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Potent and Broadly Reactive HIV-2 Neutralizing Antibodies Elicited by a Vaccinia Virus Vector Prime-C2V3C3 Polypeptide Boost Immunization Strategy▽†

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Human immunodeficiency virus type 2 (HIV-2) infection affects about 1 to 2 million individuals, the majority living in West Africa, Europe, and India. As for HIV-1, new strategies for the prevention of HIV-2 infection are needed. Our aim was to produce new vaccine immunogens that elicit the production of broadly reactive HIV-2 neutralizing antibodies (NAbs). Native and truncated envelope proteins from the reference HIV-2ALI isolate were expressed in vaccinia virus or in bacteria. This source isolate was used due to its unique phenotype combining CD4 independence and CCR5 usage. NAbs were not elicited in BALB/c mice by single immunization with a truncated and fully glycosylated envelope gp125 (gp125t) or a recombinant polypeptide comprising the C2, V3, and C3 envelope regions (rpC2-C3). A strong and broad NAb response was, however, elicited in mice primed with gp125t expressed in vaccinia virus and boosted with rpC2-C3. Serum from these animals potently neutralized (median 50% neutralizing titer, 3,200) six of six highly divergent primary HIV-2 isolates. Coreceptor usage and the V3 sequence of NAb-sensitive isolates were similar to that of the vaccinating immunogen (HIV-2ALI). In contrast, NAbs were not reactive on three X4 isolates that displayed major changes in V3 loop sequence and structure. Collectively, our findings demonstrate that broadly reactive HIV-2 NAbs can be elicited by using a vaccinia virus vector-prime/rpC2-C3–boost immunization strategy and suggest a potential relationship between escape to neutralization and cell tropism.

Human immunodeficiency virus type 2 (HIV-2) infection affects 1 to 2 million individuals, most of whom live in India, West Africa, and Europe (17). HIV-2 has diversified into eight genetic groups named A to H, of which group A is by far the most prevalent worldwide. Nucleotide sequences of Env can differ up to 21% within a particular group and by over 35% between groups.

The mortality rate in HIV-2-infected patients is at least twice that of uninfected individuals (26). Nonetheless, the majority of HIV-2-infected individuals survive as elite controllers (17). In the absence of antiretroviral therapy, the numbers of infected cells (39) and viral loads (36) are much lower among HIV-2-infected individuals than among those who are HIV-1 infected. This may be related to a more effective immune response produced against HIV-2. In fact, most HIV-2-infected individuals have proliferative T-cell responses and strong cytotoxic responses to Env and Gag proteins (17, 31). Moreover, autologous and heterologous neutralizing antibodies (NAbs) are raised in most HIV-2-infected individuals (8, 32, 48, 52), and the virus seems unable to escape from these antibodies (52). As for HIV-1, the antibody specificities that mediate HIV-2 neutralization and control are still elusive. The V3 region in the envelope gp125 has been identified as a neutralizing target by some but not by all investigators (3, 6, 7, 11, 40, 47, 54). Other weakly neutralizing epitopes were identified in the V1, V2, V4, and C5 regions in gp125 and in the COOH-terminal region of the gp41 ectodomain (6, 7, 41). A better understanding of the neutralizing determinants in the HIV-2 Env will provide crucial information regarding the most relevant targets for vaccine design.

The development of immunogens that elicit the production of broadly reactive NAbs is considered the number one priority for the HIV-1 vaccine field (4, 42). Most current HIV-1 vaccine candidates intended to elicit such broadly reactive NAbs are based on purified envelope constructs that mimic the structure of the most conserved neutralizing epitopes in the native trimeric Env complex and/or on the expression of wild-type or modified envelope glycoproteins by different types of expression vectors (4, 5, 29, 49, 58). With respect to HIV-2, purified gp125 glycoprotein or synthetic peptides representing selected V3 regions from HIV-2 strain SBL6669 induced autologous and heterologous NAbs in mice or guinea pigs (6, 7, 22). However, immunization of cynomolgus monkeys with a subunit vaccine consisting of gp130 (HIV-2BEN) micelles offered

