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Priming with a Potent HIV-1 DNA Vaccine Frames the Quality of Immune Responses prior to a Poxvirus and Protein Boost

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ABSTRACT The use of heterologous immunization regimens and improved vector systems has led to increases in immunogenicity of HIV-1 vaccine candidates in non-human primates. In order to resolve interrelations between different delivery modalities, three different poxvirus boost regimens were compared. Three groups of rhesus macaques were each primed with the same DNA vaccine encoding Gag, Pol, Nef, and gp140. The groups were then boosted with either the vaccinia virus strain NYVAC or a variant with improved replication competence in human cells, termed NYVAC-KC. The latter was administered either by scarification or intramuscularly. Finally, macaques were boosted with adjuvanted gp120 protein to enhance humoral responses. The regimen elicited very potent CD4$^+$ and CD8$^+$ T cell responses in a well-balanced manner, peaking 2 weeks after the boost. T cells were broadly reactive and polyfunctional. All animals exhibited antigen-specific humoral responses already after the poxvirus boost, which further increased following protein administration. Polyclonal reactivity of IgG antibodies was highest against HIV-1 clade C Env proteins, with considerable cross-reactivity to other clades. Substantial effector functional activities (antibody-dependent cell-mediated cytotoxicity and antibody-dependent cell-mediated virus inhibition) were observed in serum obtained after the last protein boost. Notably, major differences between the groups were absent, indicating that the potent priming induced by the DNA vaccine initially framed the immune responses in such a way that the subsequent boosts with NYVAC and protein led only to an increase in the response magnitudes without skewing the quality. This study highlights the importance of selecting the best combination of vector systems in heterologous prime-boost vaccination regimens.

IMPORTANCE The evaluation of HIV vaccine efficacy trials indicates that protection would most likely correlate with a polyfunctional immune response involving several effector functions from all arms of the immune system. Heterologous prime-boost regimens have been shown to elicit vigorous T cell and antibody responses in nonhuman primates that, however, qualitatively and quantitatively differ depending on the respective vector systems used. The present study evaluated a DNA prime and poxvirus and protein boost regimen and compared how two poxvirus vectors with various degrees of replication capacity and two different delivery modalities—conventional intramuscular delivery and percutaneous delivery by scarification—impact several immune effectors. It was found that despite the different poxvirus boosts, the overall immune responses in the three groups were similar, suggesting the potent DNA priming as the major determining factor of immune responses. These findings emphasize the importance of selecting optimal priming agents in heterologous prime-boost vaccination settings.

About 0.5% of the world population is infected with human immunodeficiency virus type 1 (HIV-1), and it is estimated that 5,700 new infections occur each day (1). Despite the success of combination antiretroviral therapy in controlling the infection, a cure has not been achieved so far. Accordingly, there is a demand for a vaccine preventing HIV acquisition or at least progression to disease.

Increasingly, more insight is gained from the analyses of HIV vaccine candidates in nonhuman primates and especially from the efficacy trials that have so far been performed in humans (2). Based on these analyses, the immune correlate that is hypothesized to provide protection actually consists of a complex mixture of individual immune effectors (3), and knowledge about the required composition is most likely still incomplete. Therefore, it is necessary that a vaccine elicits polyfunctional responses from all arms of the immune system but with the right characteristics. For instance, antibodies that can mediate antibody-dependent cellular cytotoxicity contributed to lowering the infection risk in the RV144 efficacy trial (4) but only in the absence of serum IgA responses (5). Binding antibodies toward the V1V2 loops of the envelope (Env) protein also proved to be associated with a decreased risk of infection (6), especially when the antibody isotype was IgG3 (7). Although no vaccine efficacy was observed in the VAX004 trial (8), follow-up analyses showed that antibody-dependent cell-mediated virus inhibition (ADCVI) activity of serum was inversely associated with the rate of infection, with the ADCVI activity significantly higher in uninfected vaccinees (9). Moreover, T cell responses can also contribute to a protective immune profile. Detailed analysis of CD4$^+$ T cell responses of RV144 vaccinees implicated polyfunctional cells secreting several cytokines to have contributed to vaccine efficacy (10). In the HVTN 505 trial (11), both the magnitude and polyfunctionality of Env-specific CD8$^+$ T cell responses were strongly associated with reduced infection risk, whereas there was only a moderate association for CD4$^+$ T cells (12). Collectively, these results point toward a model for an efficacious immune control that consists of a complex mixture of additively or synergistically acting mechanisms that together could help to protect from infection albeit some properties may also be detrimental.

Therefore, vaccine design should aim at concepts that activate multiple effector responses. Yet there is currently a strong focus on improving antibody responses, especially regarding the capacity to neutralize challenging tier 2 envelope proteins (13). That such a response would be able to protect from infection is expected because of the efficacy of passively transferred broadly neutralizing antibodies (bnAbs) in nonhuman primate models (14–16). However, although much progress has been made by employing engineered soluble Env antigens mimicking the native Env spike that gives rise to antibodies capable of neutralizing autologous tier 2 isolates, only limited tier 2

neutralization breadth has been achieved so far (17–20). In contrast, delivery of simian immunodeficiency virus (SIV) antigens via a rhesus cytomegalovirus vector gave rise to a broad T cell response of effector memory phenotype that was able to suppress SIV replication early after infection of several macaques and prevent the formation of a latent reservoir (21). Consequently, vaccine candidates should not be focused on eliciting exclusively humoral or cellular responses but should harness both. In fact, development of high-quality and durable antibody responses is dependent on T cell help from follicular helper T cells (22, 23). Conversely, CD8$^+$ T cell responses can also benefit from good antibody responses, as has been shown for macaques that were treated with bnAbs early after infection with simian-human immunodeficiency virus (SHIV). Here, the initial suppression of viremia facilitated development of potent cytotoxic T lymphocyte (CTL) responses that subsequently suppressed virus replication (24).

Parameters that influence the outcome of immune responses include, among others, the antigen, the choice of the delivery system, route of administration, and the number and spacing of immunizations. For ease of real-world applicability, a regimen should consist of as few immunizations as possible and use a well-established route of administration, such as the most commonly used intramuscular (i.m.) injection. Nevertheless, implementation of an alternative method would be warranted if it leads to significantly enhanced immunity. For instance, scarification has traditionally been used to percutaneously deliver attenuated poxviruses as a highly effective smallpox vaccine (25). Moreover, combining different vector systems in heterologous prime-boost regimens often results in more potent immune responses (26). This often includes the use of DNA plasmids as the priming agent because it has been shown that DNA vaccines are safe and induce potent CTL responses, whereas there is no vector immunity (27).

