


Induction of Heterologous Tier 2 HIV-1-Neutralizing and Cross-Reactive V1/V2-Specific Antibodies in Rabbits by Prime-Boost Immunization

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ABSTRACT

Poxvirus prime-protein boost used in the RV144 trial remains the only immunization strategy shown to elicit a modest level of protection against HIV-1 acquisition in humans. Although neutralizing antibodies (NAb) were generated, they were against sensitive viruses, not the more resistant “tier 2” isolates that dominate circulating strains. Instead, risk reduction correlated with antibodies recognizing epitopes in the V1/V2 region of HIV-1 envelope glycoprotein (Env). Here, we examined whether tier 2 virus NAb and V1/V2-specific non-NAb could be elicited by a poxvirus prime-gp120 boost strategy in a rabbit model. We studied two clade B Envs that differ in multiple parameters, including tissue origin, neutralization sensitivity, and presence of the N197 (N7) glycan that was previously shown to modulate the exposure of conserved epitopes on Env. We demonstrate that immunized rabbits generated cross-reactive neutralizing activities against >50% of the tier 2 global HIV-1 isolates tested. Some of these activities were directed against the CD4 binding site (CD4bs). These rabbits also generated antibodies that recognized protein scaffolds bearing V1/V2 sequences from diverse HIV-1 isolates and mediated antibody-dependent cellular cytotoxicity. However, there are subtle differences in the specificities and the response rates of V1/V2-specific antibodies between animals immunized with different Envs, with or without the N7 glycan. These findings demonstrate that antibody responses that have been correlated with protection against HIV-1 acquisition in humans can be elicited in a preclinical model by a poxvirus prime-gp120 boost strategy and that improvements may be achievable by optimizing the nature of the priming and boosting immunogens.

IMPORTANCE

The only vaccine approach shown to elicit any protective efficacy against HIV-1 acquisition is based on a poxvirus prime-protein boost regimen (RV144 Thai trial). Reduction of risk was associated with nonneutralizing antibodies targeting the V1/V2 loops of the envelope protein gp120. However, the modest efficacy (31.2%) achieved in this trial highlights the need to examine approaches and factors that may improve vaccine-induced responses, including cross-reactive neutralizing activities. We show here that rabbits immunized with a novel recombinant vaccinia virus prime-gp120 protein boost regimen generated antibodies that recognize protein scaffolds bearing V1/V2 sequences from diverse HIV-1 isolates and mediated antibody-dependent cellular cytotoxicity. Importantly, immunized rabbits also showed neutralizing activities against heterologous tier 2 HIV-1 isolates. These findings may inform the design of prime-boost immunization approaches and help improve the protective efficacy of candidate HIV-1 vaccines.

While many vaccine approaches have been tested in the clinic, all but one have failed to protect against HIV-1 acquisition (1, 2). Only the RV144 trial achieved a modest efficacy of 31.2% using a prime-boost strategy with nonreplicative recombinant canarypox virus and bivalent gp120 protein (3). Antibodies against variable loops 1 and 2 (V1/V2) and high levels of antibody-dependent cellular cytotoxicity (ADCC) activities were found to inversely correlate with the risk of HIV-1 acquisition (4–6). Neutralizing antibodies (NAb) were generated but were primarily against tier 1 isolates, with little or no tier 2 neutralizing activity detected (7). Despite these limitations, results of the RV144 trial provide a starting point to examine factors in the prime-boost strategy that may improve vaccine efficacy, including the generation of antibodies that may neutralize tier 2 viruses.

Passively administered NAb have been shown to protect against primate lentivirus infection in animal models (1, 2, 8–11); therefore, it remains a major goal for HIV-1 vaccines to elicit these

antibodies. Recent studies described vaccine-induced tier 2 virus NAb in immunized animals; however, these responses are limited, sporadic, and primarily against the autologous tier 2 isolates (12–14). Novel immunogens are being examined in the hope that they may elicit cross-reactive tier 2 NAb (1, 2, 15, 16). We previously

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reported that removal of a single N-linked glycan at amino acid N197 (N7) of gp120 enhanced the ability of Env to generate cross-reactive neutralizing responses (17). This study was based on a single isolate, 89.6. Since the N7 glycan and its effect on Env antigenicity are highly conserved (17–21), it is of interest to determine if the effects of the N7 glycan on Env immunogenicity can be observed in isolates other than 89.6.

In the present study, we sought to examine whether antibody responses that have been correlated with protection against HIV-1 acquisition in humans can be elicited in a preclinical model by a poxvirus prime-gp120 protein boost strategy. Specifically, we used a replication-competent vaccinia virus vector for priming and two clade B Envs (JR-FL or PVO.4) for boosting. These Envs differ in multiple parameters, including tissue origin, neutralization sensitivity, and presence of the N7 glycan, which modulates the exposure of variable loop 3 (V3) and CD4 binding sites (CD4bs) on Env (17, 21–23). Using this prime-boost immunization regimen, we were able to induce cross-reactive binding antibodies against V1/V2 fusion proteins and neutralizing responses against heterologous tier 2 isolates. However, in contrast to our previous finding with 89.6 Env (17), results from the present study showed that the absence of the N7 glycan had little impact or a negative one on Env immunogenicity, indicating the need for further improvements in immunization strategy by optimizing the nature of the priming and boosting immunogens.

MATERIALS AND METHODS

Envelope genes. A plasmid encoding the Env of JR-FL (22) was a gift from Paul Clapham, while a plasmid encoding SF162 Env was a gift from Leonidas Stamatatos and Cecilia Cheng-Mayer (24). The following Env-encoding plasmids, including the standard clade B (23) and the global tier 2 (25) env panels, were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: TRO.11 (23, 25), 6535.3, SS1196, QH0692.42, SC422661.8, AC10.0.29, and PVO.4 from David Montefiori and Feng Gao (23); RHPA4259.7, REJO4541.67, and WITO4160.33 from B. H. Hahn and J. Salazar-Gonzalez (23); THRO4156.18 and CAAN5342.A2 from B. H. Hahn and D. L. Kothe (23); TRJO4551.58 from B. H. Hahn, X. Wei, and G. M. Shaw (23); Bal.26 (26) and DJ263.8 (27) from John Mascola; CRF02_AG clone 271 from Dennis Ellenberger, Bin Li, Margaret Callahan, and Salvatore Butera; CE1176_A3 from Ronald Swanstrom, Li-Hua Ping, Jeffrey Anderson, and David Montefiori (25); and murine leukemia virus (MuLV) from Nathaniel Landau and Dan Littman (28).

Construction of N-linked glycosylation site mutants. Generation of N7 glycan mutants was described in previous publications (17, 21). Briefly, we used the QuikChange mutagenesis kit (Stratagene) to mutate or to restore the codon for the asparagine (N) residue in the potential N-linked glycan (PNLG) sequon (N-X-S/T). In the case of PVO.4, the asparagine (N) at amino acid residue 197 (HXB2 numbering) was substituted with glutamine (Q) to generate N7 deglycosylated PVO.4 (PVO.4 N7–) Env. In the case of JR-FL, the N7 PNLG sequon was restored by replacing the aspartic acid (D197) with N to generate N7 glycosylated JR-FL (JR-FL N7+) Env.

Cells. TZM-bl cells (HeLa origin, catalog no. 8129, NIH AIDS Reagent Program), an African green monkey kidney cell line (BSC40; ATCC catalog no. CRL2761), 293T cells (ATCC catalog no. 11268), and a human osteosarcoma cell line that lacks a functional TK gene (TK– 143B; ATCC catalog no. CRL-8303) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (complete DMEM). TK– cells were cultured in complete DMEM containing 25 µg/ml 5-bromodeoxyuridine.

Recombinant vaccinia virus (rVV). A plaque-purified, replication-competent vaccinia virus (VV) generated from the New York City Board of Health strain (v-NY) was used as the vector to express full-length Env gp160 or subunit gp120 protein. The latter was generated by introducing one or two stop codons at the primary cleavage site between gp120 and gp41. Recombinant vaccinia viruses expressing either N7+ or N7– versions of JR-FL or PVO.4 Env gp160 and gp120 were generated as previously described (29–31). In brief, Env-encoding transgenes were inserted into the thymidine kinase (TK) gene of the v-NY strain of VV by homologous recombination. Two rounds of plaque purification were performed under negative-selection conditions using medium containing 24 µg/ml 5-bromodeoxyuridine on TK– 143B cells before a third round of purification was performed under nonselective conditions on BSC40 cells. Plaques were screened for transgenes by PCR using a primer set specific to the TK gene and Env. Recombinant viruses were expanded and propagated on BSC40 cells to generate crude virus stocks. Virus stocks that expressed gp160 were subsequently concentrated by sucrose density sedimentation as previously described (30, 32). Subunit gp120s were purified from BSC40 cells infected with gp120-expressing recombinant vaccinia viruses as previously described (33).

Expression of the transgene, under the control of a synthetic early-late promoter of VV, was verified by Western blotting as previously described (33, 34). Briefly, monolayers of BSC40 cells were grown to confluence and subsequently infected with recombinant or v-NY parental virus at a multiplicity of infection of 3. At 48 h postinfection, cells were harvested and resuspended in PBSAM buffer (PBS, 10 mM MgCl₂, 0.01% bovine serum albumin [BSA]). After two freeze-thaw cycles, the cells were sonicated, lysed with NuPage reducing agent (Invitrogen; catalog no. NP0004), and fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrotransfer to a nitrocellulose membrane, immunoreactive proteins were detected with pooled HIV-positive serum (NABI).

Immunization. Twenty-four female New Zealand White rabbits were purchased and maintained at Covance (Denver, PA). All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee. Rabbits were divided into four groups, sedated with ketamine (40 mg/kg) and xylazine (5 mg/kg), and then primed with ~10⁸ PFU of recombinant VV at weeks 0 and 8 by scarification. Virus was applied to two shaved sites on the back of each animal. Each area was pricked 20 times, penetrating the skin, with a bifurcated needle. Animals remained sedated until both scarification sites were dry. At weeks 35 and 48, animals were boosted intramuscularly at two sites (50 µg each) with 100 µg of the cognate gp120 formulated in alum. Blood was collected from each rabbit on the day of, and 2 weeks after, each immunization event. Blood samples were processed to serum according to standard operating procedures at Covance. Vaccine take was verified by visualization of vaccinia virus lesions, vaccinia neutralization assays, and seroconversion.