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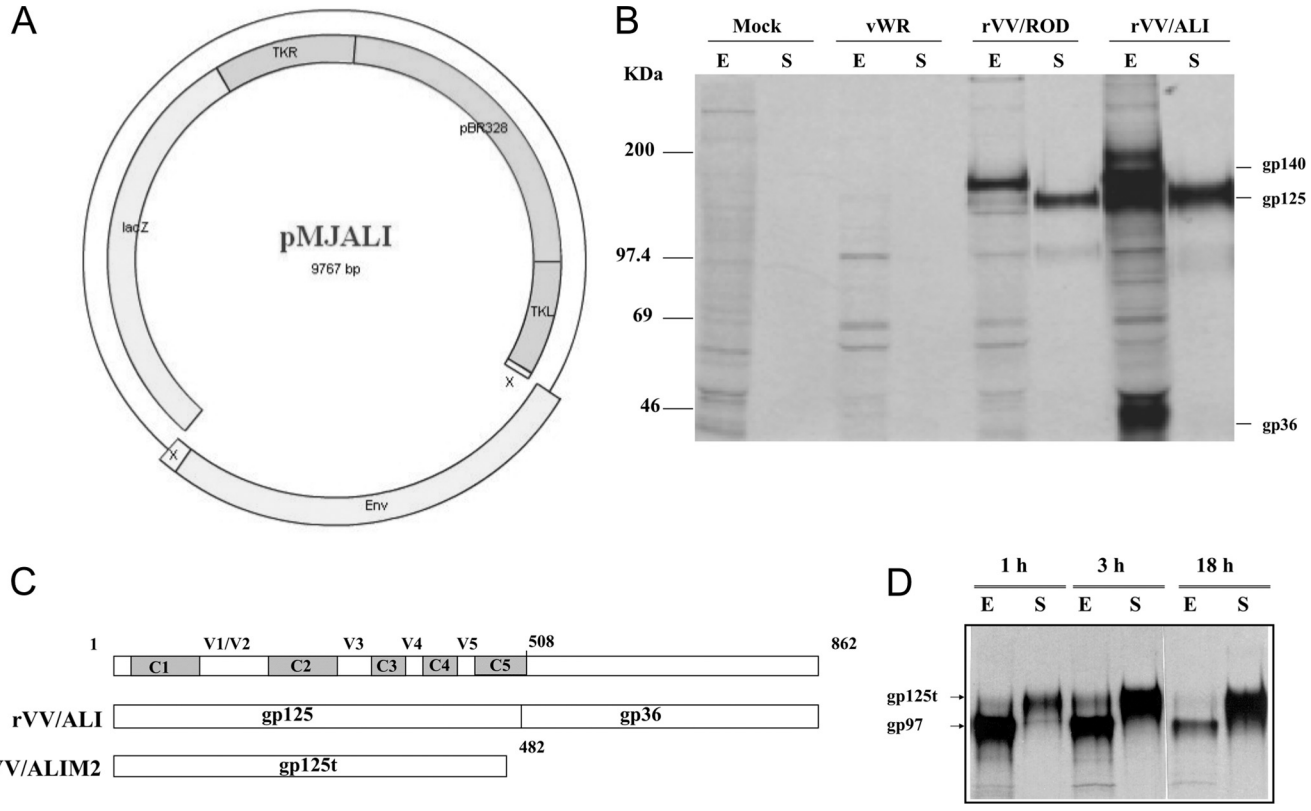

FIG. 1. Immunoprecipitation analysis of HIV-2ALI envelope glycoproteins expressed by recombinant vaccinia viruses. (A) Schematic representation of the pMJALI plasmid used to produce rVV/ALI. The *env* gene from HIV-2ALI was cloned into the unique SalI site of the pMJ601 plasmid, which is located adjacent to the synthetic late promoter within the X region (16). (B) HIV-2ALI and HIV-2ROD envelope glycoproteins expressed in HeLa cells by recombinant vaccinia viruses rVV/ALI and rVV/ROD (positive control), respectively. (C) Schematic representation of gp125t protein expressed by rVV/ALIM2, with the sizes and approximate locations of the conserved and variable domains indicated. (D) Pulse-chase analysis of gp125t expression. HeLa cells infected with rVV/ALIM2 were metabolically labeled for 30 min with [^{35}S]methionine and chased for 1, 3, and 18 h. In panels B and D, viral glycoproteins were immunoprecipitated from cell lysates (E) and supernatant (S) with antiserum from an HIV-2-infected individual and analyzed by SDS-PAGE and fluorography. Mock, uninfected cells; vWR, cells infected with the WR strain of vaccinia virus (negative control). Standard molecular mass markers are indicated in kilodaltons.

little protection against autologous or heterologous challenge (34). Immunization of rhesus (19, 44, 45) and cynomolgus (1) monkeys with canarypox or attenuated vaccinia virus expressing several HIV-2 SBL6669 proteins, including the envelope glycoproteins, in combination with booster immunizations with gp160, gp125, or V3 synthetic peptides, elicited a weak neutralizing response and partial protection against autologous HIV-2 challenge. Likewise, vaccination of rhesus monkeys with immunogens derived from the historic HIV-2ROD strain failed to generate neutralizing antibodies and to protect against heterologous challenge (55). Finally, baboons inoculated with a DNA vaccine expressing the *tat*, *nef*, *gag*, and *env* genes of the HIV-2UC2 group B isolate were partially protected against autologous challenge without the production of neutralizing antibodies (33). These studies illustrate the urgent need for new vaccine immunogens and/or vaccination strategies that elicit the production of broadly reactive NAbs against HIV-2. The present study was designed to investigate in the mouse model the immunogenicity and neutralizing response elicited by novel recombinant envelope proteins derived from the reference primary HIV-2ALI isolate, when administered alone or in different prime-boost combinations.

MATERIALS AND METHODS

Cells, plasmids, viruses, and antibodies. HeLa and Rat-2 (TK$^-$) cells were purchased from American Type Culture Collection (Rockville, MD). The Western Reserve strain of vaccinia virus (vWR), recombinant vaccinia virus expressing HIV-2ROD Env (rVV/ROD) (43), and GHOST/CD4/CXCR4 and GHOST/CD4/CCR5 cells were provided by the AIDS Research and Reference Reagent Program, National Institutes of Health. T-cell lines and peripheral blood mononuclear cells were grown in RPMI 1640 medium with 10% fetal calf serum. HeLa, Rat2, GHOST/CD4/CXCR4, and GHOST/CD4/CCR5 cells were grown in Dulbecco minimal essential medium (DMEM) with 10% fetal calf serum and antibiotics. Plasmid pMJ601 was a gift from Bernard Moss (16). HIV-2 antisera were obtained from infected individuals.