Taking these observations together, while improved antigens and delivery systems are still being developed, it is crucial to also assess the potency and safety of appropriate vector systems and establish suitable vaccination schedules. In this context, we have previously described that, for HIV-1 antigens, a DNA prime and poxvirus and protein boost regimen (DNA-poxvirus-protein regimen) elicits high-magnitude, broad, and polyfunctional T cell responses, as well as high-titer IgG antibody responses that also exhibit high levels of functional Fc-mediated responses in rhesus macaques (28). The aim of the current study was to assess how a more immunogenic variant of the New York vaccinia virus (NYVAC) strain, termed NYVAC-KC (see the companion paper by Kibler et al. in this issue [29] and reference 30) would affect the magnitude and quality of immune responses following DNA priming. In this regard, a side-by-side comparison of three different boost modalities was performed in nonhuman primates. The data show that the DNA priming immunizations frame the quality of the immune responses in such a way that subsequent booster immunizations increase only the magnitude of these responses without altering the underlying profile. This emphasizes the importance of employing optimal priming immunizations in HIV vaccination trials.

RESULTS

Study plan. Rhesus macaques were assigned to three groups of eight animals each. To obtain comparable groups, the weight and Mamu allele status of the macaques were considered during randomization. In the end, three animals in each group were Mamu-A*01-positive, and one of these from group B was in addition Mamu-B*17 positive, whereas all were Mamu-B*08 negative. As depicted in Fig. 1, all animals received three priming immunizations with the DNA vaccine 4 weeks apart at weeks 0, 4, and 8. Twelve weeks later (at week 20) a single NYVAC boost was carried out. Either the replication-competent NYVAC-C-KC vector was applied by scarification in the case of group A or by intramuscular (i.m.) injection in the case of group B or the replication-deficient NYVAC-C vector was applied by i.m. injection in the case of group C. For the subsequent boost with adjuvanted gp120 protein, animals from all groups received a first i.m. injection 8 weeks later (at week 28) and a second one another 4 weeks later (at week 32). While the study was ongoing, it was decided to extend the schedule by
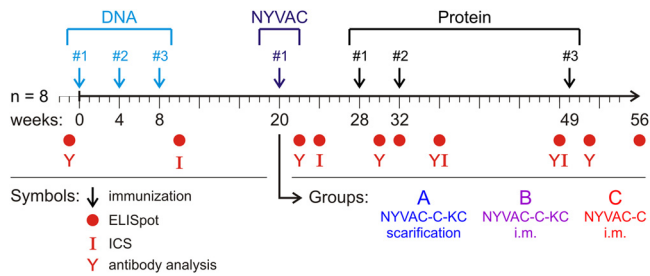
FIG 1 Immunization schedule for the DNA prime and NYVAC and protein boost regimen. Three groups of 8 macaques each were immunized three times with DNA, followed by a single immunization with an NYVAC vector and three immunizations with protein. NYVAC-C-KC (enhanced replication competence) was administered by scarification in group A, and by i.m. injection in group B. In group C, NYVAC-C (restricted replication competence) was administered by i.m. injection. Blood was collected for ELISpot analysis, intracellular cytokine staining (ICS), or antibody analysis at the indicated time points.

appending an additional late protein boost 1 week after the initially scheduled study end date (week 48). Thus, 25 weeks after the second protein immunization, the macaques received a third immunization at week 49. Peripheral blood mononuclear cells (PBMCs) and serum or plasma samples were obtained at selected time points, as shown in Fig. 1, for analysis of T cell and antibody responses.

Animals were continuously monitored for adverse events. In line with earlier results (28, 31, 32), the vaccine administrations were well tolerated. Mild erythemas (grade 1) at the inoculation site following the first NYVAC immunizations were noted in one animal of group A, one animal of group B, and two animals of group C, and all resolved within 1 or 2 days. Scarification expectedly led to pock formation, with diameters ranging from 5 to 11 mm at 2 weeks after the application. No other adverse clinical signs, such as reduced food intake, pain, or lethargy, were observed. Hematology mainly showed incidental and mild (grade 1) changes across groups and time points. There were one case of a grade 2 (group B) and two cases of a grade 4 (groups B and C) leukocytosis 8 weeks after the NYVAC boost that resolved before the next examination and that were most likely unrelated to the immunization.

Magnitude of T cell responses. The overall magnitude of T cell responses along the immunization course was assessed by determining the fraction of gamma interferon (IFN-γ)-producing T cells upon restimulation of PBMCs with peptide sets covering the whole antigens. Remarkably, 2 weeks after completing the DNA prime immunization, median responses of 1,625 (group A), 1,848 (group B), and 1,683 (group C) spot-forming units (SFUs) per 10^6 PBMCs were measured, with individual animals even exceeding 2,500 SFUs/10^6 cells (Fig. 2). Boosting with the NYVAC vector led to a vigorous increase, with median values of 4,463 (group A), 4,565 (group B), and 6,600 (group C) SFUs/10^6 cells 2 weeks later at week 22. Five animals across the groups exceeded 10,000 SFUs/10^6 cells, with a peak of 14,680 SFUs/10^6 cells. Over the following 2 weeks, median responses declined by trend, yet this was not statistically significant. Responses 2 weeks after the first protein boost, at week 30, did not show an increase above the levels of week 24 and were, by trend, lower than the peak responses of the whole time course at week 22. Similarly, the responses 4 weeks after the second protein boost mimicked those observed beforehand. After the late protein boost at week 49, the responses showed a slight increase, yet this was significant only in the case of group A. The final levels at week 56 were again lower, with median values of 878 (group A), 1,843 (group B), and 1,490 (group C) SFUs/10^6 cells. Overall, there was a slow but steady decline in the responses from the peak after the NYVAC boost onwards until the end of the study, which was interrupted only by the small increase after the late protein boost, which might have been anamnestic in nature. Nevertheless, at all time points, all animals could clearly be classified as responders. In general, there were no statistically significant differences between the three groups at any time point. Thus, the different modes of administration of NYVAC-C-KC or the choice of the respective NYVAC variant had no impact on the overall T cell responses in the present study.
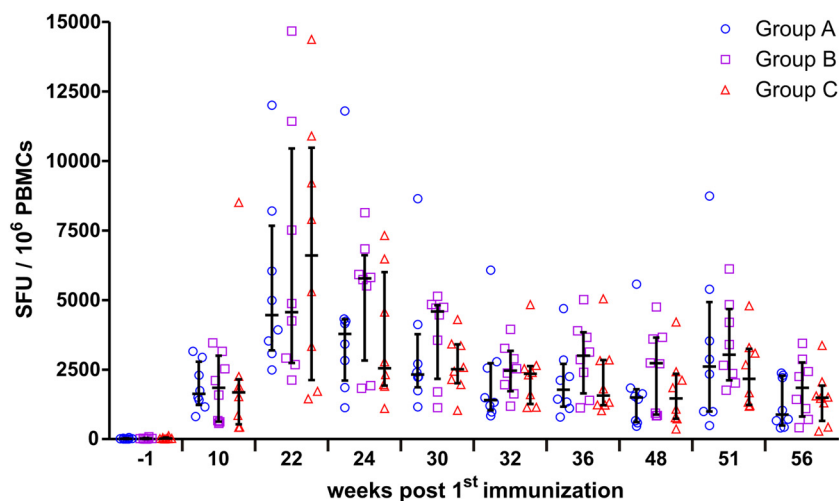
FIG 2 The DNA prime and NYVAC boost regimen elicits vigorous HIV-1-specific T cell responses. PBMCs were freshly isolated at the indicated time points and restimulated in parallel with nine different peptide pools in an ELISpot assay. The numbers of IFN-γ-producing cells per million PBMCs were enumerated as spot-forming units (SFUs) and summed for all the peptide pools. Results are shown per animal, along with the median and interquartile range per group.