Antibody capture assays. HIV-1 Env-specific antibody titers in serum were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (35). Wild-type (WT) JR-FL or WT PVO.4 gp120 produced from Chinese hamster ovary (CHO) cells (kind gift from Shan Lu) or WT SF162 gp120 (Immune-tech catalog no. 001-0028P) were used as the capture antigen (100 ng gp120 per well). The endpoint ELISA titer of binding antibodies is defined as the reciprocal of the serum dilution that resulted in optical density (OD) readings greater than the mean OD readings of the preimmune serum at a 1:100 dilution plus three times the standard deviation. The limit of detection was considered the starting dilution (1:100) of the test sera. All experiments were repeated three times in duplicate.

Bioplex assays against peptides were performed as previously described (36). Briefly, 15-mer peptides overlapping by 11-mers corresponding to the variable regions 1 to 3 (V1 to V3) or conserved region 1 or 5 (C1 or C5, respectively) were obtained through the HIV-1 Consensus Subtype B Env Peptide Set from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Ten micrograms of each peptide was coupled

to Bio-Plex COOH beads (Bio-Rad) by primary amine coupling according to the manufacturer's protocol. Immune and prebleed sera were diluted 1:20 in 1% Blotto-PBS, mixed with peptide-coupled beads (~500 beads per peptide), and incubated at room temperature for 1.5 h with shaking. To avoid potential competition, only beads coupled to peptides with no overlapping segments were included in the same well. After washing, phycoerythrin-labeled secondary antibody diluted 1:100 in 1% Blotto was added for 1 h with shaking. Following washing, the beads were analyzed for binding on a Bioplex 100 system (Bio-Rad), and the net mean fluorescence intensity (MFI) against individual peptides from each pool, minus the net MFI of matched prebleed samples, is reported. The reactivity of 447-52D (from the NIH ARRRP and Susan Zolla-Pazner) to V3 peptide (37–42) and coupled beads with no antibody or serum added were used as positive and negative controls, respectively. All experiments were repeated at least twice in duplicate.

Serum antibodies to a panel of V1/V2 scaffolds were measured by a binding antibody multiplex assay (BAMA) similar to that previously described (4, 43), with the following modifications: secondary antibody was goat anti-rabbit IgG, biotinylated (Thermo Fisher Scientific, Rockford, IL). Antibody measurements were acquired on a Bio-Plex instrument (Bio-Rad, Hercules, CA) using software compliant with 21 CFR Part 11, with the primary readout as MFI. Samples with MFI values that were (i) at least 100, (ii) higher than the antigen-specific cutoffs (95%ile MFI of all preimmune sera for each V1/V2 scaffold), and (iii) >3-fold that of the preimmune serum from the same animal were considered positive.

Assays for ADCC activity. ADCC activity in rabbit sera was determined by two methods: a flow-based assay using gp120-coated cell targets and a luciferase-based assay using virus-infected cell targets. These methods are based on the report of Pollara et al. (44).

Flow-based assay. CEM.NKRCCR5 cells (45) coated with recombinant HIV-1 SF162 gp120 (Immune Technology Corp., New York, NY) were used as targets. The SF162 gp120s share the same subtype B assignment as the immunogen used in the vaccine but represent a heterologous antigen for all vaccine groups. Cryopreserved human peripheral blood mononuclear cells (PBMC) from an HIV-seronegative donor heterozygous for 158F/V polymorphic variants of Fcγ receptor 3A were used as the source of effector cells (46). Rabbit sera were tested at six 4-fold serial dilutions starting at 1:100. Effector and target cells (at a ratio of 30:1) were incubated in the presence of rabbit sera or assay controls for 1 h. ADCC activity was measured as the frequency of events recorded in the granzyme B (GzB)-positive gate, with the cutoff value for positivity at $\geq 8\%$ GzB. Results are reported either as peak ADCC activity, representing the maximum percent GzB activity at any given dilution, or as ADCC titers, calculated as the dilution at which the responses were $\geq 8\%$ GzB.

Luc-based assay. CEM.NKRCCR5 cells were infected with an infectious molecular clone (IMC) of HIV-1 encoding the SF162 (accession number EU123924) *env* gene within an NL4-3 backbone that also expresses the *Renilla* luciferase (Luc) reporter gene (47). ADCC activity was determined as the change in the relative luciferase units (RLU) resulting from the loss of intact targets in test wells (containing effector cells, target cells, and the test serum) compared to RLU in control wells (containing only target cells and effector cells). ADCC activity was reported as percent specific killing, calculated as [(number of RLU in control well – number of RLU in test well)/(number of RLU in control well)] $\times 100$. The results were considered positive if ADCC activity was $\geq 15\%$ specific killing after subtracting the background observed in preimmune sera. ADCC activities are reported either as the maximum percent specific killing observed for each test serum at any dilution or as ADCC titers, defined as the serum dilution that intersects the positive cutoff at $\geq 15\%$ specific killing.

Neutralization assays. Stocks of Env-pseudotyped viruses were prepared by cotransfection of Env-encoding and backbone plasmids in 293T cells (23). pSG3ΔEnv backbone was used to generate clade B Env-pseudotyped virus, and Q23ΔEnv backbone was used to generate clade A and AG Env-pseudotyped virus. All assay stocks were titrated in TZM-bl cells as previously described (23). Infectivity of Env-pseudotyped virus was

measured by luciferase reporter gene expression in TZM-bl cells. Neutralization as measured by the reduction of luciferase gene expression was performed as previously described (20, 48). Briefly, indicator virus containing 150 50% tissue culture infective doses (TCID₅₀) was incubated with a single dilution or serial 3-fold dilutions of serum samples in duplicate in a total volume of 150 μl for 1.5 h at 37°C in 96-well flat-bottom culture plates before being added to TZM-bl cells (10,000 cells in 100 μl of growth medium containing DEAE dextran). One set of control wells received cells plus virus (virus control), and another set received cells only (background control) in addition to wells that received antibodies with known ID₅₀ values (positive control). After a 72-h incubation, 100 μl of cells was transferred to a white 96-well solid plate (Costar) for assays of luciferase activities using BrightGlo substrate solution as described by the supplier (Promega). In peptide competition experiments, the same neutralization protocol described above was applied, with the exception that before the exposure of the sera to pseudovirus, the sera were incubated for 30 min at 37°C with 30 μg/ml of overlapping peptides spanning the V3 loop of a consensus B (ConB) HIV-1 subtype Env or scrambled V3 peptide. Percent neutralization was determined by measuring the reduction in number of RLU relative to that of wells that contain the same dilution of the corresponding preimmune sera.

Neutralization of the global tier 2 virus panel and mutant CE1176_A3 was performed as previously described (25, 49). The neutralization titer was expressed as the reciprocal serum dilution that resulted in 50% reduction of RLU (ID₅₀).

Generation of pseudotyped viruses with mutant Env of CE1176_A3. Mutations in an epitope(s) targeted by a panel of broadly neutralizing monoclonal antibodies (bNMAb) were introduced into the CE1176_A3 *env* gene using the QuikChange mutagenesis kit (Stratagene) (21). The epitopes targeted and the specific mutations introduced were as follows: (i) CD4bs epitopes, N280D and G458Y; (ii) V2 epitopes, N160K; (iii) V3 epitopes, N332A; (iv) membrane-proximal external region (MPER) epitopes, W672A; and (v) gp120/gp41 epitopes, N611A and N625A. Since pseudotyped viruses bearing a mutated epitope(s) showed increased resistance only to the bNMAb targeting these sites, this panel of mutants was used to characterize the nature of neutralizing activities against CE1176_A.

Statistical analysis. Prism (GraphPad Software, Inc.) was employed for all statistical analyses, all using two-sided tests. The Wilcoxon matched-pairs signed-rank test was used to compare MFI binding and ID₅₀ values against the global tier 2 panel. The Mann-Whitney test was used to compare pairwise reciprocal serum ID₅₀ values and percent neutralization against tier 1 viruses. Differences between three or more groups were analyzed by the Kruskal-Wallis analysis of variance (ANOVA) test. The percentages of the global tier 2 panel neutralized were compared within the same groups by using McNemar's test for proportions for paired values.

RESULTS

Immunogens and immunization regimen. To determine if antibody responses elicited by a poxvirus prime-gp120 protein boost strategy are affected by the choice of the Env immunogen used, we studied two clade B Envs (JR-FL and PVO.4) that differ in multiple parameters, including tissue origin, neutralization sensitivity, and presence of the N7 glycan previously shown to modulate the exposure of the CD4 and coreceptor binding sites on Env (17, 21–23). JR-FL is a brain isolate that lacks the N7 glycan sequon (22) and shows tier 2 neutralization sensitivity, whereas PVO.4 is a blood isolate that retains the highly conserved N7 glycan sequon and shows a tier 3 neutralization-resistant phenotype (21, 23, 50).

To increase the efficacy of priming with these Env immunogens, we took advantage of an attenuated but replication-competent vaccinia virus vector, v-NY, which was used in the first clinical

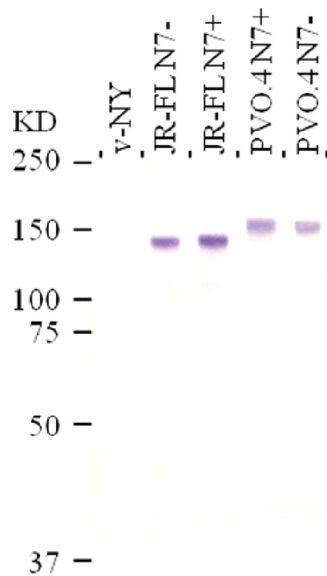


FIG 1 Western blot analysis of gp160 expressed by recombinant vaccinia virus. Lysates of African green monkey kidney cells (BSC-40) infected with parental vaccinia virus, v-NY, or recombinant viruses expressing the full-length Env of HIV-1 JR-FL or PVO.4 were resolved by SDS-PAGE, and immunoreactive protein gp160 was detected by HIV-positive human serum. N7⁻ and N7⁺ denote the absence and presence, respectively, of the sequon for the N-linked glycan at amino acid 197.

trials of the poxvirus-protein boost strategy (29, 51). We generated recombinant vaccinia viruses that express full-length Envs with (N7⁺) or without (N7⁻) the sequon of the N197 glycan (Fig. 1). As reported previously, overexpression of full-length HIV-1 Env resulted in the accumulation of unprocessed gp160. However, these Envs are processed, albeit inefficiently, and are fully functional in engaging the CD4 receptor and mediating membrane fusion (34, 52).