Cloning of native and mutated HIV-2ALI *env* genes. The HIV-2ALI *env* gene was amplified by PCR as described previously (53) and cloned into the SalI site of the vaccinia virus insertion vector pMJ601. This procedure generated the recombinant plasmid pMJALI (Fig. 1A). In this vector, protein expression is driven by a strong synthetic late vaccinia virus promoter (16). Using the megaprimer method of site-directed mutagenesis (50), a TAG stop codon was introduced at position 8143 in the HIV-2ALI *env* gene. In the first PCR the left outside primer was 5′-ATTGGGGATTATAAATTAG-3′ (nucleotides [nt] 8124 to 8143 of HIV-2ALI) and the right outside primer was 5′-TCACAGGAGGG CAATTTCTGC-3′ (nt 9262 to 9282). PCR product (megaprimer) was isolated and purified by agarose gel electrophoresis. For the second PCR amplification, the left outside primer was 5′-CGAAAGGGCAGACGAAGAAGGACTCC-3′ (nt 6623 to 6648, in the *rev* gene) and the right outside primer was the mega-

primer. The vaccinia virus insertion plasmid containing HIV-2ALI mutated *env* gene was named pMJALIM2.

Production of recombinant vaccinia viruses. Recombinant vaccinia viruses were obtained as previously described (35). In brief, pMJALI or pMJALIM2 were transfected into the thymidine kinase-negative Rat2 (TK^-) cells by the calcium orthophosphate method, and cells were simultaneously infected with vaccinia virus strain WR. Recombinant vaccinia viruses, both 5-bromodeoxyuridine resistant and β-galactosidase positive, were selected for further studies. The recombinant vaccinia viruses were named rVV/ALI and rVV/ALIM2.

Radiolabeling and immunoprecipitations. Confluent monolayers of HeLa cells were infected with 5 PFU of recombinant vaccinia virus/cell and labeled for 16 to 18 h with 100 μCi of [^{35}S]methionine/ml. For pulse-chase studies, the cells were pulsed with 100 μCi of [^{35}S]methionine/ml for 30 min and chased for various periods with DMEM containing 10% fetal calf serum. Labeled cells were lysed, and proteins from the cell lysate and from the cell-free supernatant were immunoprecipitated with 10 μl of human anti-HIV-2 sera and adsorbed to protein A-Sepharose. Immunoprecipitated proteins were characterized by SDS–7.5% PAGE and visualized by autoradiography after fluorographic enhancement (Amersham Amplify; GE Healthcare).

Mouse immunizations. Four groups (I, II, III, and V) of 6-week-old female BALB/c mice were immunized intraperitoneally (i.p.) with 2×10^7 PFU of vaccinia virus vWR or rVV/ALIM2 in 100 μl of phosphate-buffered saline (PBS). Another two groups (IV and VI) of mice were immunized by the i.p. route with 10 μg of the rpC2-C3 polypeptide (37) or soluble gp125t emulsified in complete Freund adjuvant (priming) or incomplete Freund adjuvant (boosts). Four mice were used in each group. For all mice, the schedule of immunization included one priming and two boosts at days 14 and 28 (see Table S1 in the supplemental material). Mice were bled 14 days after each immunization to assay for binding and neutralizing antibodies.

ELISAs. Enzyme-linked immunosorbent assay (ELISA) plates were coated with the rpC2-C3 polypeptide as described previously (37). To produce gp125t for ELISAs, 293 cells were infected with rVV/ALIM2 (4 PFU per cell). The infecting virus was replaced with serum-free DMEM at 3 h postinfection. The medium containing gp125t was collected at 24 h postinfection, clarified by centrifugation at 2,000 × *g* for 5 min, and passed through a 0.2-μm-pore-size filter. Immuno MaxiSorp 96-well microplates (Nunc) were coated with the gp125t supernatant (1:5 dilution) or recombinant polypeptide rpC2-C3 (2.5 μg/ml) both diluted in 0.05 M bicarbonate buffer (pH 9.4) and blocked with 1% gelatin (Bio-Rad). Mouse antiserum at serial 2-fold dilutions (starting at 1:100) was added to the microplates and, after 1 h of incubation at room temperature, alkaline phosphatase (AP)-conjugated goat anti-mouse IgG was added as a secondary antibody. Colorimetric reaction was developed with *p*-nitrophenylphosphate (pNPP) and read at 405 nm on a microplate reader. Negative controls were serum from preimmune mice and mice immunized with vWR. Positive control was serum from HIV-2-infected individuals. In this case, the secondary antibody was AP-conjugated anti-human IgG. Sera with an optical density (OD) above the cutoff (mean OD of the preimmune mice serum plus two times the standard deviation) were considered positive.

