preparations (including NK cells) at an effector/target ratio of 30:1 in the presence of plasma dilutions from the immunized macaques. The amount of granzyme B released by NK cells was measured using a fluorescent substrate. The plasma dilution was calculated to match the granzyme B activity cutoff, and the reciprocals are given as titers for values above the assay's cutoff value of 100, with median and interquartile range also shown. (B) ADCVI assay. CEM.NKRCCR5 cells were infected with infectious HIV-1 strain DU156 or DU422 and then coincubated with PBMC effector cells in the presence of plasma at an effector/target ratio of 10:1. The amount of virus released was measured by a p24 ELISA, and the inhibition of virus production compared to that of samples lacking plasma is given as a percentage.

protein boost) and were similar again at week 51 following the final boost (e.g., IgG responses) (Fig. 6). In contrast, in the DNA-primed groups, responses peaked only at the final time point assessed; however, by then they often achieved levels similar to those of this study. In IgG binding, the week 14 results of this study, after one protein boost, were similar to the week 36 results in the companion study after two protein boosts. In this report, with this regimen, we observed a boosting effect of p24 binding even after the fifth and final virus administration at week 49, suggesting that multiple doses of the poxvirus vector did not prevent expression of the antigens (Fig. 4A). The companion study did not include administration of p24 after week 20. For the V1-V2 data, the response in the ELISA for NYVAC-C-KC at week 26, after two protein boosts, was comparable to that of the highest group of the companion study at week 51 (after three protein boosts). In the neutralization assay, the response to pseudoviruses carrying MW965.26 (the highest responder in both studies) was 1 log higher at week 26 with NYVAC-C-KC, after two protein boosts, than at week 51 for the groups in Asbach et al. (16) after three protein boosts. Interestingly, robust ADCC activity was observed in this poxvirus-primed study already after the second boost in which protein was included, while there was no consistent activity for the DNA-primed groups following the second protein boost. However, at week 51 after the last protein boost, the ADCC activity was conversely slightly higher in the DNA-primed groups than in the NYVAC-C-KC-group (1.2-fold) and the NYVAC-C-group (2.3-fold). ADCVI activity also showed a trend to be slightly superior in the poxvirus/protein groups to that in the DNA-primed groups for week 26 (second protein boost) of this study in comparison to that of week 36 (second protein boost) of the DNA-primed study (1.4-fold for NYVAC-C-KC). One notable distinction between the two studies that should be considered is the contrast

in the regimens: there were a total of seven immunizations done in the companion study (one DNA prime and two DNA boosts, one virus boost, and three protein boosts), while only five were done in the study reported here (one virus prime and one virus boost, followed by three virus/protein boosts); the greater humoral responses in this study were achieved in most cases with only two protein boosts (week 26), while the peak responses in the DNA-primed study required a third protein boost (week 51).

The immunogenicity profile was undoubtedly influenced by the adjuvant used in this study, which was MF59. However, other adjuvants might also be appropriate for use in similar studies conducted in the future. An example is an NHP study that demonstrated greater efficacy with alum than with MF59 (39).

For most assays in this study, not only were the responses greater in the replication-competent NYVAC-C-KC immunized animals than in the replication-deficient NYVAC-C immunized animals in magnitude (T cells, binding, neutralization, and ADCC), but they were also greater in breadth, with more animals responding or with a greater range of response. We already know that NYVAC-KC is attenuated in the newborn-mouse model (8), the most sensitive pathogenesis model we have for poxviruses, and that NYVAC-KC's reactogenicity in rabbits is comparable to that of NYVAC (B. L. Jacobs, K. V. Kibler, and K. Denzler, unpublished data). In this study, neither of the vectors caused any adverse effects in the NHPs. Future studies will ascertain if the differences in immunogenicity in this study compared to levels of previous HIV-1 vaccine candidates (40–43) will contribute to vaccine efficacy. Though no macaque SHIV challenge study was done in connection with this immunogenicity study, there is an NHP challenge study currently taking place that includes an appropriate group size for measuring efficacy and a regimen that is partially based on the results of this study and that of the companion paper.

Together, the two outcomes of improved immunogenicity and apparent safety suggest that NYVAC-KC is a promising candidate for an HIV vaccine as well as for vaccines against other infectious agents.

MATERIALS AND METHODS

Data availability. Assay data presented here can be accessed through the DataSpace sharing platform of the Collaboration for AIDS Vaccine Discovery (https://dataspace.cavd.org).

Ethics statement. The study was performed with male Indian rhesus macaques (*Macaca mulatta mulatta*) that were housed, fed, given environmental enrichment, and handled at ABL, Inc.'s animal facility (Rockville, MD), as described previously (10). The study was approved by the Advanced BioScience Laboratories, Inc., Institutional Animal Care and Use Committee (animal use protocol number 444). All procedures were carried out as previously described (10). A total of 16 animals were divided into two groups of 8 animals each.