Characteristics of T cell responses. Next, we assessed the quality of the elicited T cell responses against HIV-1 antigens in terms of the balance between CD4+ and CD8+ responses, polyfunctionality, and peptide pool reactivity. For this, PBMCs from selected time points were stimulated with nine different peptide pools and then stained for surface markers and intracellularly for production of the cytokines IFN-γ, interleukin-2 (IL-2), and tumor necrosis factor (TNF). The fraction of reactivated CD4+ and CD8+ T cell subsets was then measured by flow cytometry (Fig. 3A). There were no significant differences between the three groups. The responses peaked by trend at week 24, i.e., after the NYVAC boost, in all groups except for the CD8+ T cell responses of group A. Here, the responses measured earlier after the last DNA priming immunization were slightly higher. However, except for the CD4+ T cell responses of group A, the increase after the NYVAC boost was not statistically significant. On average, median values of 1.5% HIV-specific CD4+ T cells and 1.7% HIV-specific CD8+ T cells were measured at week 24, thus proving the responses to be well balanced.

The data were also analyzed regarding the number and type of cytokines produced by the restimulated T cells (Fig. 3B). On average over all time points, the majority of CD4+ T cells (57%) produced all three cytokines assessed (IFN-γ, IL-2, and TNF), whereas 28% produced a combination of two cytokines, and only 15% produced only one. Polyfunctionality of CD8+ T cells was slightly inferior, with 28% trifunctional, 37% bifunctional, and 35% monofunctional cells.

Figure 3C shows the data broken down according to the nine peptide pools used that cover the whole Gag-Pol, Env, and Nef antigens. The overall CD4+ T cell responses, averaged for all groups and all time points, were mainly directed toward Gag-Pol peptides with 56%. This was closely followed by Env peptides with 42% of the responses. Nef-directed responses comprised only about 2%. CD8+ T cell responses were on average mostly directed toward Gag-Pol with 76%, while those directed toward Env were only 21% and those toward Nef were 3%. Whereas there was only little difference between the three groups for the CD4+ T cell responses, group B consistently showed higher Gag-specific CD8+ T cell responses, which were exceptionally high at week 24.

Magnitude of antibody responses. Elicitation of antibody responses to HIV-1 antigens was assessed by analysis of serum obtained 2 weeks after the NYVAC boost (week 22) and 2 weeks after the first protein boost (week 30), as well as at 4 and 16 weeks after the second protein boost (weeks 36 and 48). Moreover, the responses
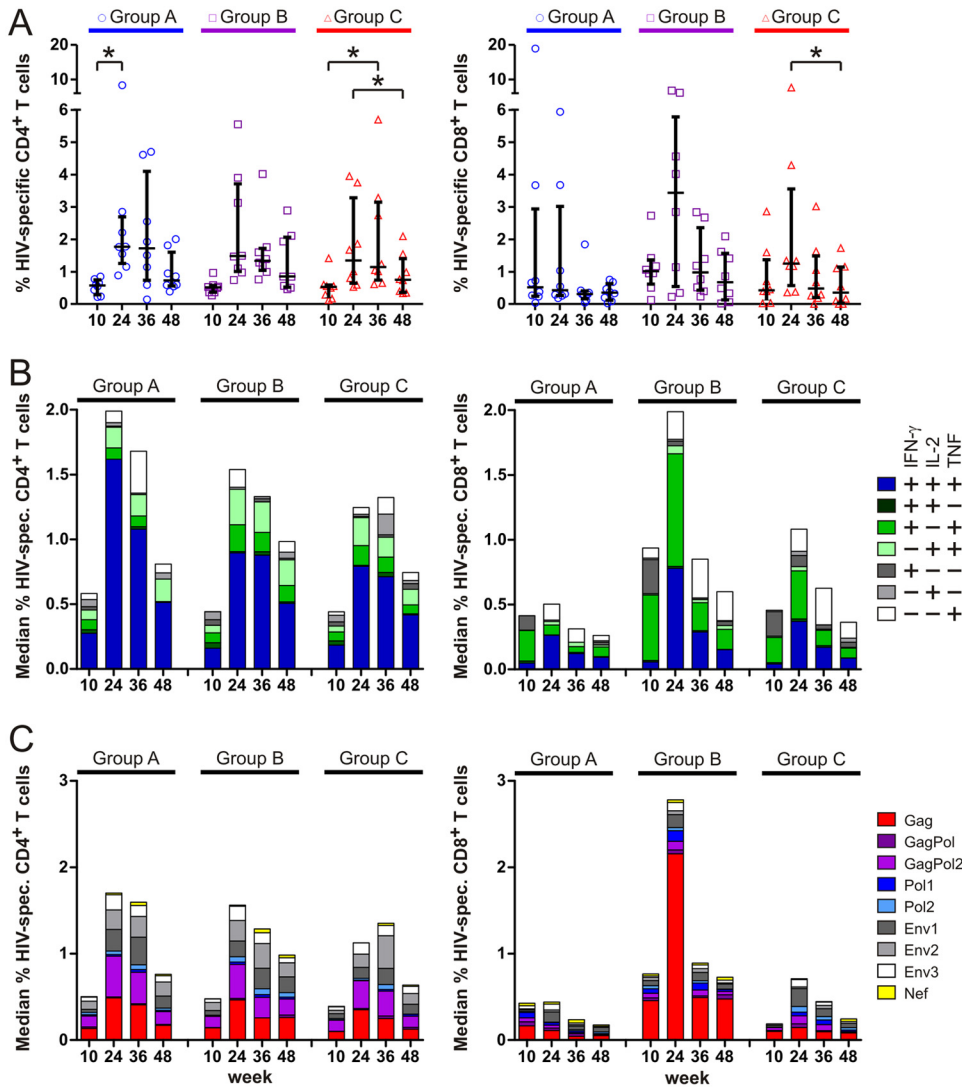
FIG 3 The combined immunizations elicited balanced, polyfunctional and broad CD4^{+} and CD8^{+} T cell responses to HIV-1 antigens. PBMCs obtained at the indicated time points (weeks after the first immunization) were stimulated with the nine different peptide pools and stained for CD3, CD4, and CD8 and intracellular IFN-γ, IL-2, and TNF. Responding CD4^{+} and CD8^{+} T cells were quantified by flow cytometry, as indicated. (A) Fractions of restimulated CD4^{+} and CD8^{+} T cells summed for all the peptide pools and any cytokine are shown with median and interquartile ranges. Significant differences between time points in a paired Wilcoxon signed rank test are marked with an asterisk ($P < 0.05$, Bonferroni corrected). (B) Median values of the summed fractions of T cells restimulated with any peptide pool, broken down according to the cytokines produced by the cells. (C) Median values of the summed fractions of T cells secreting any combination of the three cytokines, broken down according to the peptide pools used.