Neutralization assays. Primary virus isolates were obtained from nine unrelated HIV-2-infected Portuguese patients by using the cocultivation method as described previously (13). The neutralizing activity of mice serum against these HIV-2 primary isolates was analyzed in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells (56, 57). The cells (15,000 cells in 100 μl of complete growth medium (GM) that consists of DMEM supplemented with 10% fetal bovine serum (heat inactivated), 25 mM HEPES, and 50 μg of gentamicin/ml were added to each well of 96-well flat-bottom culture plates (Nunc) and allowed to adhere overnight before addition of equals parts of serum and virus dilutions. Next, 100-μl portions of 5-fold serial dilutions (beginning at 1:40) of heat-inactivated mouse sera were mixed with 100 μl of each virus (corresponding to 5 to 15 ng of capsid p26, as quantified by Innotest HIV antigen MAb [Innogenetics N.V., Belgium]) and incubated for 1 h at 37°C in a total volume of 200 μl of GM containing Polybrene (20 μg/ml). After 48 h, culture medium was removed from each well, and cells were lysed directly in the plate during 2 min with 100 μl of One-Glow luciferase assay substrate reagent (Promega, Madison, WI). Plates were immediately analyzed for luciferase activity on a luminometer (TECAN). Background luminescence was measured by using control wells that contained only target cells and medium. Virus neutralization titer was defined as the maximal dilution of plasma required to reduce virus production by 50% on day 2 after infection. Neutralizing titer was displayed as the percent inhibition of viral infection (luciferase activity) at each serum dilution: % inhibition = [1 − (luciferase serum samples/luciferase without serum samples)] × 100. To monitor the amount of neutralization activity that is not HIV-2 specific, each serum sample was also tested against a pseudovirus carrying the vesicular stomatitis virus (VSV) envelope protein. These VSV envelope proteins are able to mediate virus entry into the target cells used but are not inhibited by anti-HIV Env antibodies. The reduction of VSV infection by different sera was 15% at a 1:40 dilution, 5% at a 1:100 dilution, and 0% at a dilution of >1:100. The different mice sera were also tested against an HIV-1 isolate (HIV-1BAL), and no reduction in virus infectivity was observed.

Molecular and evolutionary analysis of C2-V3-C3 Env sequences. Maximum likelihood phylogenetic trees were constructed with alignments of C2, V3, and C3 consensus nucleotide sequences obtained from all primary isolates used in the present study. GenBank accession number for PTHCC6.03 is GU591163. Accession numbers for all other sequences can be found elsewhere (10). Reference HIV-2 sequences were obtained from GenBank. Evolutionary distances between sequences were calculated by using the HKY model of nucleotide substitution. Maximum-likelihood tree searches were conducted using Treefinder (28) with bootstrap resampling. Three-dimensional (3D) structural homology modeling of C2, V3, and C3 amino acid sequences from HIV-2 isolates was performed using SWISS-MODEL (2). This tool maps linear amino acid sequences to 3D structures of proteins. This is done by comparing the source protein sequence to that of proteins with known 3D structures in the Protein Data Bank (PDB). The tool generates an alignment between the query sequence and a homologous sequence from the PDB and allows visualizing the result.

Data analysis. The statistical significance of differences between groups was determined by using the Mann-Whitney test within the program GraphPad Prism (version 4.00). A P value of <0.05 was considered statistically significant.

RESULTS

Expression of native and truncated HIV-2ALI Env glycoproteins in vaccinia virus. HIV-2 ALI, considered the prototypic group A primary isolate, uses predominantly the CCR5 coreceptor to enter cells and is relatively CD4 independent (46). The full-length *env* gene of this isolate was cloned into the pMJ601 vaccinia virus insertion vector generating pMJALI (Fig. 1A) and recombinant vaccinia virus rVV/ALI was produced as described previously (35). Immunoprecipitation assays with human HIV-2 antiserum showed that rVV/ALI expresses high levels of HIV-2 precursor envelope glycoprotein (gp140) that is processed to originate the surface (gp125) and transmembrane (gp36) glycoproteins that are then transported to the cell surface (Fig. 1B). In these assays, recombinant vaccinia virus expressing the Env glycoproteins of the historical HIV-2ROD isolate (rVV/ROD) was used as a positive control. Cell-to-cell fusion assays showed that rVV/ALI only induced syncytium formation in CCR5-positive cells (GHOST/CD4/CCR5) (see Table S2 in the supplemental material). To be able to produce high amounts of gp125, a stop codon was inserted by site-directed mutagenesis at the end of the corresponding *env* coding region. The mutated *env* gene should code for a truncated gp125 (gp125t) lacking 26 amino acids at the carboxyl terminus of the C5 region (Fig. 1C). As anticipated, recombinant vaccinia virus rVV/ALIM2 secreted gp125t into the cell supernatant in high levels (95% of total amount after 18 h of chase, as determined by densitometric analysis of the bands), and no gp140 or gp36 was produced (Fig. 1D). Secreted gp125t bound poorly to cellular CD4 (data not shown). Together, these results are consistent with the relative CD4 independence and CCR5 usage of HIV-2ALI (46), indicating that the envelope glycoproteins expressed in vaccinia virus maintain the structure and function of the original viral isolate.

