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Heterologous Envelope Immunogens Contribute to AIDS Vaccine Protection in Rhesus Monkeys

Norman L. Letvin,[1,2]* Yue Huang,[1] Bimal K. Chakrabarti,[1] Ling Xu,[1] Michael S. Seaman,[2] Kristin Beaudry,[2] Birgit Korioth-Schmitz,[2] Faye Yu,[2] Daniela Rohne,[2] Kristi L. Martin,[2] Ayako Miura,[2] Wing-Pui Kong,[1] Zhi-Yong Yang,[1] Rebecca S. Gelman,[3] Olga G. Golubeva,[3] David C. Montefiori,[4] John R. Mascola,[1] and Gary J. Nabel[1]

Vaccine Research Center, National Institutes of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20892-3005[1]; Beth Israel Deaconess Medical Center[2] and Dana Farber Cancer Institute,[3] Harvard Medical School, Boston, Massachusetts 02215; and Duke University Medical Center, Durham, North Carolina 27710[4]

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Because a strategy to elicit broadly neutralizing anti-human immunodeficiency virus type 1 (HIV-1) antibodies has not yet been found, the role of an Env immunogen in HIV-1 vaccine candidates remains undefined. We sought to determine whether an HIV-1 Env immunogen genetically disparate from the Env of the challenge virus can contribute to protective immunity. We vaccinated Indian-origin rhesus monkeys with Gag-Pol-Nef immunogens, alone or in combination with Env immunogens that were either matched or mismatched with the challenge virus. These animals were then challenged with a pathogenic simian-human immunodeficiency virus. The vaccine regimen included a plasmid DNA prime and replication-defective adenoviral vector boost. Vaccine regimens that included the matched or mismatched Env immunogens conferred better protection against $CD4^+$ T-lymphocyte loss than that seen with comparable regimens that did not include Env immunogens. This increment in protective immunity was associated with anamnestic Env-specific cellular immunity that developed in the early days following viral challenge. These data suggest that T-lymphocyte immunity to Env can broaden the protective cellular immune response to HIV despite significant sequence diversity of the strains of the Env immunogens and can contribute to immune protection in this AIDS vaccine model.

The diversity of envelope (Env) proteins in human immunodeficiency virus (HIV) isolates worldwide poses a challenge for the development of an effective AIDS vaccine. The failure of traditional vaccine strategies to provide protection against HIV infection is attributable, at least in part, to the genetic heterogeneity of Env (11). Env diversity underlies many of the problems associated with eliciting antibody responses that neutralize a variety of HIV isolates (12). This diversity also poses difficulties for generating T-lymphocyte responses through vaccination that recognize genetically varied viruses (11). In fact, the problems associated with Env diversity have raised questions about the utility of including an Env immunogen in candidate HIV vaccines.

Nonhuman primates have been powerful models for evaluating HIV vaccine strategies. Studies with macaques have provided evidence for the critical contribution of cellular immunity in controlling AIDS virus replication (9, 20) and have illustrated the ability of vaccines to modify the clinical course of disease even when such vaccines cannot confer frank protection against infection with an AIDS virus isolate (1, 3). Moreover, the rationale for advancing a number of vaccine modalities into early-phase human trials derives from studies in nonhuman primates (10, 21).

* Corresponding author. Mailing address: Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215. Phone: (617) 667-2766. Fax: (617) 667-8210. E-mail: nletvin@bidmc.harvard.edu.

Recent studies with nonhuman primates have suggested that vaccine-elicited Env-specific immune responses can contribute to containment of simian immunodeficiency virus (SIV) and simian-human immunodeficiency virus (SHIV) replication (2, 15, 17, 18). However, the experiments were performed with envelopes in the immunogens and challenge viruses that were genetically matched, raising questions about the practical relevance of those observations. The present studies were initiated in the SHIV-rhesus monkey model to evaluate a plasmid DNA prime–recombinant replication-defective adenovirus (ADV) boost immunization strategy for an HIV vaccine. Further, these experiments were done to evaluate the contribution to protection of envelope immunogens that are genetically disparate from the challenge virus. The findings in these studies demonstrate the potency of this vaccine regimen and suggest that T-lymphocyte immunity to Env can broaden the protective cellular immune response to an AIDS virus isolate independent of the sequence of the Env immunogen.