2 weeks after the week 49 late protein boost were also evaluated. The overall magnitude of IgG and IgA antibodies specifically binding to a set of eight different HIV Env-derived and two Gag-Pol-derived readout antigens was determined with a Luminex assay. Antibody responses were readily detected at all time points and exhibited similar kinetics, as exemplarily shown for the binding to group M consensus gp140, subtype C consensus gp140, and TV1 gp120 (Fig. 4A). Two or 4 weeks after the first NYVAC or the first protein immunization, responses were similar for most antigens and declined over the following weeks. Levels of antibodies binding to TV1 clearly tended to increase further after the first protein boost, likely because of TV1 gp120 being a component of the protein boost. The late protein boost was able to quickly restimulate the memory B cell responses and by trend caused a further increase in antibody levels that was also statistically significant for some groups and some readout antigens. The
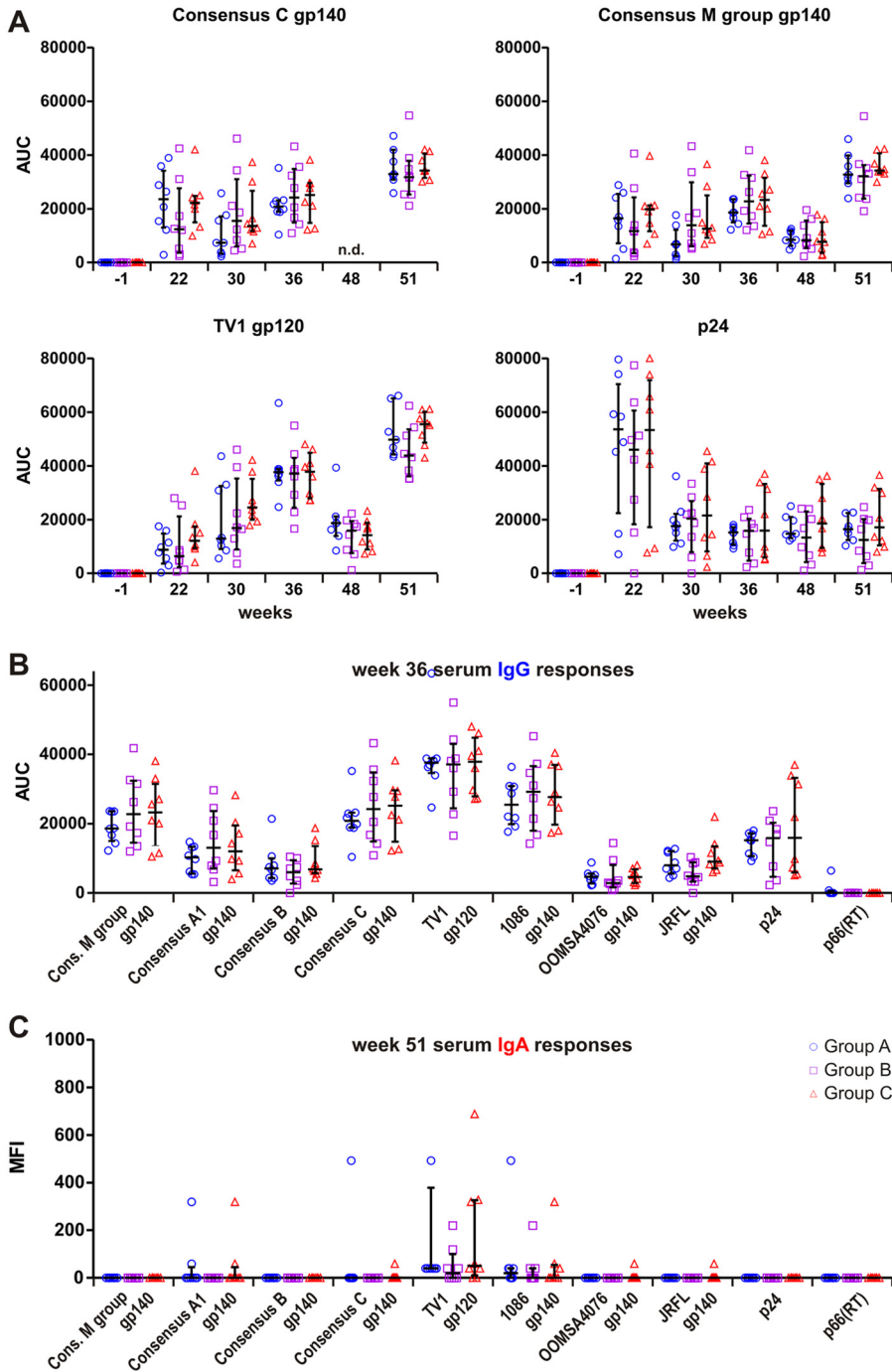
FIG 4 The DNA/NYVAC/protein immunization course elicited substantial IgG antibody responses and negligible IgA antibody responses to HIV-1 antigens. (A and B) IgG antibodies from serum dilutions binding to the indicated readout antigens coated on beads were quantified using a customized Luminex assay. The magnitudes of the antibody binding responses are given as values of the area under the titration curve (AUC), and medians and interquartile ranges are shown. (A) Representative time courses of responses toward the indicated antigens. n.d., not done. (B) Comparative responses of the week 36 sera toward all antigens assessed. (C) IgA antibodies from the lowest serum dilution binding to the indicated readout antigens were quantified using the Luminex assay. The magnitudes are given as mean fluorescence intensity (MFI) values, and medians and interquartile ranges are shown.