HIV-2ALI Env-based immunogens elicit a strong and broad NAb response in mice. To investigate the humoral immunogenicity of HIV-2ALI-derived envelope gp125t and rpC2-C3 polypeptide (37), BALB/c mice were inoculated with one or more of the following immunogens: rVV/ALIM2, gp125t or

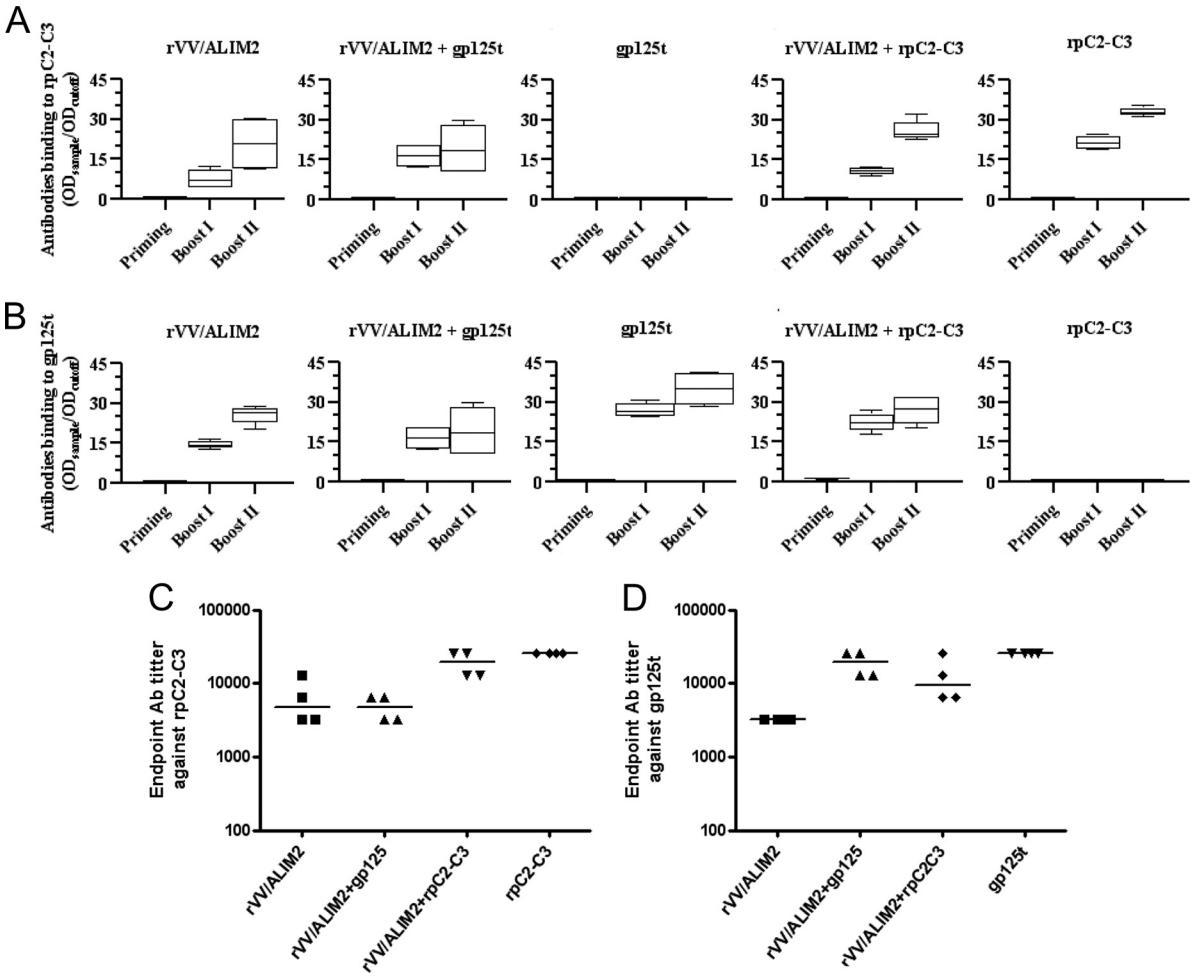

FIG. 2. Binding IgG response against the rpC2-C3 polypeptide and gp125t in BALB/c mice immunized with different HIV-2ALI antigens. Four mice were immunized with each vaccine regimen. For all mice, the schedule of immunization included one priming and two boosting at days 14 and 28. Fourteen days after each immunization, sera were collected and assayed for the presence of binding IgG antibodies to rpC2-C3 polypeptide and soluble gp125t. Mice antiserum at a 1:100 dilution was added to rpC2-C3 polypeptide (A)- or gp125t (B)-coated microplates, and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG was added as a secondary antibody. (C) Endpoint antibody titers against rpC2-C3 after the last immunization (boost II). (D) Endpoint antibody titers against gp125t after the last immunization (boost II). The boxes in panels A and B indicate the median antibody responses and standard deviations. The lines in panels C and D indicate the median antibody responses.

rpC2-C3 polypeptide (see Table S1 in the supplemental material). The binding antibody response to each immunogen was analyzed with the ELISA-HIV2 assay (37), which uses the rpC2-C3 polypeptide as antigen, and with a newly derived ELISA using gp125t as a capture antigen. All mice produced IgG antibodies reacting with gp125t or rpC2-C3 polypeptide (Fig. 2). Mice immunized solely with rpC2-C3 produced the strongest rpC2-C3-specific binding antibody responses (endpoint titer, 1:25,600) but did not produce antibodies against gp125t. Conversely, mice immunized solely with gp125t produced a strong IgG response against gp125t (endpoint titer, 25,600) but not against rpC2-C3 (Fig. 2). Importantly, mice primed with rVV/ALIM2 and boosted with rpC2-C3 produced IgG antibodies that bound strongly to rpC2-C3 (median titer, 19,200; range, 12,800 to 25,600) and gp125t (median titer, 9,600; range, 6,400 to 25,600).