New Zealand White rabbits were primed at weeks 0 and 8 with recombinant vaccinia virus expressing one of four full-length gp160s: JR-FL N7 deglycosylated (JR-FL N7⁻), JR-FL N7 glycosylated (JR-FL N7⁺), PVO.4 N7 glycosylated (PVO.4 N7⁺), or PVO.4 N7 deglycosylated (PVO.4 N7⁻) Env (Fig. 1). Animals were boosted with the cognate gp120 Env protein formulated in alum at weeks 35 and 48 (Fig. 2). Animal sera collected 2 weeks

after the second vaccinia immunization (week 10) and after each gp120 boost (weeks 37 and 50) were tested for antibody binding, ADCC, and neutralizing capabilities in comparison to pre-immune sera.

All immunized animals generated Env-specific antibodies.

In order to determine the potency and cross-reactivity of the Env-specific antibodies, we tested the ability of week 37 and week 50 sera to bind homologous wild-type (WT) JR-FL and PVO.4 gp120 protein as well as heterologous SF162 gp120 protein in an ELISA (Fig. 3A). Immune sera showed endpoint binding titers of $\sim 10^5$ against SF162 gp120 and titers up to $\sim 10^6$ against homologous gp120. All sera demonstrate similar endpoint binding titers to heterologous gp120. Maximal endpoint titers were achieved after the first gp120 boost, with no further increase obtained after the second boost. This indicates that the prime-boost immunization regimen, regardless of the Env used in this study, was able to generate high titers of cross-reactive Env-specific antibodies.

Effect of gp120 boost on the specificity of anti-Env responses.

Previous studies with subunit and prime-boost immunization have elicited higher antibody titers against V3, constant region 1 (C1), and constant region 5 (C5) than against V1 and V2 (53–55). To determine the epitope specificity of the antibody responses in immunized animals, we used a Bioplex assay to examine the ability of week 37 and week 50 sera to bind overlapping peptides derived from consensus B (ConB) Env spanning C1, C5, V1, V2, and V3 epitopes (Fig. 3B). Consistent with previous studies and regardless of which immunogen was used, high levels of serum reactivity were detected against C1, C5, and V3 peptides compared to V1 and V2 peptides. All animals generated antibodies that recognize peptides that span the midregion of the V2 loop (HXB2 numbering sequence 169 to 184; data not shown), a region recognized by antibodies generated by protected RV144 vaccinees (56). While there was no significant difference in the overall gp120-binding antibody titer between week 37 and week 50 sera (Fig. 3A), the second gp120 immunization significantly boosted antibody reactivity to all peptides tested (Fig. 3B), indicating a preferential expansion of antibodies that recognize linear epitopes resulting from the gp120 boosts.

Cross-reactive antibodies that recognize recombinant V1/V2 fusion proteins. Antibodies against the V1/V2 loop, specifically those that recognize V1/V2-gp70 fusion proteins, have been shown to correlate with protection in the RV144 trial (4–6). Since

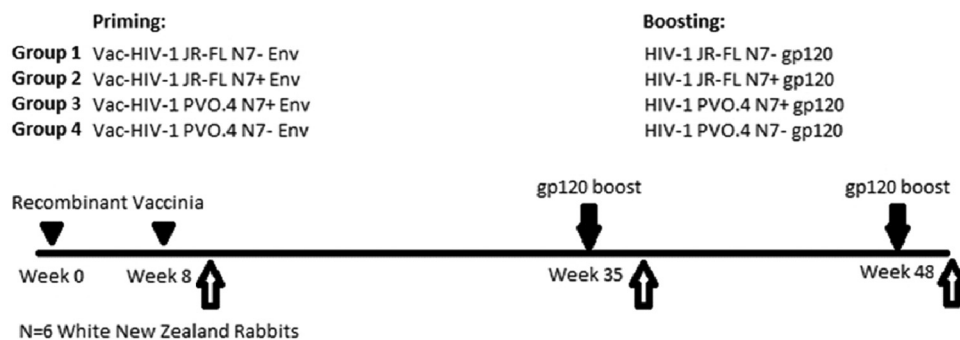


FIG 2 Study design and immunization schedule. Rabbits were immunized with recombinant vaccinia virus expressing full-length gp160 at weeks 0 and 8, followed by a cognate gp120 protein boost formulated in alum at weeks 35 and 48 as described in Materials and Methods. Sera were collected before and 2 weeks after each immunization event. Time points at which sera were collected for the analyses described in this study are marked by open arrows (weeks 10, 37, and 50).

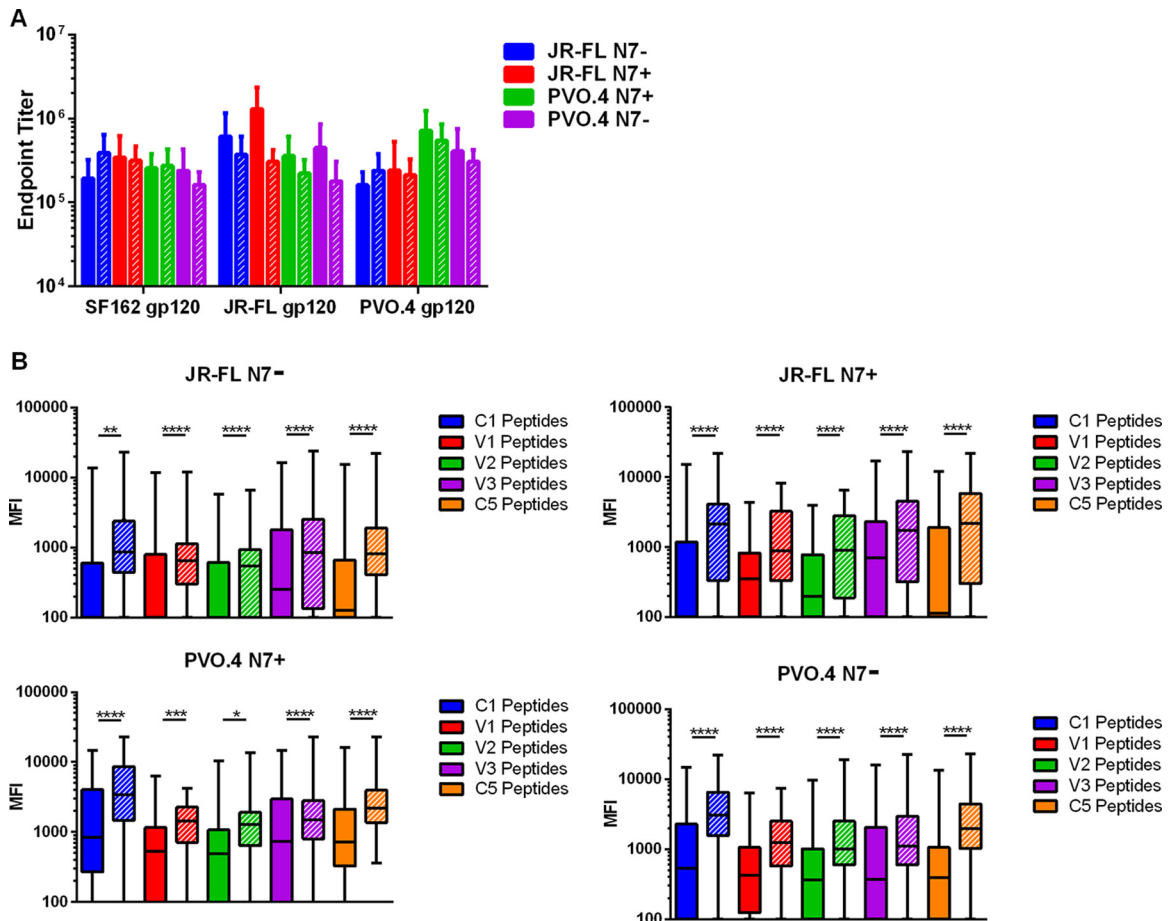


FIG 3 gp120-specific antibody response in immunized animals. (A) Endpoint titers of Env-specific antibodies in week 37 (solid boxes) or week 50 (hatched boxes) sera were determined by ELISA against a heterologous gp120 (WT SF162 gp120) or a homologous gp120 (WT JR-FL or PVO.4 gp120) as denoted on the x axis. Error bars indicate standard deviations within the group. (B) Reactivity of antibody responses targeting single, overlapping linear peptides corresponding to the variable (V1 to V3) or conserved (C1 and C5) regions of gp120 as indicated was reported as the mean fluorescence intensity (MFI) in the Bioplex assay. Solid boxes indicate week 37 serum, and hatched boxes represent week 50 serum. The boxplots indicate the median as well as the range of responses against individual peptides in each pool, including the maximum, minimum, and the 75% and 25% quartiles as indicated. *P* values (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$) were calculated by the Wilcoxon matched-pairs signed-rank test.

all animals generated antibodies against peptides corresponding to V1 and V2 (Fig. 3B), we tested the rabbit serum's ability to recognize a cross-clade panel of V1/V2 fusion proteins derived from different Envs in a multiplex binding antibody assay (Fig. 4). By week 37, all 12 JR-FL-immunized animals and 9 of 12 PVO.4-immunized animals generated antibodies that recognize V1/V2 fusion proteins derived from multiple isolates of different clades. Reactivities to V1/V2 fusion proteins are comparable between week 37 and week 50 sera, indicating that the priming followed by one gp120 boost was sufficient to generate V1/V2 binding antibodies. The animals immunized with JR-FL N7+ generated some of the highest mean fluorescence intensity (MFI) values against clade B V1/V2 fusion proteins, compared to other groups. Regardless of the presence or absence of the N7 glycan, immunization with JR-FL Env consistently generated V1/V2 fusion binding antibodies capable of recognizing clade B, C, and AE Envs. This contrasts with the more sporadic reactivity observed with PVO.4 Env immunization. These results indicate that the prime-boost strategy effectively induced V1/V2-directed antibodies, but the profile of their V1/V2 reactivity

differed between animals that received the JR-FL or the PVO.4 immunogens.