Antigens. The Gag sequence is derived from the HIV-1 C-clade isolate 96ZM651 (GenBank accession number AF286224). The Pol-Nef sequence is derived from the HIV C/B-clade isolate 97CN54 (GenBank accession number AX149647.1) and consists of p6* (amino acids [aa] 1 to 56), followed by protease (aa 57 to 155; inactivated by mutation D81N), the N-terminal part of reverse transcriptase (RT) (aa 156 to 320 and 357 to 360), scrambled Nef (aa 101 to 206 followed by aa 1 to 100), the C-terminal part of RT (aa 361 to 715), and finally the middle part of RT (aa 321 to 356). The GagFSPolNef (where FS is frameshift) cassette inserted into NYVAC (see below) contains identical sequences on the amino acid level though the two reading frames are connected, employing the natural ribosomal frameshift (thus keeping the wild-type codon usage for the region from the slippery site to the stop codon of Gag), leading to expression of Gag and Gag-Pol-Nef presumably in a ratio of about 10:1 (32). The gp140 sequence is derived from strain 96ZM651 and consists of amino acids 1 to 673, including the autologous signal sequence (incorporation of a strong Kozak initiation site leads to the mutation R2G within the signal sequence), and contains a mutated gp120-gp41 cleavage site (R516S).

All antigen open reading frames were codon optimized for human expression using the GeneOptimizer algorithm (44), supplemented with a strong Kozak initiation site, and synthesized by GeneArt AG (Regensburg) with suitable restriction sites for insertion into the respective plasmids.

Gag-Pol-Nef and gp140 were inserted into the New York vaccinia virus (NYVAC) (45) as described in Kibler et al. (8). Previously, we reported that cells infected with replication-deficient NYVAC produced trimeric gp140 and Gag-derived VLPs, which activate HIV-1-specific B and T cell immune responses in mice (9). Recombinant clade C gp120 protein (isolate TV1) (46) was expressed from stably transfected CHO cell lines, purified, and characterized as previously described (47).

Vaccines and immunizations. NYVAC-based recombinant viruses were grown in BHK-21 cells and purified by sucrose cushion centrifugation twice. Titers were determined by plaque immunostaining in BSC-40 cells. Each animal received two recombinant viruses, one expressing Envgp140(96ZM651) and one expressing Gag(96ZM651)-Pol-Nef(97CN54), each at 1×10^8 PFU in a total volume of 1 ml of 1×

Tris-buffered saline (TBS). Group 1 received the replication-competent NYVAC-C-KC combined viruses [NYVAC-KC-Envgp140(96ZM651) plus NYVAC-KC-Gag(96ZM651)-Pol-Nef(97CN54)], and group 2 received the replication-deficient NYVAC-C combined viruses [NYVAC-Envgp140(96ZM651) plus NYVAC-Gag(96ZM651)-Pol-Nef(97CN54)]. One milliliter was injected intramuscularly into the deltoid of the right arm of each animal. The subtype C gp120 protein boost of HIV-1 consisted of 100 μg of the TV1 isolate with MF59 as an adjuvant, at a 0.1-mg/ml final protein concentration. One milliliter was injected intramuscularly into the deltoid of the left arm.

Blood samples were taken at the indicated time points shown in Fig. 1 as described previously (10). Briefly, EDTA-blood samples were collected for T cell assays, and plasma or clotted blood was collected for the antibody analyses.

Immunological analyses. (i) Peptides. Nine different peptide pools were used for T cell stimulations (described in reference 10).

(ii) IFN-γ ELISpot. Freshly isolated PBMCs were stimulated and processed as described previously (10). Briefly, the stimulated PBMCs were used to probe IFN-γ-coated plates. Results were visualized and counted with an automated ELISpot reader (ImmunoSpot v5 Reader; CTL). Animals with more than 50 SFC/10^6 cells and four times the week 0 background values for any one of the peptide pools were considered responders (40).

(iii) Intracellular cytokine staining (ICS). The assay was carried out by the Nonhuman Primate Immunogenicity Core (M. Roederer lab), Vaccine Research Center, NIAID, as described previously (10, 48). Briefly, cells were stained with fluorescence-labeled antibodies directed against CD3, CD4, CD8, IFN-γ, IL-2, and TNF-α for flow cytometric analysis.

(iv) HIV-1-specific binding antibody assay. HIV-1-specific IgG and IgA antibodies to gp120/gp140 proteins, p24, and p66 (RT) were measured by an HIV-1 binding antibody multiplex assay (G. D. Tomaras lab [10]) as previously described (22, 24). All assays were run under conditions compliant with good clinical laboratory practice, including tracking of positive controls by Levy-Jennings charts. Briefly, positive controls included an HIVIG and CH58 monoclonal antibody (MAb) IgG titration. Negative controls included in every assay were blank beads and HIV-1-negative sera. Antibody measurements were acquired on a Bio-Plex instrument (Bio-Rad, Hercules, CA) using 21CFR Part 11-compliant software, and the primary readout is in mean fluorescence intensity (MFI). Samples not matching the assay's predefined positivity criteria were considered nonresponders of value zero. For IgG, MFI data were transformed to values of the area under the curve (AUC) (41). The following antigens were examined: (i) consensus gp140 proteins of group M (Con S gp140 CF [49, 50]), clade A (A1.con.env03 140 CF), clade B (B.con.env03 140 CF), and clade C (C.con.env03 140 CF); (ii) the primary Env variants 1086 gp120 (clade C), TV1 gp120 (clade C), JRFL gp140 (clade B), MSA4076 gp140 (clade A1, also called OOMSA); (iii) Gag p24 and RT p66.