The neutralizing properties of mice antiserum were tested against nine highly divergent heterologous isolates of HIV-2 group A (see Fig. S1 in the supplemental material), of which six used the CCR5 coreceptor and three used the CXCR4 coreceptor (Table 1). The genetic diversity of the primary virus isolates was significantly higher compared to the reference isolates (median number of nucleotide substitutions per site, 0.2399 versus 0.1657 [$P = 0.0013$]) (see Table S3 in the supplemental material). The median divergence from the vaccinating ALI strain was also very high (0.2146 nucleotide substitutions per site), and this was independent of coreceptor usage. Mice inoculated with rVV/ALIM2 alone and mice inoculated with prime-boost regimens containing rVV/ALIM2 and rpC2-C3 polypeptide or rVV/ALIM2 and gp125t, generated antibodies neutralizing the R5 isolates (Table 1). In contrast, mice immunized solely with rpC2-C3 or gp125t did not produce neutralizing antibodies. The strongest and broadest neutralizing responses were found in mice primed with rVV/ALIM2 and boosted with rpC2-C3. Sera from these animals potently neutralized (50% median neutralizing titers, 3,200;

TABLE 1. Neutralization of primary HIV-2 isolates by antiserum from mice immunized with envelope proteins derived from HIV-2ALI

Primary virus isolate	Coreceptor use	V3 net charge (no. of amino acids)	Evolutionary distance from HIV-2ALI[a]	NAb titer elicited in mice inoculated with different immunogens[b]				
				rVV/ALIM2	gp125t	rVV/ALIM2 + gp125t	rpC2-C3	rVV/ALIM2 + rpC2-C3
PTHCC19.03	R5	7 (34)	0.1521	40	<40	1,280	<40	1,280
PTHCC6.03	R5	7 (34)	0.1582	40	<40	160	<40	10,240
PTHCC7.03	R5	7 (34)	0.1760	160	<40	1,280	<40	5,120
PTHCC12.03	R5	7 (34)	0.1821	40	<40	80	<40	5,120
PTHCC2.03	R5	7 (34)	0.2146	40	<40	160	<40	1,280
PTHCC17.03	R5	7 (34)	0.2323	40	<40	320	<40	1,280
PTHSM9.03	X4	9 (35)	0.2301	<40[c]	<40	<40	<40	<40
PTHSM10.03	X4	9 (35)	0.2214	<40	<40	<40	<40	<40
PTHCC20.03	X4	11 (37)	0.2187	<40	<40	<40	<40	<40

[a] That is, the number of nucleotide substitutions per site in C2, V3, and C3 under maximum likelihood.
[b] Endpoint titters measured in TZM-bl cells are the reciprocal serum dilutions that reduced virus replication by >50%.
[c] Non-neutralizable at 1/40 serum dilution.

range, 1,280 to 10,240) all primary isolates of the CCR5 phenotype. However, none of the immunogens generated neutralizing antibodies reactive against the X4 isolates. Together, these results demonstrate that a potent and broad HIV-2 neutralizing response can be elicited with a vaccinia virus vector-prime/rpC2-C3-boost vaccination strategy that directs the antibody response to the central C2, V3, and C3 envelope regions in the native HIV-2 envelope complex.

Sequence and structure markers of viral susceptibility to antibody-mediated neutralization. Considering the C2, V3, and C3 regions, ALI differed from the other isolates by a median of 16 amino acids (magnitude range, 11 to 30) (Fig. 3). C2 was the most conserved region (median number of amino acid replacements, 3; range, 2 to 5), whereas C3 was the most divergent region (median, 11; range, 8 to 17). This was unrelated with the tropism of the virus isolates. However, the V3 loop of neutralization-sensitive R5 (NS-R5) isolates differed only by 1 amino acid from ALI (magnitude, 0 to 1), whereas those from neutralization-resistant X4 (NR-X4) isolates differed by an average of 8 amino acids (magnitude, 7 to 9) ($P = 0.0238$). Compared to ALI and with the NS-R5 isolates, the V3 loop of the NR-X4 isolates was longer, due to the insertion of 1 to 3 amino acids at the tip of the loop, and had a higher number of charged amino acids, mostly arginine, leading to a higher overall net charge (Fig. 3 and Table 1). Superimposed 3D structures of C2, V3, and C3 regions derived from the primary isolates and HIV-2ALI were obtained by homology modeling with the conformational structure of an unliganded SIV gp120 envelope glycoprotein (PDB ID 2bf1) (14). Not surprisingly, the V3 loop structure from NS-R5 isolates was similar to ALI and differed significantly from the V3 loop structure of the NR-X4 isolates (Fig. 4). Taken together, these results suggest that the V3 loop is a major neutralization determinant in the HIV-2 envelope. Since X4 usage evolves from R5 usage, the results also suggest a potential relationship between HIV-2 escape to neutralization, coreceptor usage and cell tropism.