Antibody-dependent cellular cytotoxicity in immune sera. Prime-boost immunization in the RV144 trial induced robust HIV-1-specific ADCC responses (4), some of which were linked to the protective V1/V2 Ab response (6, 57). Since Env-specific ADCC activity can be elicited in rabbits (58), we examined if antisera from the present study were able to mediate ADCC. As shown in Fig. 5, immune sera from all experimental groups showed robust ADCC activities against target cells expressing a heterologous clade B (SF162) Env in the infected cell assay (Fig. 5A and B) and target cells coated with SF162 gp120 (Fig. 5C and D). It is notable that with the exception of JR-FL N7- Env-immunized animals, close to maximal levels of ADCC activities were elicited in animals from all immunization groups 2 weeks after recombinant vaccinia virus immunization alone (week 10, Fig. 5). ADCC titers were significantly lower in JR-FL N7- Env-immunized animals than in JR-FL N7+ and/or PVO.4 N7+ Env-immunized groups after vaccinia priming ($P \leq 0.05$, Kruskal-Wallis test) (Fig. 5B and D, week 10). However, after gp120 boosts, comparable ADCC

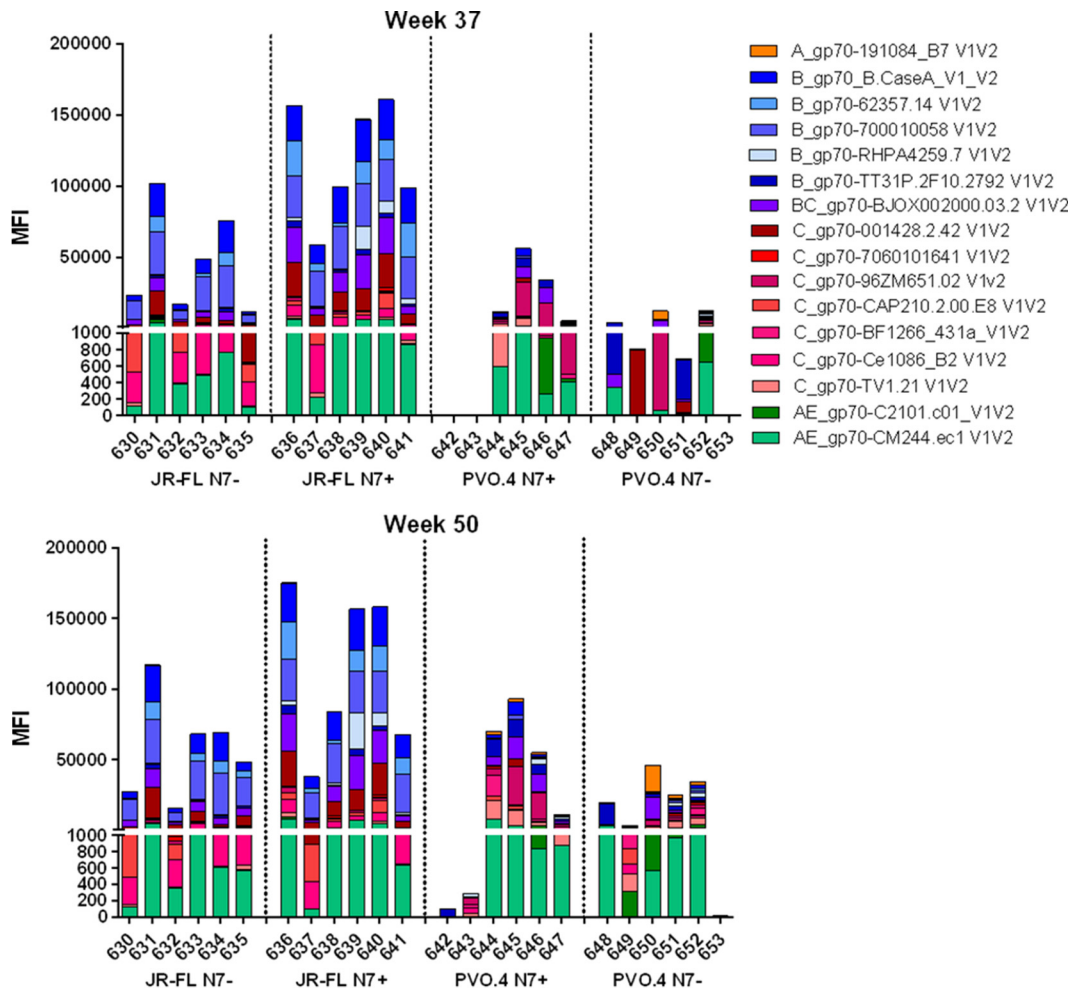


FIG 4 Cross-reactivity of anti-V1/V2 antibodies in immunized animals. V1/V2-specific antibodies in week 37 and week 50 sera at a 1:500 dilution were measured against a cross-clade panel (color coded by clade) of gp70-V1/V2 MuLV scaffold proteins in a binding antibody multiplex assay (BAMA). The animal number and immunization group are indicated on the x axis. Dotted lines separate the groups.

titers were achieved in all immunization groups by the end of the study (Fig. 5B and D, week 50).

Neutralization of standard tier 1B and tier 2 clade B isolates.

In order to determine their neutralization capabilities, we tested immune sera against a number of tier 1 HIV-1 isolates and a standard panel of primary clade B isolates (Table 1). At a 1:15 dilution, all sera neutralized SF162, a tier 1A isolate, and most sera were capable of neutralizing some tier 1B isolates, including DJ263.8, a clade A isolate (27), indicating the presence of cross-neutralizing activities. However, CRF02_AG_271, a clade AG isolate, was not as readily neutralized by the sera. Sera from 4 of 6 animals immunized with JR-FL N7+ and from 3 of 6 animals immunized with PVO.4 N7+ neutralized one or more of the tier 2 and tier 3 isolates on the standard clade B panel, whereas those from N7- Env-immunized animals failed to do so. Notably, two animals in the PVO.4 N7+ group, animals 643 and 645, showed neutralizing activities against nearly the entire panel of tier 1, 2, and 3 isolates tested.

Previous studies have shown that sera neutralizing tier 1 isolates are primarily directed against the V3 loop. Therefore, we tested whether linear ConB V3 peptides would compete with the

ability of immune sera to neutralize tier 1 viruses (Fig. 6). Regardless of the immunogen used, neutralizing activities of immune sera against SF162 and Bal.26 were fully or partially competed out with the addition of V3 peptides (Fig. 6A and B), indicating that V3-directed antibodies contributed to the neutralizing activities in the sera. However, neutralization of DJ263.8 was unchanged with the addition of V3 peptides (Fig. 6C), indicating that the neutralizing activities could not be competed with the V3 peptides used, or the NAb may recognize epitopes other than V3 on the clade A Env.

Cross-clade neutralization of a panel of global tier 2 isolates.

To further test the breadth of the neutralizing reactivity of the immune sera, we tested the ability of week 37 sera to neutralize a standard panel of global tier 2 viruses (Fig. 7). Interestingly, each virus on the panel was neutralized by at least one animal sera tested. All animals, except one in the JR-FL N7- group (animal 631) (Fig. 7A, CE1176_A3 panel), generated sera capable of neutralizing at least one virus on this global panel. Notably, a tier 2 clade C isolate, CE1176_A3, was neutralized by sera from all but one animal, with titers reaching ~1:100 for some sera (Fig. 7A). JR-FL N7+ and PVO.4+ Env-immunized animals generated

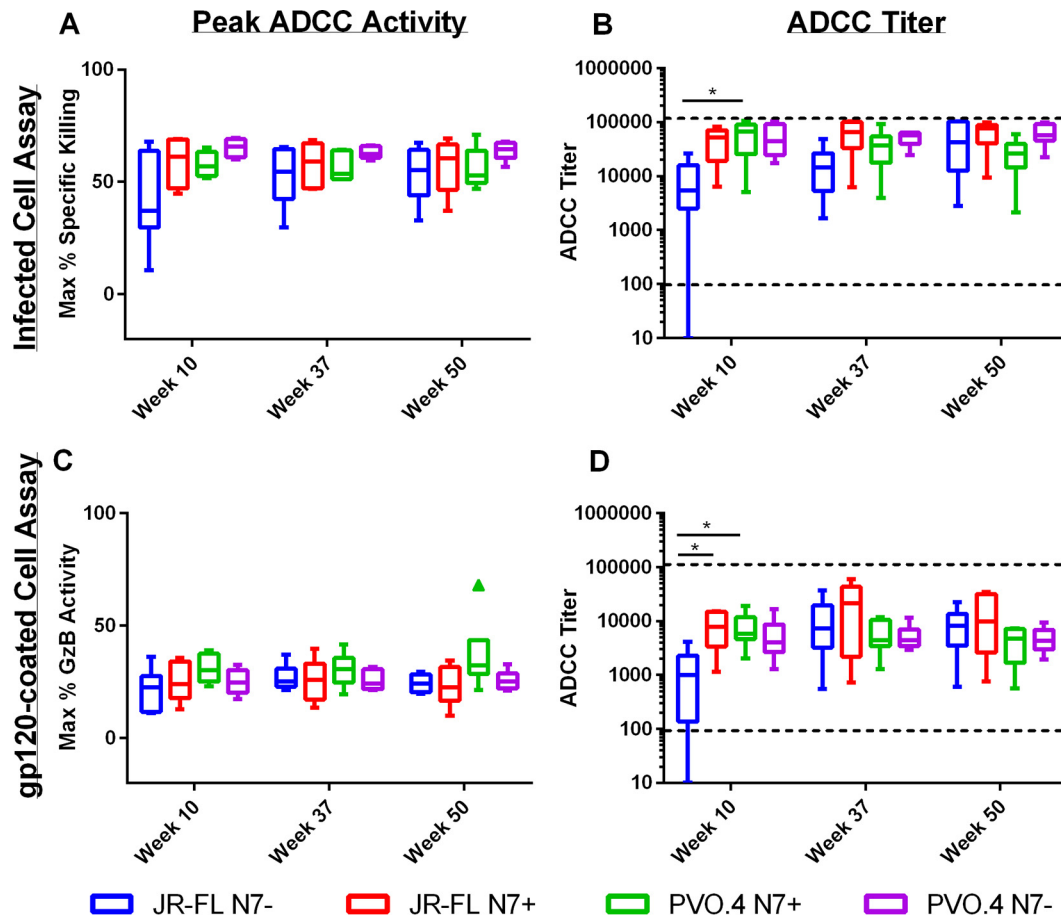


FIG 5 ADCC activity in rabbit sera. ADCC activity was measured by two methods: a luciferase-based assay using CEM.NKRCR5 cells infected with HIV-1 SF162 infectious molecular clone as targets (A and B), or a flow-based assay using the same target cells but coated with SF162 gp120 (C and D). Results are expressed in the left panels as the peak ADCC activity, which is the maximum percent specific killing (A) or the maximum percent GzB activity (C) observed for each serum tested at any dilution. Alternatively, activities are reported in the right panels as the ADCC titer, which is the serum dilution at which the last positive ADCC response was observed ($\geq 15\%$ specific killing for panel B; $\geq 8\%$ GzB activity for panel D). Activities in preimmune sera were subtracted from all values reported for immune sera collected at weeks 10, 37, and 50. The median, range, and upper and lower quartile of values within each group are indicated by the boxplots, and statistically significant differences between groups are indicated by horizontal bars above the respective boxes (*, $P \leq 0.05$, Kruskal-Wallis test).

higher ID₅₀ titers against the global tier 2 virus panel than the JR-FL N7⁻ Env-immunized animals, while PVO.4 N7⁺ Env-immunized animals showed a trend ($P = 0.08$) toward higher ID₅₀ titers against the same panel of viruses in comparison to PVO.4 N7⁻ Env-immunized animals (Fig. 7B). Dose dependence of the neutralizing activities of week 37 sera against a subset of the tier 2 viruses is shown in Fig. 8. To demonstrate the specificity of viral inhibition activities in the immune sera, we summarize in Table 2 the ID₅₀ values for all the virus and serum combinations (including MuLV as the negative control and sera from weeks 37 and 50) without background subtraction. Notably, the percent neutralization against the global panel diminished after a second gp120 boost for animals in the JR-FL N7⁺ and PVO.4 N7⁻ groups (Table 2 and Fig. 7C), consistent with the notion that repeated immunization with gp120 did little to boost the neutralizing antibody response against heterologous tier 2 isolates tested.