MATERIALS AND METHODS

Antibody binding and neutralization assays. HIV-1 gp120-specific binding antibodies were quantified by enzyme-linked immunosorbent assay as described previously (5). Immunoplates (MaxiSorb F96) (Nunc, Roskilde, Denmark) were coated with BaL-gp120 (Quality Biological, Inc., Gaithersburg, Md.), IIIB-gp120 (Advanced Biotechnologies, Inc., Columbia, Md.), or KB9-gp120 (kindly provided by Patricia Earl, National Institutes of Allergy and Infectious Diseases, Bethesda, Md.). Antibody detection was accomplished with alkaline phosphate-conjugated, goat anti-monkey immunoglobulin G (IgG) (whole molecule; Sigma Chemical Co, St. Louis, Mo.). Neutralizing antibodies were measured in MT-2 cells as described previously (5). Briefly, 50 μl of cell-free SHIV-89.6P virus

containing 500 50% tissue culture infective doses and grown in human peripheral blood mononuclear cells (PBMCs) was added to multiple dilutions of test plasma in 150 μl of growth medium in triplicate. These mixtures were incubated for 1 h before the addition of 5×10^4 MT-2 cells. Infection led to extensive syncytium formation and virus-induced cell killing in approximately 6 days in the absence of neutralizing antibodies. Neutralizing titers were calculated as the reciprocal dilution of plasma required to protect 50% of cells from virus-induced killing as measured by neutral red uptake.

Construction of synthetic SIV and HIV-1 genes. The synthetic SIVmac239 *gag-pol-nef* gene was prepared by using a strategy similar to that used to contruct a previously described HIV vaccine vector (8). Briefly, the protein sequences of Gag, Pol, and Nef from SIVmac239 (GenBank accession no. M33262) were reverse translated with the GCG package (Genetics Computer Group, Inc., Madison, Wis.) with codons typically utilized in human cells. Oligonucleotides covering 5169 DNA bp of the theoretical gene with 5′ SalI and 3′ BamHI sites and a consensus Kozak sequence were synthesized (GIBCO Life Technologies) from multiple fragments, each 75 bp long with 25 nucleotides (nt) of overlap. The codon-modified *gag-pol-nef* gene was assembled by PCR with Pwo (Boehringer Mannheim) and Turbo Pfu (Stratagene) high-fidelity DNA polymerase. The PCR conditions were optimized with a PCR optimization kit (Stratagene) on a gradient Robocycler (Stratagene). The full-length synthetic *gag-pol-nef* gene was cloned into the SalI and BamHI site of the mammalian expression vector, pVR1012, and confirmed by DNA sequencing.

A synthetic 89.6P gp145DCFI Env gene was made analogously to a previous HIV vector (8, 23). Briefly, the protein sequence of the 89.6P envelope (GenBank accession no. U89134) was reverse translated as described above. Oligonucleotides covering 1,950 DNA bp of the theoretical gene, with a 5′ XbaI, a consensus Kozak sequence, and 3′ BamHI site, were synthesized (GIBCO Life Technologies): each fragment was 60 bp in length with 20 nt of overlap. In this modified envelope gene, the sequence from nt 1501 (amino acids [aa] 501, R) to 1602 (aa 534, T) and nt 1771 (aa 591, M) to 1851 (aa 617, V) with respect to start codon ATG (A as nt 1) were deleted. This deletion removes the cleavage site and fusion peptide for the envelope as well as part of the interspace between the two heptad repeats. The protein was terminated at nt 2124 (aa 702, I). The amino acid at 617 was changed to E from D due to the creation of XhoI cloning sites. The codon-modified gp145DCFI gene was assembled by PCR as described above. The synthetic gp145DCFI gene was cloned into the XbaI and BamHI sites of the mammalian expression vector pVR1012, and the sequence was confirmed by DNA sequencing. The synthetic 89.6Pgp140DCFI gene was derived from the gp145DCFI plasmid with introduction of a termination codon after nt 2046 (aa 676, W).