p24-specific responses, elicited by the produced Gag virus-like particles (VLPs), were initially even higher than the Env responses but declined continuously in accordance with Gag being absent from the protein boosts. A comparison of the responses for all tested antigens at week 36 (Fig. 4B) shows that the sera had by trend the best reactivity
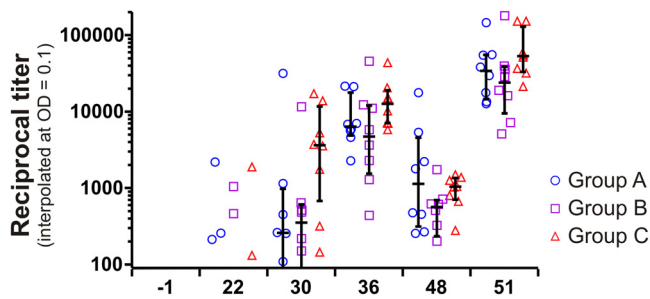
FIG 5 High-titer V1V2-directed responses were elicited after the protein boosts. IgG antibodies directed toward an MLV gp70-scaffolded V1V2 peptide from isolate Case-A2 were measured by ELISA. The reciprocal titer matching an optical density (OD) threshold value of 0.1 interpolated from the titration curve is given for each animal along with group medians and interquartile ranges.

toward the autologous antigen used for the protein immunizations (TV1). This was followed by the subtype C isolate 1086, as well as the subtype C consensus, thus matching expectations as the immunization antigens were derived from subtype C isolates. The B and A1 subtype consensus proteins, as well as selected isolates from these subtypes, were inferior targets yet showed substantial cross-reactivity. Responses to the p66 subunit of reverse transcriptase were negligible. There were no statistically significant differences between the three groups at any time point and for any antigen tested.

In addition, serum IgA responses were evaluated in parallel at weeks 22, 48, and 51. These were very low and hardly detectable, with only few macaque sera showing reactivity above the background level (Fig. 4C). No group had responses that were significantly different from the prebleed level at any time point.

Finally, the IgG antibody titers toward a V1V2 peptide from isolate Case-A2, scaffolded on murine leukemia virus (MLV) gp70, were assessed by a classical enzyme-linked immunosorbent assay (ELISA) (Fig. 5). The response kinetics were generally similar to the antibody responses described above though responses were only significantly above the prebleed values from week 36 onwards, i.e., after the second protein boost. As observed for the other Env readout antigens, responses declined over time but were readily rebooted by the final protein administration to titers exceeding 1:10,000.

Functional characteristics of antibody responses. Blood samples were also assessed for their capacity to neutralize HIV-1 virus-like-particles pseudotyped with various Env proteins of several tier 1 and tier 2 isolates in a classical TZM-bl assay (Fig. 6A and B). In general, the neutralizing activity was low but detectable for the nine Env proteins evaluated in the TZM-bl assay in at least some of the macaques. The best neutralization was obtained for MW965.26, which is a clade C tier 1A variant that is highly neutralization sensitive. Clade B tier 1A variants were neutralized to a lower degree, whereas tier 1B and tier 2 isolates were not neutralized. Exemplary time courses for neutralization of MW965.26 and MN.3 (Fig. 6A) show that the responses tended to increase over time. In all cases, highest neutralization titers were measured for the week 51 samples, which are depicted in Fig. 6B for comparison. No differences between groups were observed. The full, primary neutralization data are available in the supplemental material as Data Set S1.

Finally, the capacity of plasma samples to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and ADCVI, which are measures of various functional antibody Fc-mediated responses, was tested. ADCC was assessed by determining the lysis of target cells, coated with gp120 from isolate TV1, by NK cells in the presence of macaque plasma dilutions. As shown in Fig. 7A, ADCC activity was near the assay's cutoff level for the early time points (weeks 22, 30, and 36) and exhibited a significant increase to a median value of 1.3×10^4, on average, at week 51. There were no statistically significant differences between the three groups at any time point.
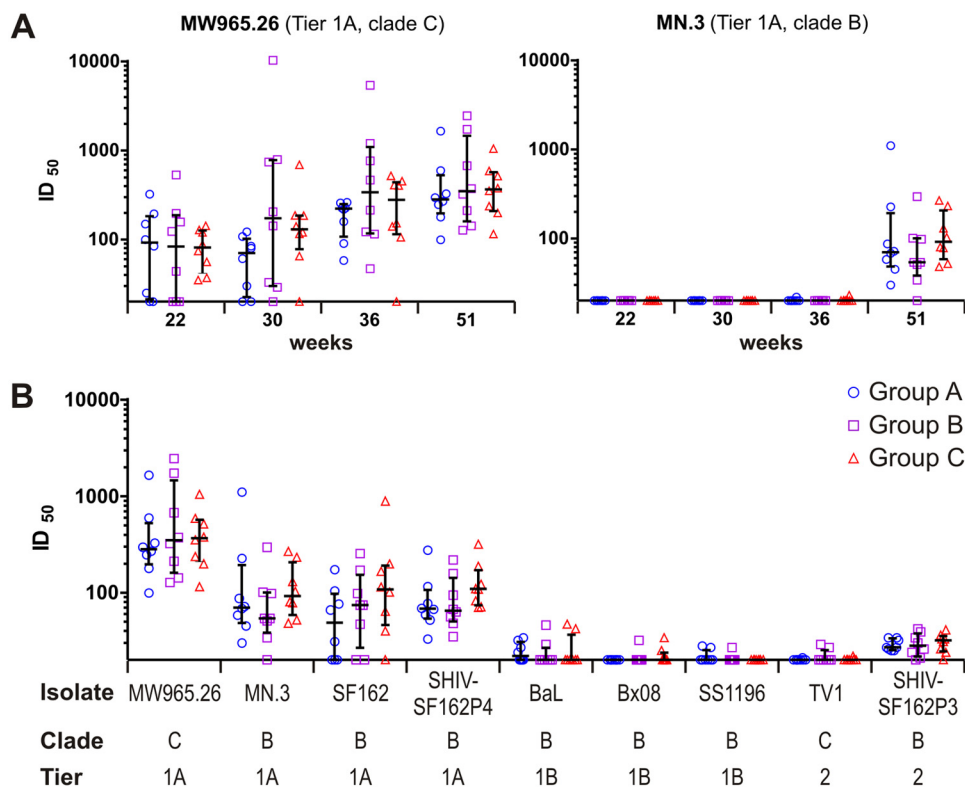
FIG 6 Macaque sera had low neutralization activity in the TZM-bl assay. Serum dilutions were tested for neutralization of pseudoviruses, and the reciprocal dilution at which 50% inhibition (ID_{50}) was observed is given for each animal, with medians and interquartile ranges. (A) Time courses of neutralization of the pseudoviruses carrying MW965.26 or MN.3. (B) ID_{50} values of the week 51 serum samples for pseudoviruses carrying Envs from the indicated isolates.