Characterization of neutralizing activities in immune sera against CE1176_A3. To determine the epitope specificity of the tier 2 neutralizing activities in the immune sera, we generated a

panel of site-specific mutants of CE1176_A3 that are resistant to one or more broadly neutralizing monoclonal antibodies (bNMAbs) of known specificities. CE1176_A3 env was used to generate these mutants because all animals but one could neutralize this isolate better than other isolates on the global panel (Fig. 7A). Previous work has defined the epitopes targeted by bNMAb by mutational analysis: the N280D and G458Y mutants are resistant to CD4bs-directed antibodies VRC01, CH31, and 3BNC117 (59), the N160K mutant is resistant to V2-directed antibodies CH01, PG9, and PG16 (60–62), the N332A mutant is resistant to V3 glycan-directed antibody PGT128 (63), the W672A mutant is resistant to MPER-directed antibody 4E10, 2F5, or 10E8 (64, 65), and the N611A N625A double mutant is resistant to antibody PGT151 directed to gp120/gp41 interface epitopes (66, 67). Based on this information, a panel of single-site and multiple-site mutants was generated and their sensitivity profile was tested against a panel of bNMAb listed above. Results in Fig. 9 confirm the expected neutralization sensitivity profiles of the following mutants: (i) N280D and G458Y single-site mutants were resistant to CD4bs-directed monoclonal antibodies (MAb) VRC01, CH31,

TABLE 1 Neutralization of tier 1 and tier 2 viruses

| Group | Virus | Percent Neutralization of HIV -1 Pseudovirus ^a by Week 37 Serum at 1:15 dilution | | | | | | | | | | | | | | | | | | | | |
|-----------|-------|---|----------------------|---------|---------|----------|---------------------|---------|-------------------------|--------------------------|----------------------|-------------------------|--------------------------|---------------------------|---------------------------|---------------------------|--------------------------|---------------------|---------------------------|--------|--------|--------|
| | | SF 162 | 653 5.3 ^b | BAL .26 | SS 1196 | DJ 263.8 | CRF0 2_AG Clone 271 | JR-FL | QH069 2.42 ^b | SC422 661.8 ^b | TRO.1 1 ^b | AC10. 0.29 ^b | RHPA 4259.7 ^b | THRO4 156.18 ^b | REJO45 41.67 ^b | WITO41 60.33 ^b | CAAN5 342A2 ^b | PVO. 4 ^b | TRJO45 51.58 ^b | Mu LV | | |
| | | Clade | B | B | B | B | A | AG | B | B | B | B | B | B | B | B | B | B | B | B | B | |
| | | Animal Number | Tier 1A | Tier 1B | Tier 1B | Tier 1B | Tier 1B | Tier 1B | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 3 |
| JR-FL N7- | 630 | 95 | 71 | 91 | 84 | 65 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 631 | 96 | 63 | 93 | 80 | 78 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 632 | 95 | - | 96 | 81 | 65 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 633 | 94 | 57 | 79 | 75 | 59 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 634 | 93 | 63 | 95 | 81 | 80 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 635 | 95 | 57 | 87 | 78 | 79 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| JR-FL N7+ | 636 | 95 | 57 | 92 | 66 | 84 | - | 55 | - | - | - | - | - | 51 | - | - | - | - | - | - | - | - |
| | 637 | 90 | - | 93 | 75 | 51 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 638 | 98 | - | 98 | 82 | 78 | 56 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 639 | 94 | 81 | 88 | 72 | 85 | - | 52 | 55 | - | - | - | - | - | 51 | 55 | - | - | - | - | - | - |
| | 640 | 97 | 91 | 96 | 83 | 90 | 53 | 90 | 70 | - | - | 73 | 60 | - | - | 60 | - | - | - | - | - | - |
| | 641 | 99 | 58 | 99 | 86 | 93 | 57 | - | 58 | - | - | - | - | - | - | 51 | - | - | - | - | - | - |
| PVO.4 N7+ | 642 | 97 | - | 94 | 75 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 643 | 98 | 84 | 96 | 86 | 87 | 69 | 87 | 83 | 78 | 50 | 75 | 81 | 72 | 74 | 62 | - | 68 | - | - | - | - |
| | 644 | 96 | - | 90 | 64 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 645 | 98 | 92 | 57 | 56 | 86 | - | 81 | 81 | 78 | 72 | 81 | 84 | 74 | 76 | 63 | 63 | 59 | 56 | - | - | - |
| | 646 | 93 | - | 55 | - | - | - | 70 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 647 | 99 | 90 | 98 | 84 | - | 58 | 66 | 51 | - | - | - | - | 56 | - | - | - | - | - | - | - | - |
| PVO.4 N7- | 648 | 73 | - | - | - | - | - | 50 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 649 | 96 | - | 89 | 63 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 650 | 97 | 67 | 94 | 68 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 651 | 90 | - | 58 | - | 54 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 652 | 98 | - | 97 | 60 | 59 | 56 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 653 | 89 | - | - | - | - | - | 69 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

^a Input virus was normalized by infectivity at 150 TCID₅₀ in TZM-bl cells. The column subheadings specify the indicator viruses and their respective clades. Percent neutralization was determined in TZM-bl cells by measuring the reduction in number of RLU relative to that of wells that contain the same dilution of corresponding preimmune sera. Pink highlighting, 50% to 74% neutralization; red highlighting, $\geq 75\%$ neutralization. Results are the average of two or more experiments, each performed in duplicate.

^b Isolates included in the standard clade B panel are indicated (23).

and 3BNC117 but sensitive to MAb directed to all other sites (Fig. 9A and B); (ii) a quintuple mutant (N160K N332A N611A N625A W672A) retained sensitivity to CD4bs antibodies but was resistant to antibodies directed to all other sites (Fig. 9C); (iii) a quadruple mutant (N332A N611A N625A W672A) was sensitive to CD4bs- and V2-directed antibodies but resistant to V3, MPER, and gp120/

gp41 interface antibodies (Fig. 9D); and (iv) a triple mutant (N160K N332A W672A) was sensitive to CD4bs- and gp120/gp41 interface-directed antibodies but resistant to V2-, V3-, and MPER-directed antibodies (Fig. 9E). Thus, these mutants demonstrated the expected neutralization sensitivity profile against the panel of bNMAb tested.

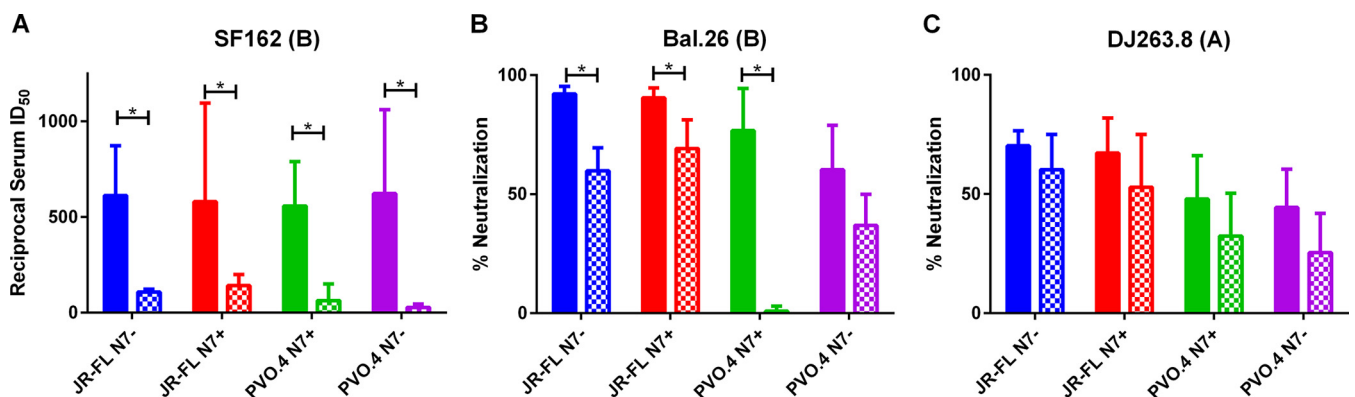


FIG 6 Competition of tier 1 virus-neutralizing activities by V3 peptides. The effect of V3 peptide on the neutralizing activity of week 37 sera against tier 1A (SF162) and tier 1B (Bal.26 and DJ263.8) viruses was evaluated. The clade designation of the indicator viruses is shown in parentheses. Neutralizing activities are shown either as the reciprocal serum dilution that resulted in 50% reduction of infectivity (ID₅₀) (A) or as the percent neutralization of virus infectivity by test sera at a 1:15 dilution (B and C). Neutralizing activities detected in the presence of a scrambled V3 peptide are represented by solid boxes, and activities detected in the presence of overlapping peptides corresponding to the consensus clade B V3 sequence are represented by checkered boxes. Error bars indicate standard deviations of data from at least two independent experiments performed in duplicate. *P* values (*, *P* \leq 0.05) were calculated by the Wilcoxon matched-pairs signed-rank test.