DISCUSSION

We show here, for the first time, that a potent and broad HIV-2 neutralizing response can be elicited in mice using a vaccinia virus vector-prime/rpC2-C3-polypeptide-boost vaccination strategy. All immunogens used in the present study were derived from the envelope gene of HIV-2ALI. We used this source isolate for three main reasons. First, because it is the prototypic primary isolate of HIV-2 group A, the most prevalent group worldwide. Second, because it uses predominantly the CCR5 coreceptor to enter cells (46), a phenotypic feature that is shared by most HIV-2 isolates found in asymptomatic patients (9) and that is commonly found in transmitted HIV isolates (30). Third, because HIV-2 ALI is CD4 independent as many other primary HIV-2 isolates are (46), and this has been associated with an increased likelihood of exposition of broadly neutralizing epitopes *in vivo* (58). Based on the published information, we reasoned that the combination of these three features would make this an appropriate source isolate for the production of Env-based vaccine immunogens aimed at eliciting neutralizing antibodies targeting the most commonly transmitted strains of HIV-2. A new recombinant

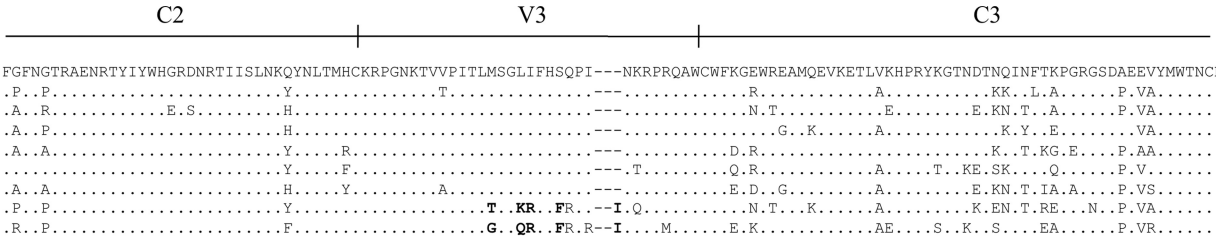

FIG. 3. Alignment of C2, V3, and C3 amino acid sequences from the reference HIV-2ALI strain and the other primary HIV-2 isolates analyzed in the present study. Amino acids only present in the V3 region of the X4 neutralization-resistant isolates are shown in boldface letters.

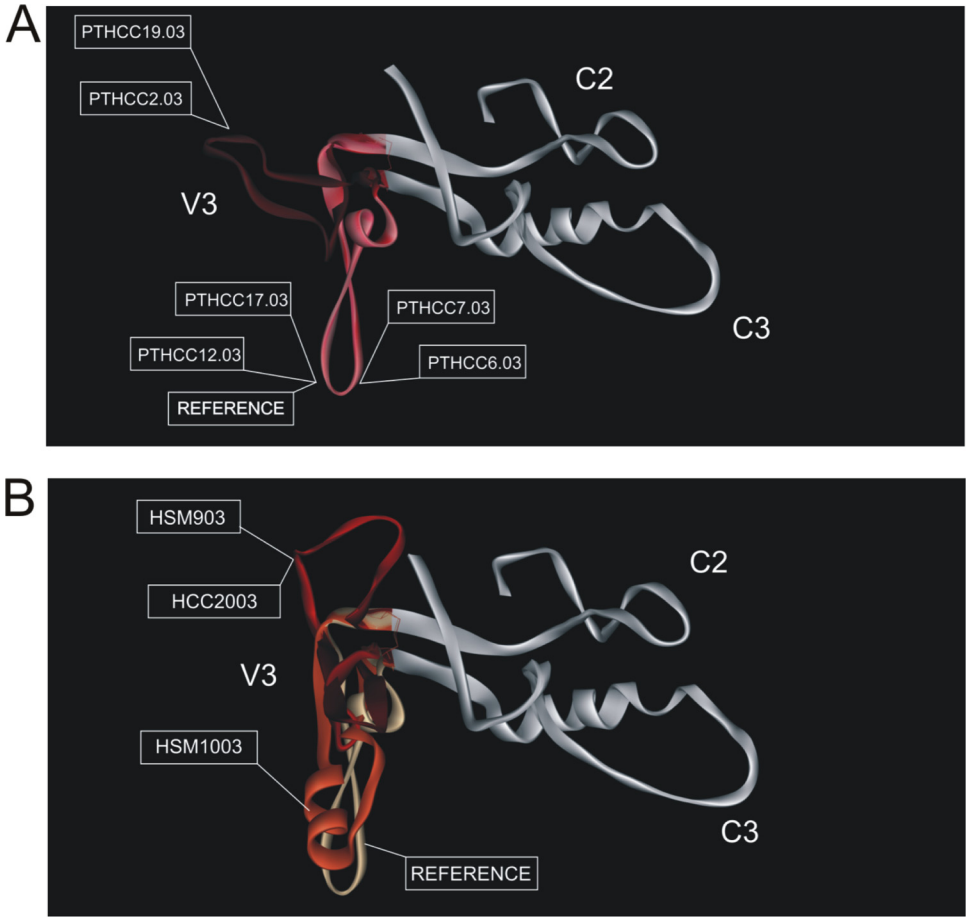

FIG. 4. Conformational structure of the C2-V3-C3 domains from the primary HIV-2 isolates, as determined by homology modeling. (A) V3 loop conformation of reference HIV-2ALI and neutralization sensitive isolates (PTHCC2.03, PTHCC6.03, PTHCC7.03, PTHCC12.03, PTHCC17.03, and PTHCC19.03). Two different patterns are predicted: a conformational pattern similar to the reference HIV-2 ALI (PTHCC6.03, PTHCC7.03, PTHCC12.03, and PTHCC17.03) and a pattern slightly more exposed than the reference (PTHCC2.03 and PTHCC19.03). (B) V3 loop structure conformation of reference and of neutralization resistant isolates (PTHCC20.03, PTHSM9.03, and PTHSM10.03). All of the conformational patterns are structurally different from the reference HIV-2ALI. The V3 loop is highlighted by a red color gradient, while the C2 and C3 domains are shown in white.