The synthetic CCR-5-tropic clade B immunogen was derived from both HXB2 and Bal strain envelopes. The protein sequence of the clade B Env glycoprotein (gp160) from HXB2 (X4-tropic; GenBank accession no. K03455) was used to create a synthetic version of the gene (X4gp160/h). The nucleotide sequence of X4gp160/h shows little homology to the HXB2 gene, but the protein encoded is the same, with the following amino acid substitutions: aa 53 (phenylalanine→leucine), aa 94 (asparagine→aspartic acid), aa 192 (lysine→serine), aa 215 (isoleucine→asparagine), aa 224 (alanine→threonine), aa 346 (alanine→aspartic acid), and aa 470 (proline→leucine). These seven amino acid substitutions were present in the Los Alamos sequence database at the time those genes were synthesized. To produce an R5-tropic version of the envelope glycoprotein (R5gp160/h), the region encoding HIV-1 envelope glycoprotein aa 205 to 361 from X4gp160/h (VRC-3300) was replaced with the corresponding region from the BaL strain of HIV-1 (GenBank accession no. M68893, again using human preferred codons). The full-length R5-tropic version of the envelope gene from pR5gp160/h (VRC-3000) was terminated after the codon for aa 704. The truncated envelope glycoprotein (gp145) contained the entire SU protein and a portion of the TM protein, including the fusion domain, the transmembrane domain, and regions important for oligomer formation. (H1 and H2 and their interspace are required for oligomerization.) Subsequently, the fusion and cleavage domains from aa 503 to 536 were deleted. The interspace between H1 and H2 from aa 593 to 620 was also deleted. The gp140 DCFI version was derived from this sequence by introduction of a termination codon as previously described (4).

Construction and purification of the rADVs. Recombinant ADVs (rADVs) were generated by a modification of a previously published method (14, 22). Briefly, the synthetic SIVmac239 *gag-pol* adapted from the sequence described above (terminated at aa 1451) was cut with SalI, blunted, and then digested with BamHI, after which it was subcloned into the blunted EcoRV and BamHI sites of the shuttle plasmid pAdAdaptCMVmcs. Synthetic HIV-1 gp140DCFI adapted from the sequence described above was subcloned into the shuttle vector by using the XbaI and BamHI sites. 293T cells were plated onto six-well plates and cultured to about 30% confluence, and then cotransfected with 2 μg of twice-cesium chloride-purified and linearized shuttle plasmid with ADV cosmid by the calcium phosphate method. After 7 to 12 days, the supernatant containing recombinant adenovirus was collected from the cell lysate with freezing and thawing at least three times in 0.6 ml of Tris-HCl, pH 8.0. The production of recombinant adenovirus was scaled up by infection of 293T cells with the virus-containing supernatant. The viruses were purified by cesium chloride, aliquoted as 10^{12} particles/ml, and stored in phosphate-buffered saline (PBS) with 13% glycerol at −20°C for future use.

Expression of plasmid and rADV Env vaccine constructs. Expression of plasmids encoding gp145DCFI(R5) and gp145DCFI(89.6P) was measured after transfection of 293T cells (in a six-well-dish) with a calcium phosphate transfection reagent (Invitrogen) with 2 μg of each plasmid. Forty-eight hours after transfection, cells were collected, lysed in cell lysis buffer (50 mM HEPES, 150 mM NaCl, 1% NP-40, 1× protease inhibitor cocktail [Roche]), and resolved by 4 to 15% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad), followed by Western blot analysis with human HIV IgG as the primary antibody at a 1:2,000 dilution. For comparison of the rADVs expressing these Env immunogens, A549 cells were infected at 5,000 particles/cell. Forty-eight hours after infection, cell lysates were prepared and Western blotting was performed as described above.