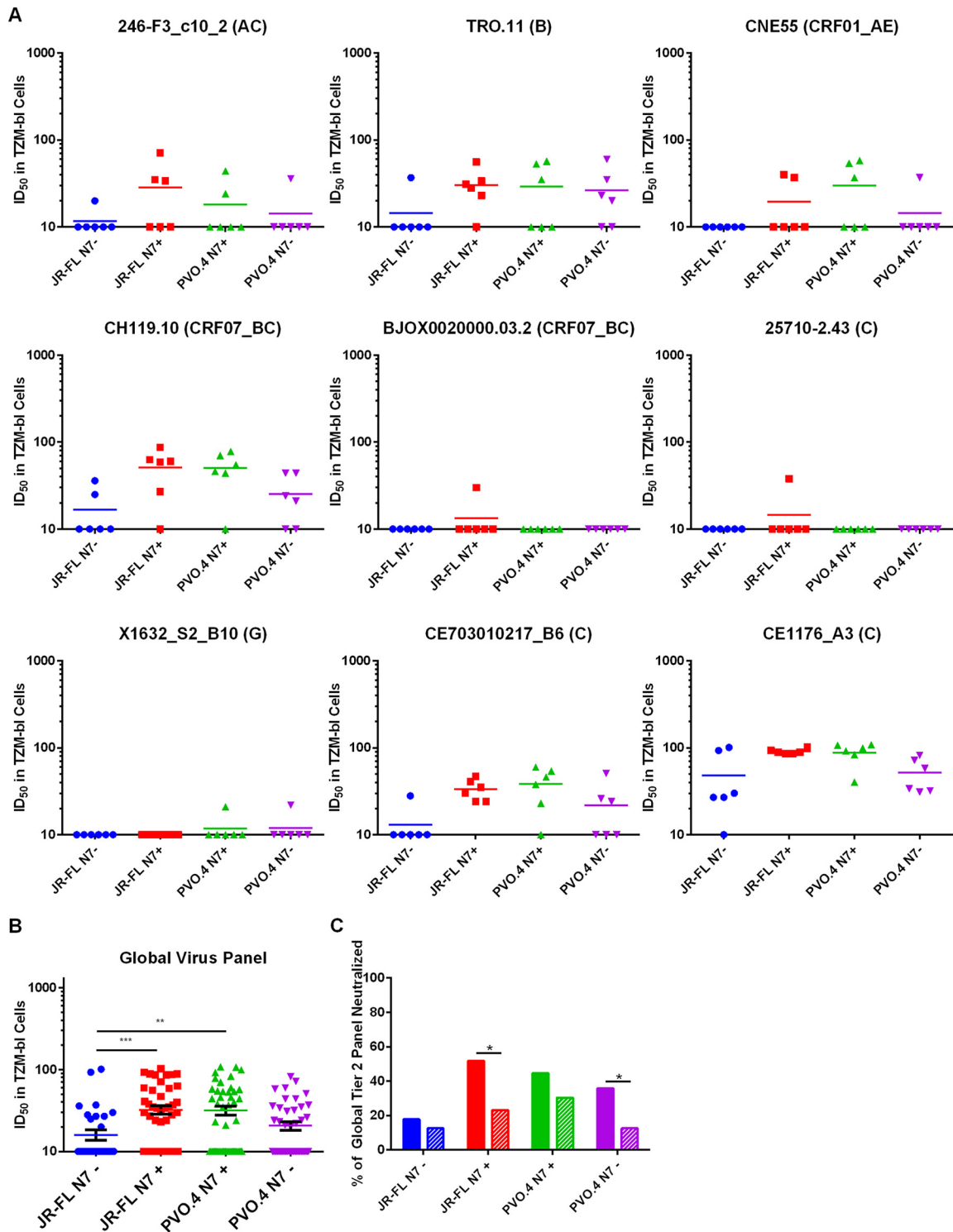


FIG 7 Neutralization of global tier 2 viruses. (A) Each graph shows the reciprocal dilution of week 37 serum from each immunized animal that resulted in 50% reduction of infectivity (ID_{50}) against the indicator virus shown at the top of each graph. The clade designation of the indicator viruses is shown in parentheses. Values above 10 were considered positive neutralization, as nonspecific activities measured in the same assay against virus pseudotyped with the MuLV envelope were subtracted as background for each serum sample before the ID_{50} values were reported here. See Table 2 for ID_{50} values before background subtraction. (B) The neutralization titer (ID_{50}) for each animal serum from each immunization group collected at week 37 was determined against the entire global tier 2 virus panel. The means are indicated by colored bars, and the standard errors are indicated by black error bars. P values ($*$, $P \leq 0.01$; $***$, $P \leq 0.001$) were calculated by the Mann-Whitney test for paired values. (C) Percent positive neutralization is defined as the percentage of the total number of serum samples and indicator virus combinations that resulted in positive neutralization in the TZM-bl assay. This value was determined for all the animals in each immunization group after the first (week 37) and second (week 50) gp120 boost as indicated. The difference in results between week 37 and week 50 samples was determined by McNemar's test for proportions for paired values ($*$, $P \leq 0.05$).

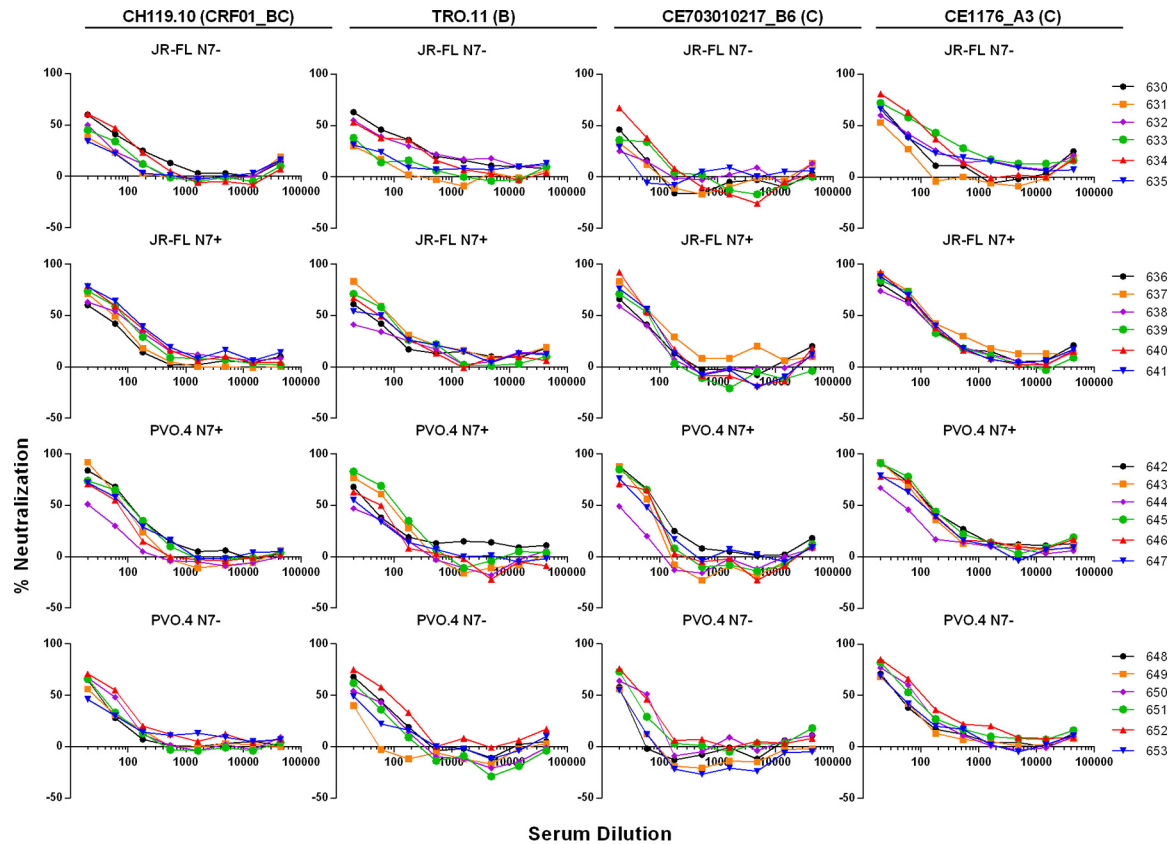


FIG 8 Dose-dependent neutralization of tier 2 viruses by week 37 sera. The *x* axis indicates the reciprocal dilutions of the immune sera tested (3-fold serial dilution starting at 1:20). The *y* axis indicates the percentage of viral infectivity neutralized by a given serum dilution against the indicator viruses. The four groups of experimental animals are aligned horizontally and designated as indicated on each panel. The four indicator viruses are aligned vertically, and their designations and clade origin (in parentheses) are given at the top of each column. Please note that the same color scheme denotes different animals in the four immunization groups (see legends on the right).

Having characterized the neutralization sensitivity profile of these mutants, we examined the epitope specificity of the neutralizing activity in a subset of immune sera against WT and mutant CE1176_A3. Most sera had similar neutralizing potencies against WT CE1176_A3 and mutant viruses, thus showing no identifiable specificity but confirming their neutralizing activities against this tier 2 virus. Notably, two animals, 640 and 647, showed clearly reduced neutralization potency when tested against CD4bs-resistant mutants (Fig. 10). Serum from animal 640 was unable to neutralize the CE1176_A3 N280D and G458Y mutants, both of which are resistant to CD4bs-directed antibodies, while retaining neutralizing activities against all other mutants tested (Fig. 10A). Serum from animal 647 also showed reduced neutralization potency against the N280D mutant, but not the G458Y mutant, consistent with the idea that the antibodies in this animal's serum targeted an epitope(s) dependent on N280 but not G458 (Fig. 10B). Together, these data indicate that CD4bs-directed antibodies may contribute to the neutralizing activities in the immune sera from these rabbits against a heterologous tier 2 virus, CE1176_A3. Interestingly, animal 647 serum also showed reduced potency against three multiple-site mutants tested (Fig. 10B), indicating neutralizing activities directed against multiple targets.

DISCUSSION

We demonstrate here a poxvirus prime-gp120 boost immunization regimen that elicited, in a rabbit model, immune responses

that have been shown to correlate with protection against HIV-1 acquisition in humans or other preclinical animal models. Specifically, we showed that immunized rabbits generated cross-clade V1/V2-specific antibodies (Fig. 4), as well as those that mediate ADCC activities (Fig. 5), both responses found to correlate with risk reduction of HIV-1 acquisition in the RV144 trial (4, 5, 56). In addition, all the immunized rabbits in this study showed neutralizing activities against one or more of the HIV-1 isolates tested, including a number of heterologous tier 2 isolates in the standard TZM-bl assay (Tables 1 and 2; Fig. 7 and 8), albeit at low titers (1:20 to ~1:100). These observations, if confirmed in parallel studies in humans or nonhuman primates, may provide a starting point to examine factors in the prime-boost immunization strategy that may improve upon the modest efficacy achieved by the RV144 trial.