vaccinia virus, rVV/ALIM2, was produced expressing high levels of a truncated version of gp125 from HIV-2ALI. Mice immunized with this virus elicited a binding IgG antibody response that is similar to natural HIV-2 infection (37, 38) and elicited the production of low levels of NAbs. In contrast, animals vaccinated solely with monomeric gp125t or rpC2-C3, despite eliciting a binding IgG antibody response that was even stronger than that attained in natural infection (4.4 $\log_{10}$ versus 3.3 $\log_{10}$) (38), could not raise the production of NAbs. These results indicating that NAb epitopes are not formed or presented effectively by our monomeric Env immunogens are consistent with previous data showing poor induction of HIV NAbs by envelope subunits (12, 18, 51). Strikingly, however, elicitation of NAbs by the same monomeric Env proteins was highly effective in animals primed with rVV/ALIM2 expressing truncated gp125t. To our knowledge, this is the first demonstration that a robust and broad HIV neutralizing response can be elicited with a prime-boost vaccination strategy based on replicating competent poxvirus vectors and monomeric Env subunits.

Replication-competent poxvirus vectors, in contrast to nonreplicating poxvirus such as MVA, NYVAC, or canarypox, allows sustained and high-level transgene expression *in vivo*, and this has been related with strong $CD8^+$ (21, 27) and $CD4^+$ (27) T-cell responses. Recent evidence indicates that the generation and persistence of a strong antiviral antibody response depends on the previous induction of a strong Th2 type-specific cellular immune response (20). Thus, the high-level expression of Env glycoproteins ascribed by the strong late promoter present in our recombinant vaccinia virus vector may have been determinant for the generation of a strong Th2 type-specific response and B-cell activation, which then enabled an effective NAb production following the booster immunizations with either gp125t or rpC2-C3. Of note, the specific cellular immune response triggered by nonreplicating poxvirus vectors is of the Th1 type, which may explain the weak antibody response elicited by these vectors (23, 25). Our results therefore provide support for the use of replicating competent recombinant vaccinia virus as a component of an HIV vaccine. To this end, new recombinant vaccinia virus vectors may need

to be produced using highly attenuated smallpox vaccine strains such as, for instance, ACAM2000 (24).

The data also demonstrate that directing the antibody response to the central C2, V3, and C3 envelope regions in the native HIV-2 envelope complex was determinant for the elicitation of high levels of broadly reactive NAbs. These core Env regions contain highly antigenic and immunodominant HIV-2 epitopes, and most HIV-2-infected patients produce IgG and IgA antibodies reacting with them (37, 38). We have recently shown that the V3 and C3 regions in the HIV-2 envelope are remarkably stable over the course of infection and that C2V3C3-specific IgG antibodies may contribute to reduce viral population size and limit the number of virus escape mutants (10). Collectively, therefore, the results suggest that a therapeutic or sterilizing vaccine strategy targeting the C2, V3, and C3 envelope regions may lead to a sustained neutralizing response and durable control of HIV-2 replication (15).

We noticed that all neutralizable HIV-2 isolates used the CCR5 coreceptor and that the V3 amino acid sequence and structure of these isolates was remarkably conserved and diverged very little from that of the vaccine isolate (HIV-2ALI). In contrast, X4-tropic viruses were resistant to neutralization and their V3 loop diverged significantly from ALI in amino acid sequence, net charge (higher charge), size (longer), and structural conformation. These results suggest that the V3 loop is the broadly neutralizing domain contained within the C2, V3, and C3 envelope regions and establish a possible link between neutralization escape and tropism in HIV-2. These results also provide important new leads for the design of new vaccine immunogens aimed at eliciting antibodies that neutralize both R5 and X4 HIV-2 isolates.

In conclusion, a prime-boost immunization strategy with recombinant vaccinia virus expressing the envelope gp125 of HIV-2ALI, a CD4-independent R5 primary isolate, and a polypeptide comprising the C2, V3, and C3 envelope regions of the same isolate induces in mice a strong and broadly neutralizing antibody response, possibly targeting the V3 region of the vast majority of primary HIV-2 isolates (R5 isolates of group A). Our findings provide support for testing these new HIV-2 immunogens in vaccine trials in other animal models. Finally, our findings provide proof-of-concept for a new type of HIV vaccine aimed at eliciting high levels of broadly NAbs, one that uses replication-competent recombinant vaccinia virus vectors to prime the cellular immune response and activate B cells and monomeric polypeptides comprising broadly neutralizing epitopes to boost the neutralizing B-cell response.

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We do not have a commercial or other association that might pose a conflict of interest.

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