ADCVI activity was measured by determining the inhibitory activity of plasma on infection of CEM.NKR$_{CCR5}$ cells with the HIV-1 isolates DU156 and DU422 in the presence of PBMCs. At week 36, after the second protein boost, plasmas caused on average median inhibition levels of 16% and 11% for DU156 and DU422, respectively (Fig. 7B). Inhibition significantly increased to vigorous levels of 71% and 86% at week 51, after the late protein boost, once again without showing significant differences between the three groups.

DISCUSSION

This preclinical NHP study evaluated the immunogenicity of a heterologous DNA prime and poxvirus and protein boost regimen delivering the HIV-1-derived antigens Gag, PolNef, and Env in rhesus macaques. The main focus was the comparison of two different boosting agents and two different delivery methods in regard to the expansion of DNA-primed immune responses. The agents were the poxviral vector NYVAC and its derivative NYVAC-KC that exhibits improved replication competence due to the reinsertion of two host range genes (30, 33, 34). NYVAC-KC was administered via two routes, i.e., by intramuscular injection and percutaneous delivery by scarification (an effective vaccine method used during the eradication of smallpox). By the latter method, various antigen-presenting cells residing in the skin are reached that potently initiate adaptive immune responses (35). Moreover, the direct comparison of intramuscular delivery and scarification of vaccinia virus in humans, indeed, had shown that the latter method led to higher immune responses (36). Finally, to differentiate the impacts of these variables on the outcome of the analyzed immune responses, the two NYVAC vectors were not coadministered with the protein, as done in a previous study using a DNA and NYVAC-plus-protein regimen (28), but given sequentially.
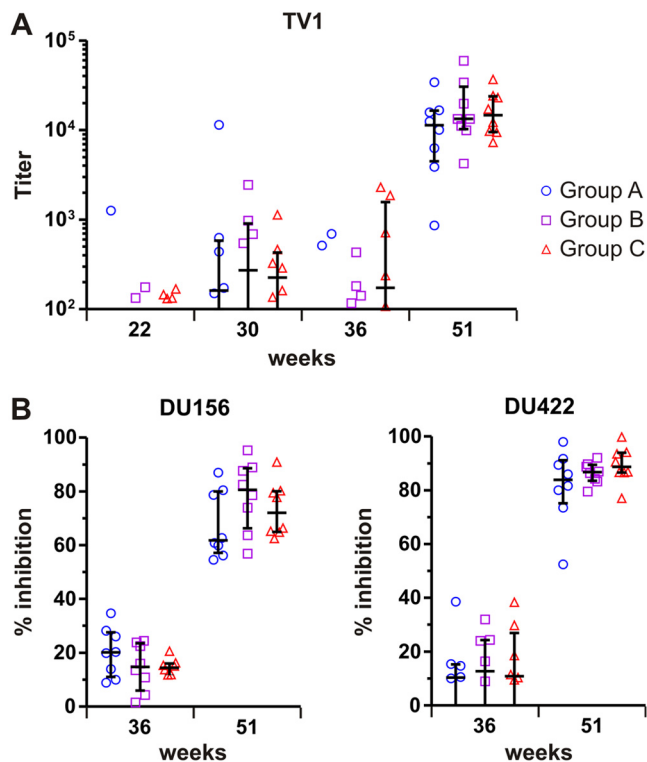
FIG 7 Sera obtained from macaques at week 51 showed vigorous ADCC as well as ADCVI activity. (A) CEM.NKR$_{CCR5}$ target cells coated with TV1 gp120 were coincubated with PBMC preparations (including NK cells) at an effector/target cell ratio of 30:1 in the presence of plasma dilutions from the immunized macaques. The amount of granzyme B released by NK cells was measured using a fluorescent substrate. The plasma dilution needed to match the granzyme B activity cutoff value was calculated, and the reciprocal is given as the titer for values above the assay cutoff of 100, along with median and interquartile ranges. (B) CEM.NKR$_{CCR5}$ cells were infected with infectious HIV-1 strains DU156 and DU422 and then coincubated with PBMC effector cells in the presence of plasma at an effector/target cell ratio of 10:1. The amount of virus released was measured by a p24 ELISA, and the inhibition of virus production compared with that in samples lacking plasma is given.

Vigorous T cell responses were obtained that peaked after the first poxvirus boost. They were well balanced regarding the fraction of HIV-specific CD4$^+$ and CD8$^+$ T cells and did not reveal any differences between the groups. Both sets of T cells exhibited broad peptide pool reactivity and had a high frequency of cells secreting multiple cytokines. An additional NYVAC boost might further augment the quality and quantity of these responses. However, the second NYVAC-plus-protein boost that was applied after two or three DNA primes in a preceding study (28) led only to an anamnestic recall response to the level that had already been obtained after the first administration of NYVAC plus protein. In contrast to the T cell responses, the humoral responses were highest after the whole vaccination schedule with the three protein boosts was completed. Among these responses, high titers of antibodies binding to the V1V2 loop were detected. Such antibodies were associated with a decreased risk of infection in the RV144 trial (5) but were absent or very low in the HVTN 505 trial that failed to show efficacy (11). Although antibody titers toward envelope proteins gradually increased over the course of the vaccination schedule, ADCC and ADCVI responses were rather low until 2 weeks after the final protein boost. This suggests that such functional responses may arise only after prolonged B cell development in germinal center reactions. Eventually, however, ADCVI activity exceeded 60% to 80% inhibition of virus replication *ex vivo*. This is a rather potent response, given that it has been estimated that per 10% increase in ADCVI activity, the hazard rate of infection with HIV drops by 6% (9). Antibodies toward p24 were highest after the poxvirus boost and subsequently declined over time. This difference in kinetics is due to p24 not being part of the protein

boost immunizations. In summary, in a comparison of the general kinetics of the responses, the DNA-poxvirus modality can simply be considered an inducer of T cell responses, in contrast to the recombinant protein that, rather, induces humoral responses. Overall, the use of either NYVAC or the NYVAC-KC derivative had no impact on the immune response profiles. Moreover, i.m. delivery and scarification were equally efficient in the context of the DNA priming immunizations.