The profiles of V1/V2-specific responses differed between animals immunized with JR-FL or PVO.4 Env (Fig. 4). All JR-FL-immunized animals generated antibodies that recognized V1/V2 fusion proteins from diverse isolates, whereas a number of PVO.4-immunized animals failed to show any detectable V1/V2 reactivity. The V1/V2 loops of different HIV-1 isolates differ significantly in their amino acid lengths and the numbers of potential N-linked glycan sites (68–70). Whether the differential immunogenicity in V1/V2 responses observed in this study reflects the accessibility or structural stability of the V1/V2 loop in these Envs is yet to be

TABLE 2 Neutralization against global tier 2 viruses

| Group | Animal | Bleed wk | ID ₅₀ in TZM-bl cells ^a | | | | | | | | | |
|-----------|--------|-------------|---|-----------------------------------|---------------|---------------------|------------------------|-------------------------------|-------------------|----------------------|-----------------------|------------------|
| | | | MuLV | | | | | | | | | |
| | | | negative control ^b | 246-F3_C10_2 (AC) ^c | TRO.11 (B) | CNE55 (CRF01_AE) | CH119.10 (CRF07_BC) | BJOX002000.03.2 (CRF07_BC) | 25710-2.43 (C) | X1632_S2 _B10 (G) | Ce703010217_B6 (C) | Ce1176_A3 (C) |
| JR-FL N7- | 630 | 37 | <20 | 28 | 47 | <20 | 35 | <20 | <20 | <20 | <20 | 40 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 |
| | 631 | 37 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 23 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 |
| | 632 | 37 | <20 | <20 | 27 | <20 | 20 | <20 | <20 | <20 | <20 | 37 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 43 |
| | 633 | 37 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 111 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 116 |
| | 634 | 37 | <20 | 30 | 25 | 25 | 46 | <20 | 22 | <20 | 38 | 103 |
| | | 50 | <20 | 27 | 34 | 38 | 31 | <20 | 22 | <20 | 21 | 51 |
| | 635 | 37 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 37 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 46 |
| JR-FL N7+ | 636 | 37 | <20 | 29 | 38 | <20 | 37 | 29 | 29 | <20 | 40 | 103 |
| | | 50 | <20 | <20 | 22 | <20 | <20 | <20 | <20 | <20 | <20 | 57 |
| | 637 | 37 | 52 | 64 | 86 | 89 | 56 | 29 | 36 | 24 | 76 | 138 |
| | | 50 | <20 | <20 | 28 | 23 | 28 | <20 | 21 | <20 | 24 | 58 |
| | 638 | 37 | <20 | <20 | <20 | <20 | 73 | <20 | 21 | <20 | 34 | 99 |
| | | 50 | <20 | <20 | <20 | <20 | 24 | <20 | <20 | <20 | <20 | 80 |
| | 639 | 37 | 23 | 57 | 79 | 63 | 83 | 53 | 61 | 46 | 64 | 112 |
| | | 50 | <20 | 36 | 69 | 53 | 69 | 31 | 39 | 23 | 50 | 118 |
| | 640 | 37 | 33 | 68 | 56 | 32 | 92 | 33 | 27 | 23 | 68 | 119 |
| | | 50 | 32 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 22 | 84 |
| | 641 | 37 | 23 | 94 | 54 | 34 | 110 | 21 | 27 | 41 | 70 | 126 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 31 |
| PVO.4 N7+ | 642 | 37 | 32 | 50 | 38 | 29 | 110 | 24 | 22 | 28 | 92 | 140 |
| | | 50 | <20 | <20 | <20 | <20 | 29 | <20 | <20 | <20 | 21 | 35 |
| | 643 | 37 | 29 | 73 | 86 | 87 | 84 | 45 | 42 | 50 | 67 | 112 |
| | | 50 | <20 | 21 | 56 | 59 | 52 | 22 | 20 | 22 | 42 | 89 |
| | 644 | 37 | <20 | <20 | <20 | <20 | 21 | <20 | <20 | <20 | <20 | 50 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 47 |
| | 645 | 37 | 57 | 81 | 110 | 111 | 103 | 47 | 54 | 55 | 80 | 149 |
| | | 50 | 25 | 33 | 83 | 180 | 75 | 27 | 31 | 20 | 61 | 116 |
| | 646 | 37 | 24 | <20 | 59 | 61 | 68 | 35 | 40 | 25 | 78 | 131 |
| | | 50 | <20 | <20 | 31 | 55 | 43 | <20 | <20 | <20 | 66 | 45 |
| | 647 | 37 | <20 | 26 | 26 | <20 | 80 | <20 | <20 | <20 | 56 | 109 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 |
| PVO.4 N7- | 648 | 37 | <20 | 29 | 45 | 23 | 31 | <20 | <20 | <20 | 23 | 41 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 31 |
| | 649 | 37 | <20 | <20 | <20 | <20 | 26 | <20 | <20 | <20 | 23 | 42 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 |
| | 650 | 37 | <20 | <20 | 30 | <20 | 54 | 21 | 25 | 32 | 61 | 82 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 |
| | 651 | 37 | <20 | 22 | 33 | 22 | 34 | 21 | 24 | 21 | 36 | 68 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 25 | 68 |
| | 652 | 37 | 27 | 63 | 87 | 64 | 71 | <20 | 21 | 31 | 51 | 109 |
| | | 50 | <20 | <20 | 42 | <20 | 60 | <20 | <20 | <20 | 31 | 81 |
| | 653 | 37 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 23 | 44 |
| | | 50 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 |

^a ID₅₀ is defined as the serum dilution that resulted in 50% reduction of infectivity as measured by RLU. The starting dilution was 1:20.

^b MuLV was used as the negative control; nonspecific activities shown in the column were not subtracted from other values in the table.

^c Clade designations of the indicator viruses used in the neutralization assay are indicated in parentheses.

determined. Nevertheless, our finding indicates that Envs from some HIV-1 isolates may be better than others at inducing antibodies that recognize the conformation-dependent epitopes in the V1/V2 loop. Our findings also indicate the importance of examining diverse Env designs, including polyvalent Envs and Envs with judiciously chosen modifications (e.g., glycans, stabilized

trimers, etc.) that may provide consistent and sufficient coverage of V1/V2 responses and other desired immunogenicity profiles.

While we have yet to demonstrate any functional role of the V1/V2-specific antibodies generated in this study, sera from all animals mediated ADCC against gp120-coated or HIV-1-infected target cells (Fig. 5). It is of interest to note that even animals that

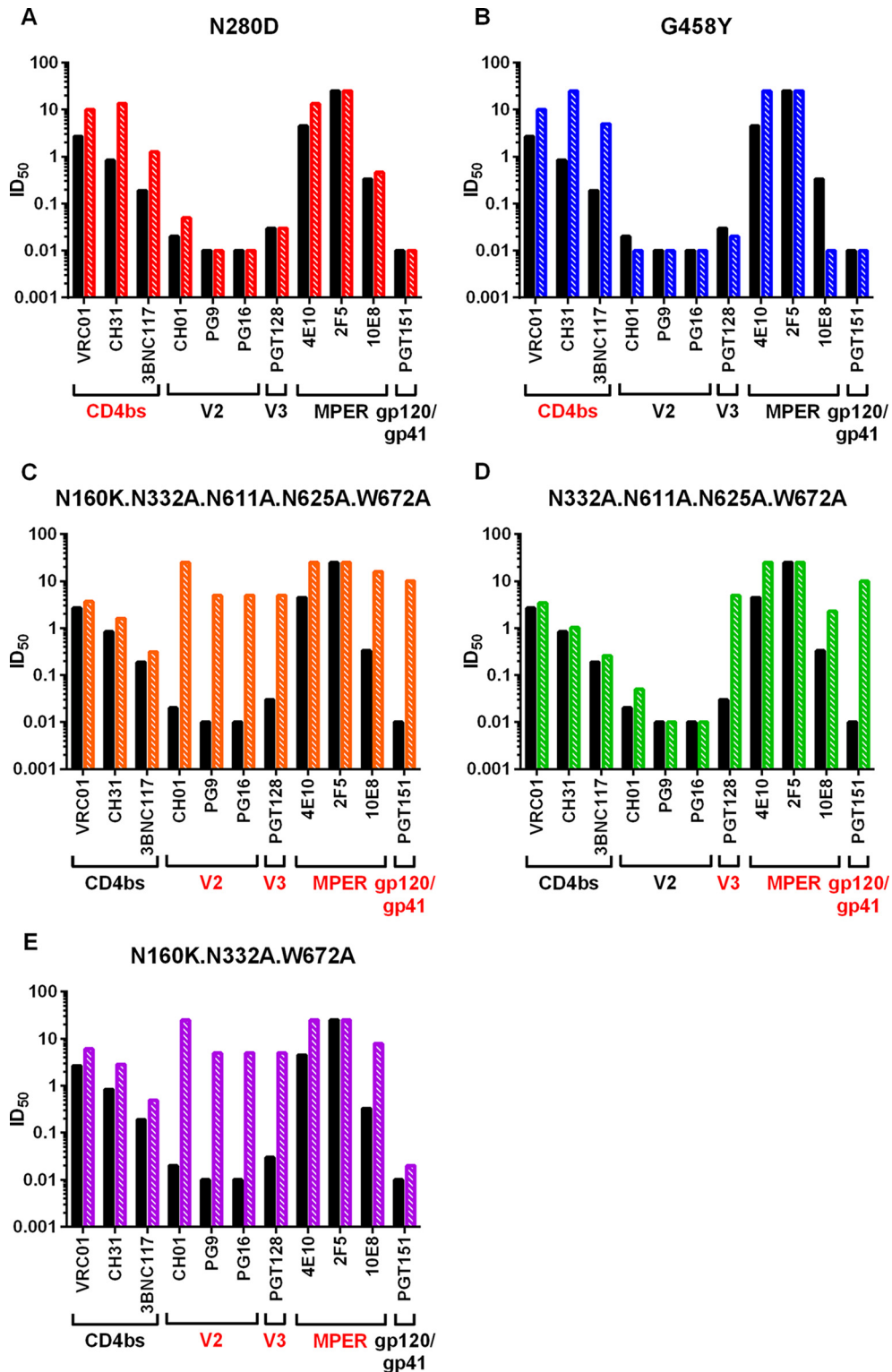


FIG 9 Neutralization sensitivity profile of CE1176_A3 mutants determined by a panel of broadly neutralizing monoclonal antibodies. The ID₅₀ values for a given neutralizing antibody are shown against the WT CE1176_A3 (solid black bar) and the indicated mutants (hatched, colored bar). The epitope recognized by each antibody is indicated at the bottom of the panel. The maximum antibody concentrations used were as follows: 10 μ g/ml for VRC01 or PGT151; 25 μ g/ml for CH31, CH01, 4E10, 2F5, or 10E8; and 5 μ g/ml for 3BNC117, PG9, PG16, or PGT128. The lower limit of detection is 0.01 μ g/ml of antibody. A greater height of the hatched, colored bar than of the black bar indicates increased resistance of the mutant virus to the antibody tested compared to that of the WT virus. Resistance of the mutant to any MAb tested is indicated by the epitopes in red letters at the bottom of the panel.