(v) Env-V1-V2-specific antibodies. Antibodies binding the variable loops 1 and 2 of Env were measured by an ELISA. ELISA plates (384-well) (3700; Costar) were coated with 2 μg/ml MLV gp70-scaffolded Case-A2 V1-V2 in 0.1 M sodium bicarbonate and blocked with assay diluent (4% [wt/vol] whey protein, 15% normal goat serum, 0.5% Tween 20, 0.05% sodium azide in phosphate-buffered saline [PBS]). Plasma samples were applied in quadruplicates as eight 3-fold serial dilutions starting at 1:90. Plates were incubated for 1.5 h and then washed with PBS–0.1% Tween 20. Ten microliters of horseradish peroxidase (HRP)-conjugated goat anti-rhesus secondary antibody (617-103-012 [Rockland],1:10,000 in assay diluent without azide) was added for 1 h. The plates were washed again and developed with 20 μl of SureBlue Reserve (53-00-03; KPL) for 15 min. The reaction was stopped by addition of 20 μl of HCl stop solution. Absorbance was measured at 450 nm.

(vi) Neutralization of HIV-1. Neutralization of sera against HIV-1 was assessed using a TZM-bl assay by the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA-VIMC). Pseudoviruses were incubated, and procedures were done as described previously (10). Neutralization titers are given as the serum dilution leading to a 50% decrease (ID_{50}) in relative light units for the respective virus compared to the value for the control after subtraction of the background values.

(vii) ADCC assay. An antibody-dependent cellular cytotoxicity (ADCC) assay was carried out by the CA-VIMC ADCC core laboratory as previously described (10, 51). Briefly, CEM.NKR$_{CCR5}$ cells were used as target cells and labeled with fluorescence markers to assess viability. The target cells were mixed with a granzyme B (GrB) substrate prior to addition of human effector cells. The plasma samples were tested starting at 1:100 dilutions and using six 4-fold dilution schemes. Cells were analyzed by flow cytometry for the fluorescent signal arising from cleavage of the GrB substrate in the target cells. The percentages of GrB-positive viable target cells minus background, represented by the effector and target cell conditions in the absence of any Ab, were calculated for each dilution. The titers of the ADCC-mediating antibodies were then calculated by interpolation of the dilution exhibiting a GrB activity matching the cutoff value for positivity of 8%.

(viii) ADCVI assay. An antibody-dependent cell-mediated virus inhibition (ADCVI) assay (D. N. Forthal lab) was carried out as described previously (10, 52). Briefly, target and effector cells were mixed at an effector/target cell ratio of 10:1; supernatants were harvested and assayed for p24 by ELISA. The virus inhibition in percent is calculated as [1 − (c(p24)_v/c(p24)_c)] × 100, where c(p24)_v is the concentration of p24 in tests with plasma from vaccinated animals and c(p24)_c is the concentration in control plasma (average of week 0 samples).

Statistics. The method of analysis is identified in each graph. Graphical distributions of the magnitude of responses were plotted for NYVAC-C-KC and NYVAC-C at each time point. The midline in the box plots denotes the median, and the ends of the whiskers denote the 25th and 75th percentiles. If

necessary, the plots were drawn on a $\log_{10}$ scale. Only significant P values (i.e., less than 0.05) are displayed above the plots in comparisons of two groups.

To determine a difference in response magnitudes between groups at specific time points, a Wilcoxon rank sum test was used, based on the exact reference distribution and using the mid-ranks method for ties (53). All tests were one-sided (alternative hypothesis [H_a], NYVAC-C-KC > NYVAC-C) and considered significant at an alpha of 0.05. Due to the exploratory nature of this analysis, multiple comparison adjustments were not made here. R (version 3.3.1) and GraphPad Prism, version 7, were used to create all box plots, and the R coin package was used for testing.

Individual-specific and group-average magnitude-breadth (MB) curves (54) were used to display the breadth of binding antibody activity in terms of the percentage of antigens with $\log_{10}$ titers of $<x$ for a range of $\log_{10}$ titers, x. The group differences of area under the MB curve (AUC-MB) were tested using a Wilcoxon rank sum test.

Intracellular cytokine staining was graphed using SPICE software (NIAID). The percentages of total responses between two groups were compared using a Wilcoxon rank sum test for each cytokine combination at each time point.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI .01513-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.

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