TV1 gp120 was used here as the antigen for the protein immunizations as opposed to a bivalent mixture of TV1 and 1086 gp120 used in a related study (28). Comparison of the binding antibody titers between the two studies after the second protein boost (compare Fig. 4 here with Fig. 5 in reference 28) shows a very similar response magnitude toward TV1 as readout antigen. However, the cross-reactivity toward the consensus C gp140 and to the more distantly related envelope variants as well was superior for the regimens using the bivalent gp120 mixture although it cannot be excluded that differences in the schedules contributed to this effect. Thus, as one would expect, vaccination with bivalent or even multivalent protein mixtures leads to improved breadth of antibody responses. Accordingly, this bivalent mixture of clade C-derived gp120 proteins (37) is currently being tested in the HVTN 702 phase IIb/III efficacy trial in South Africa (38). However, although the MF59-adjuvanted protein was very immunogenic here, other adjuvants might even be superior regarding the quality and durability of the response. For instance, in a rhesus macaque study using SIV antigens, conventional alum-adjuvanted protein resulted in higher efficacy upon challenge, despite the fact that the MF59-adjuvanted protein was more immunogenic (39).

In a companion paper (Kibler et al. in this issue [29]), a comparison of the immune responses elicited by NYVAC plus TV1 gp120 protein and NYVAC-KC plus TV1 gp120 protein without the DNA prime is described (here referred to as poxvirus-protein-only regimen). Whereas NYVAC-KC was clearly more immunogenic than NYVAC when applied in the poxvirus-protein-only regimen (29), no immunological differences were observed between the groups receiving NYVAC and NYVAC-KC in the context of the DNA-poxvirus-protein regimen (this study).

The magnitude of T cell responses at the peak time points as measured by an IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay was similar in the DNA-poxvirus-protein regimens regardless of whether the parental NYVAC vector or the NYVAC-KC derivative with enhanced replication competence was used. In quantitative terms, the peak responses in the DNA-primed groups were 5-fold and 15-fold higher than responses with the poxvirus-protein-only regimens employing NYVAC-KC and NYVAC, respectively (29). Correspondingly, HIV-specific T cells comprised 0.5% (CD4$^+$) and 0.4% (CD8$^+$) at week 24 in the poxvirus-protein-only study, as opposed to 1.5% (CD4$^+$) and 1.7% (CD8$^+$) in the DNA-poxvirus-protein study. The latter exhibited 57% (CD4$^+$) and 28% (CD8$^+$) polyfunctional T cells secreting IFN-γ, TNF, and IL-2, whereas the values were 32% (CD4$^+$) and 10% (CD8$^+$) for the NYVAC-KC-group and only 8.2% (CD4$^+$) and 6.2% (CD8$^+$) for the NYVAC group of the poxvirus-protein-only study (averages of all time points). In terms of epitope specificity, CD4$^+$ T cells showed a reactivity toward Gag-Pol/Env/Nef with a ratio of 41:53:6%, and the ratio for CD8$^+$ T cells was 58:37:5% in the poxvirus-protein-only regimen. These figures were 56:43:2% (CD4$^+$) and 76:21:3% (CD8$^+$) for the DNA-poxvirus-protein regimens (averages over all time points), thus showing that in the latter case there is a skewing of CTL responses toward Gag-Pol.

In contrast, the humoral responses peaked earlier with the poxvirus-protein-only regimen (29), most likely because the first protein immunization took place already at week 12. The same was true for the functional antibody responses. At the end of both studies, however, ADCC and ADCVI titers reached roughly similar levels with no significant differences between the three DNA-poxvirus-protein groups (this study) and the poxvirus-protein-only regimens (29).

Taken together, the results described here and in the paper by Kibler et al. (29) show that the immune responses toward the HIV-1 antigens delivered by the DNA priming immunizations define the immunogenicity profile over the whole course of the study. Accordingly, there are no differences between the groups despite the fact that

NYVAC-KC is more immunogenic than NYVAC when administered in the absence of a DNA priming immunization. The immune responses shaped by the priming are qualitatively constant in terms of the T cell cytokine secretion profile and peptide pool reactivities as well as antibody cross-reactivities. The poxvirus and protein boosts do lead to increases in magnitudes, the latter especially with respect to humoral responses, but they do not skew the quality of the initially framed immune responses; i.e., there were no statistically significant differences between the groups for matched time points. Although NYVAC-KC elicited higher-magnitude T cell responses than NYVAC in the companion study (29), this was not the case here when the vectors were used as boosting agents, most likely because the responses to the DNA priming immunizations had already been very strong. Yet the vaccinia virus boosts led to additional T cell responses, with a 3-fold-increased overall magnitude on average. In any case, the results emphasize the importance of choosing the optimal priming agent for potent immunization regimens. Therefore, it will be important to test whether the improvements in immunogenicity obtained here in comparison to immunogenicity of previous generation of vaccine candidates (40–43) also translate into improved efficacy in a macaque SHIV or SIV challenge situation. No challenge was performed here as the objective of the studies was to qualify the vaccine candidates and gain information on the optimal immunization regimen for further studies. In addition, with a group size of eight animals, the study was not sufficiently powered for evaluation of effects in a challenge situation. A challenge study in which the DNA and NYVAC-KC agents together with improved protein antigens are being tested is currently ongoing.

Finally, the only trial demonstrating some efficacy so far (RV144) (4) and the ongoing HVTN 702 phase IIb/III trial both employ a poxvirus prime and poxvirus-plus-protein boost regimen. Given the benefits of a DNA priming before the poxvirus and protein boost as shown here, further testing of the DNA component in humans in prime-boost and/or combined coimmunization regimens seems justified.