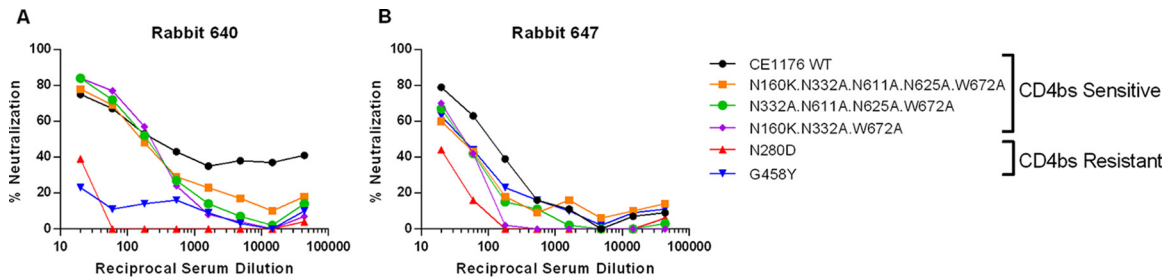


FIG 10 Specificity of neutralizing activities in immune sera determined by sensitivity to epitope-specific mutants of CE1176_A3. Week 37 sera from two immunized rabbits were tested for neutralizing activities against WT CE1176_A3 and a panel of epitope-specific mutants. The three multiple-site mutants shown retain their sensitivity to CD4bs-directed antibodies, whereas the two single-site mutants are resistant. The x axis indicates the reciprocal dilutions of the immune sera tested (3-fold serial dilution starting at 1:20). The y axis indicates the percentage of viral infectivity neutralized by a given serum dilution against the WT or mutant viruses. A decreased percent neutralization against a mutant virus compared to that of the WT indicates a loss of neutralizing activities due to the mutation(s) introduced into a specific epitope(s) in the indicator virus.

failed to generate V1/V2 antibodies against the panel of gp70 fusion proteins tested (e.g., animals 642 and 643 in the PVO.4 N7+ immunization group) (Fig. 4) showed robust ADCC activities against SF162 Env (Fig. 5 and data not shown). This might indicate differential specificity of their V1/V2 responses or the presence of ADCC activities against Env-specific targets outside V1/V2. This is also consistent with the differential ADCC activities measured by two different assay formats, as shown in Fig. 5 (panels A and B versus panels C and D). We used two different assay formats because, by virtue of the nature of the antigens expressed on the target cells, they display different epitopes. For instance, use of gp120-coated target cells allows detection of ADCC activities against CD4-induced epitopes but not activities mediated by CD4bs antibodies, since the latter is engaged by the cellular CD4 receptor. In contrast, the infected target cells provide a wide range of epitopes exposed at the time of virus budding, including the CD4bs. Therefore, not surprisingly, the two assays yielded different results, as the target cells are recognized by polyclonal Ab responses with different ADCC epitope specificities. Regardless, these findings indicate that the recombinant vaccinia prime-gp120 protein boost regimen described here was able to elicit, in a preclinical animal model, antibodies with epitope specificity (reactive to V1/V2 fusion protein) and functional activities (ADCC) that have been correlated with protection in the RV144 trial (4, 56).

Importantly, the immunized rabbits described here also generated cross-reactive neutralizing activities, not only against tier 1A and some tier 1B viruses but also against heterologous tier 2 viruses, with up to 50% neutralization of the global tier 2 panel tested (Table 1 and Fig. 7). This neutralizing activity was dose dependent (Fig. 8), reproducible (Fig. 10 and data not shown), and specific, as indicated by the minimal or undetectable activities against MuLV Env-pseudotyped virus (Tables 1 and 2). However, the nature and specificity of the neutralizing responses observed in these animals remain to be defined. Some, but not all, of the tier 1 neutralizing activities can be competed out by V3 peptides (Fig. 6), indicating that antibodies directed to non-V3 or nonlinear epitopes in V3 may contribute to the neutralization of these easy-to-neutralize isolates. Analysis with epitope-specific mutants of CE1176_A3 indicates that some animals generated CD4bs-directed antibodies, albeit with differential and overlapping specificities, that contributed to the neutralization against this tier 2 virus (Fig. 10). This is highly surprising, since cross-reactive neu-

tralizing antibodies directed against the CD4bs typically take 2 to 4 years after seroconversion to develop in infected individuals (2, 7, 9, 48). Without further substantiation, we cannot attribute the CD4bs-directed neutralizing activities in the immunized rabbits to bNAb as defined by MAb such as VRC01. Nevertheless, our findings support the notion that the prime-boost immunization approach as described here can induce NAb responses against heterologous tier 2 isolates, albeit with limited breadth and modest titers. It is noteworthy in this context that the pattern of NAb response reported here is different from those reported by others. For instance, using SOSIP Env trimers as immunogens, Sanders et al. (14) were able to elicit high titers of autologous NAb (against BG505, a tier 2 virus) in rabbits and macaques but little or no NAb against heterologous tier 2 viruses, such as CE1176_A3. In the present study, despite little or no NAb response against the autologous viruses (JR-FL and PVO.4) (Table 1), the majority of immunized rabbits generated NAb against heterologous tier 2 viruses in the global virus panel (Fig. 7 and 8; Table 2). It should also be noted that the study described by Sanders et al. (14) was done at the same contract facility (Covance) as ours and the neutralization assays were performed by the same investigators, using the same assay (pseudotyped virus assay in TZM-bl cells) and many of the same indicator viruses, including CE1176_A3 in the global tier 2 virus panel. These findings suggest that the immunization approach described here may have elicited a profile of anti-gp120 responses distinct from those described by Sanders et al. (14). Future studies will be needed to better define the nature of these responses and to determine if and how they may be improved and translated in nonhuman primates or humans.

It is interesting to note that repeated gp120 boosting increased the ability of immune sera to bind certain peptides, including those that were also recognized by RV144 vaccinees (Fig. 3B) (4–6). However, the increase in peptide-specific responses was not accompanied by increased titers to gp120 (Fig. 3A). Therefore, the second boost may preferentially increase responses to linear peptides presented by the gp120 proteins. Additionally, repeated gp120 immunization failed to boost and, in some cases, resulted in diminished neutralization responses (Fig. 7C). This observation is consistent with the idea that the first subunit protein immunization may boost antibodies that were generated after the priming event, while the second subunit protein boost preferentially expanded antibodies induced by the first protein immunization that were limited in their neutralizing activities (71). If so, gp120 may

be a suboptimal immunogen for a prime-boost immunization regimen aimed at generating neutralizing antibody responses, in line with the observation of Sanders et al. (14).

While the N7 glycan has been shown to increase the stability or exposure of the CD4bs and V3 epitopes in a conserved manner (17, 21), results reported here suggest that the N7 glycan may not play a conserved role in modulating Env immunogenicity, at least in the context of the vaccinia prime-gp120 boost regimen. As shown in Table 1 and Fig. 7, animals immunized with N7+ Env showed similar or slightly broader neutralization and higher titers of neutralizing activity than those immunized with their N7- Env counterparts. The failure to observe an improved NAb response in N7- Env-immunized animals is not due to suboptimal immunization in these animals, since sera from all experimental groups have similar vaccinia virus neutralizing (data not shown) and gp120 binding titers (Fig. 3), although we cannot exclude the possibility that any putative effect of the N7 glycan on Env immunogenicity may have been masked by responses not detected by these assays. This is in direct contrast to our previous study in macaques demonstrating that N7- 89.6 Env induced broader and more potent neutralizing serum than its glycosylated counterpart (17). While these results may be related to the Env immunogen (89.6 versus JR-FL or PVO.4) or the animal species (rabbits versus macaques) used, the potential effect of the N7 glycan on Env immunogenicity may also depend on a number of other factors. For example, while the removal of the N7 glycan enhanced the ability of 89.6 Env to mediate CD4-independent entry (17), no such effect was observed for N7- versions of JR-FL or PVO.4 Env (21). Additionally, our earlier study in macaques used 89.6 gp140 as the boosting immunogen, whereas the current study used monomeric gp120, in an attempt to better simulate the RV144 regimen. Since the N7 glycan is predicted to shield the adjacent protomer's V3 loop (72, 73), the biological property of the N7 glycan may depend on the trimeric structure of Env. In fact, our V3 peptide competition data demonstrate no difference between the ability of N7+ or N7- Env to generate V3-neutralizing antibodies in the context of gp120 boost (Fig. 6). Therefore, gp120 may not be the appropriate immunogen to determine if the N7 glycan has any impact on Env immunogenicity. Indeed, other studies have demonstrated improved neutralization responses in animals immunized with trimeric gp140 compared to those of animals immunized with gp120 monomers (74-77). Our finding that repeated gp120 boosting skewed antibody responses to linear epitopes (Fig. 3B) may also contribute to the lack of improved neutralization responses observed in N7- Env-immunized animals (Fig. 7C). It is therefore of interest to explore immunogens that can better preserve the trimer structure, such as SOSIP trimers, to test the potential role of the N7 glycan in modulating Env immunogenicity.

Despite many attempts (12-14, 78-80), generation of bNAb against tier 2 HIV-1 primary isolates by vaccination remains an unmet goal. Several recent studies have succeeded in demonstrating vaccine-induced NAb against autologous tier 2 virus or a panel of mutated tier 2 isolates with increased CD4 or V3 epitope exposure (12-14, 21). Our finding of neutralizing responses against heterologous tier 2 global isolates, albeit of modest potency and breadth, point to the possibility of generating such responses through the prime-boost immunization platform. The basis for the apparently improved response reported here is not clear. Several factors may contribute to our findings, including the possible role of a replication-competent virus for priming (29, 51). Repli-

cation-competent viruses have been shown to be more effective in protecting against certain viral infections and disease than immunization by replication-incompetent viruses or subunit vaccines (81-83). Alternatively, differences in vaccine responses may also result from the specific Env, the nature of the boosting immunogens, and the immunization regimen used. Further studies will be needed to determine the potential contribution of these factors, including the role of specific glycans, to improve the protective efficacy achievable by the prime-boost immunization regimen.

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