MATERIALS AND METHODS

Ethics statement. Male Indian rhesus macaques (*Macaca mulatta mulatta*) were housed at Advanced BioScience Laboratories (ABL), Inc. (Rockville, MD). All procedures strictly adhered to the recommendations of the 8th edition of the *Guide for the Care and Use of Laboratory Animals* (44) and the Public Health Services policy on the humane care and use of laboratory animals from the Office of Animal Welfare (part of the U.S. Department of Health and Human Services), in full compliance with Animal Welfare Act (9 CFR 3.81) regulations. ABL, Inc.'s Institutional Animal Care and Use Committee approved the study under protocol number AUP444. Anesthesia with ketamine (10 mg/kg of body weight) was given by trained personnel under the supervision of veterinary staff for all procedures. Recommendations by the Weatherall report on the use of nonhuman primates in research (45) were followed and extended by ABL's Primate Environmental Enrichment Program to enhance animal welfare and to minimize suffering.

Safety monitoring included observation for general behavior, for clinical symptoms, and for local reactions after injections twice daily in the week after immunizations. Animals were sedated for immunizations or sample collections, and body weight and temperature were measured. At certain time points, an advanced examination consisting of a physical examination by a veterinarian and including measurements of clinical chemistry and hematology parameters was performed. Each group consisted of eight macaques.

Vaccine administration. The DNA vaccine consisted of a mixture of three plasmids carrying either the *gag* or the *gp140* gene of HIV-1 clade C isolate 96ZM651 or an artificial fusion of modified *pol* and *nef* genes of isolate 97CN54 in the VRC-8400 DNA vector at 2 mg/ml in phosphate-buffered saline (PBS) (for details, see our previous study [28]). It was administered by i.m. injection of 1 ml in each of the upper legs. The poxvirus vaccines consisted of a 1:1 mixture of Gag^{FS}PolNef- and gp140-encoding NYVAC vectors (where FS is frameshift) with restricted replication competence (see references 28 and 46) or analogously generated variants of the NYVAC-KC vector with enhanced replication competence in human cells (30). All virus preparations were purified by sedimentation through two sucrose cushions (47). For i.m. delivery, 1 ml of a 2×10^8 PFU/ml solution in Tris-buffered saline was injected into the upper right arm. For percutaneous delivery by scarification, 100 μl of the NYVAC-KC mixture (2×10^9 PFU/ml) was first pipetted on the shaved and cleaned skin between the shoulder blades. Then, 20 strokes with a perpendicularly held bifurcated needle (Eclipse; BD) (cannula size, 0.965 mm) on an area of maximally 1 cm in diameter were made. Finally, the remaining liquid was allowed to dry. The protein vaccine consisted of recombinantly produced gp120 of the clade C isolate TV1 (37). The protein solution was mixed with an equal volume of MF59 adjuvant prior to administration. In total, 1 ml of the formulated protein containing 100 μg of TV1 was injected by the i.m. route into the upper left arm.

Immunological analyses. For a detailed description of the readout methods please see our previous paper (28). A brief summary follows.

(i) T cell analyses. Peripheral blood mononuclear cells (PBMCs) were stimulated with one of nine different pools of overlapping peptides spanning the whole autologous antigens, referred to as Gag1, Gag/Pol, Gag2/Pol, Pol1, Pol2, Env1, Env2, Env3, and Nef. Restimulated T cells were quantified by an IFN-γ ELISpot assay. Animals were classified as responders if more than 50 spot-forming units (SFUs) per million PBMCs and at least four times the week 0 background values for at least one peptide pool were measured. In addition, cryopreserved PBMCs were analyzed at the Nonhuman Primate Immunogenicity Core (M. Roederer lab), Vaccine Research Center, NIAID, as described previously (48). For flow cytometric analysis, cells were stained for the surface markers CD3, CD4, and CD8 and intracellularly for the cytokines IFN-γ, IL-2, and TNF.

(ii) BAMA. IgG and IgA antibodies binding to a panel of readout antigens were quantified by binding antibody multiplex assay (BAMA) at the Immunology Core (Duke University and Medical Center, G. D. Tomaras lab) as previously described (49). Binding to consensus gp140 proteins of group M (Con S gp140 CF), clade A (A1.con.env03 140 CF), clade B (B.con.env03 140 CF), and clade C (C.con.env03 140 CF) and to the primary Env variants 1086 gp140 (clade C), TV1 gp120 (clade C), JRFL gp140 (clade B), and OOMSA4076 gp140 (clade A1), as well as to Gag p24 and RT p66, was assessed.

(iii) Env V1V2-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 murine leukemia virus (MLV) gp70-scaffolded Case-A2 V1V2 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 PBS). Plasma samples were applied (eight 3-fold serial dilutions starting at 1:90) in quadruplicates, and the plates were incubated for 1.5 h. After plates were washed with PBS– 0.1% Tween 20, 10 μl 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 until the reaction was stopped by addition of 20 μl of HCl stop solution. Absorbance was measured at 450 nm.

(iv) Functional antibody responses. The neutralization capacity of serum samples was measured by a TZM-bl assay. TZM-bl cells were transduced in the presence of plasma dilutions with pseudoviruses or primary virus carrying the envelope proteins MW965.26 (clade C, tier 1A), MN.3 (clade B, tier 1A), SHIV-SF162P4 (clade B, tier 1A), SF162 (clade B, tier 1A), Bx08 (clade B, tier 1B), BaL (clade B, tier 1B), SS1196 (clade B, tier 1B), TV1 (clade C, tier 2), and SHIV-SF162P3 (clade B, tier 2). Finally, Tat-dependent luciferase expression was measured (Comprehensive Antibody Vaccine Immune Monitoring Consortium [CA-VIMC], M. S. Seaman lab). Antibody-dependent cellular cytotoxicity (ADCC) was assessed as described previously (50) by the responsible CA-VIMC lab. TV1 gp120-coated CEM.NKR$_{CCR5}$ target cells were coincubated with PBMCs as a source of NK cells in the presence of plasma samples. ADCC activity was determined by measuring cleavage of a fluorescent granzyme B substrate via flow cytometry. Antibody-dependent cell-mediated virus inhibition (ADCVI) activity was measured by infecting CEM.NKR$_{CCR5}$ with the HIV-1 isolate DU156 or DU422 in the presence of plasma dilutions and PBMCs as a source of NK cells as described previously (51). The level of virus replication was measured by a p24 ELISA (CA-VIMC, D. N. Forthal lab).

Statistical analyses. Comparisons between groups were assessed by a Wilcoxon rank sum test, and differences within groups over different time points were evaluated with a Wilcoxon signed rank test using R, version 3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria). P values of <0.05, corrected by the Bonferroni method where applicable, were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.01529-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.

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