ELISPOT assays. Ninety-six well multiscreen plates were coated overnight with 100 μl (per well) of 5-μg-ml^{-1} anti-human gamma interferon (IFN-γ) (B27; BD Pharmingen) in endotoxin-free Dulbecco's PBS (D-PBS). The plates were then washed three times with D-PBS containing 0.25% Tween 20 (D-PBS/Tween), blocked for 2 h with D-PBS containing 10% fetal bovine serum to remove the Tween 20, and incubated with peptide pools and 2×10^5 PBMCs in triplicate in 100-μl reaction volumes. Individual peptide pools covered the entire SIVmac239 Gag, Nef, and Pol proteins and both the HIV-1 HXB2/BaL and HIV-1 89.6P (KB9) Env proteins. Each pool comprised 15-aa peptides overlapping by 11 aa, except for the HIV-1 89.6P Env pool, which comprised 20-aa peptides overlapping by 10 aa. Each pool contained no more than 130 peptides. Each peptide in a pool was present at a concentration of 1 μg ml^{-1}. Following an 18-h incubation at 37°C, the plates were washed nine times with D-PBS/Tween and once with distilled water. The plates were then incubated with 2 μg of biotinylated rabbit anti-human IFN-γ ml^{-1} (Biosource) for 2 h at room temperature, washed six times with Coulter Wash (Beckman Coulter), and incubated for 2.5 h with a 1:500 dilution of streptavidin-alkaline phosphate (Southern Biotechnology). After five washes with Coulter Wash and one wash with PBS, the plates were developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate chromogen (Pierce), stopped by washing with tap water, air dried, and read with an enzyme-linked immunospot (ELISPOT) reader (Hitech Instruments) using Image-Pro Plus image processing software (version 4.1) (Media Cybernetics, Des Moines, Iowa). The number of spot-forming cells (SFC) per 10^6 PBMCs was calculated. Medium background levels were consistently less than 15 SFC/10^6 PBMCs.

CD4$^+$ T-lymphocyte counts and viral RNA levels. Counts of CD4$^+$ T lymphocytes were determined by monoclonal antibody staining and flow cytometry. Plasma viral RNA levels were measured by an ultrasensitive branched DNA (bDNA) amplification assay with a detection limit of 500 copies per ml (Bayer Diagnostics).

Statistical analysis. The Kruskal-Wallis test for three or four groups (or its equivalent Wilcoxon rank sum test for two groups) was used to compare the CD4 T lymphocytes, peak viral RNA, set point viral RNA, and ELISPOT counts between vaccine groups. The Wilcoxon test for censored data was used to compare time to detectable neutralizing antibodies between vaccine groups. The Fisher exact test was used to compare the presence of detectable neutralizing antibodies at day 20 or within the first 42 days. Linear regression (ordinary least squares) was used to relate neutralizing antibodies and ELISPOT counts to CD4 T-lymphocyte counts and (separately) to $\log_{10}$ plasma viral RNA; the Wald test was used to obtain significance levels. Power calculations for the Kruskal-Wallis and Wilcoxon tests were based on the fact that the worst asymptotic relative efficiency of these tests versus Gaussian-based tests is 0.86.

RESULTS

Twenty-four Indian-origin rhesus monkeys, none of them expressing the major histocompatibility complex class I allele *Mamu-A*01*, were analyzed in four experimental groups that received DNA priming followed by rADV vector boosting with

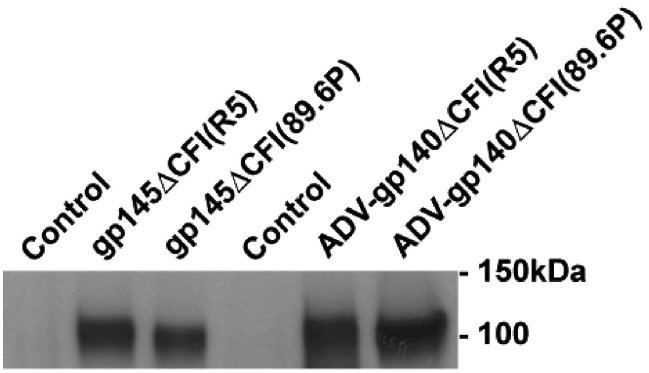

FIG. 1. In vitro expression of HXB2/Bal and 89.6P Env by both plasmids and rADV vaccine constructs. The plasmid Env [gp145Δ CFI(R5) and gp145Δ CFI (89.6P)] and rADV [ADV-gp140Δ CFI (R5) and ADV-gp140Δ CFI(89.6P)] vaccine constructs were expressed in vitro, and protein expression was assessed by Western blotting with human anti-HIV IgG.

the following immunogens: (i) control, (ii) Gag-Pol-Nef with no Env (mock), (iii) Gag-Pol-Nef with SHIV-89.6P Env (matched), or (iv) Gag-Pol-Nef with HXB2/BaL Env (mismatched). The DNA plasmid used in this study encoded a Gag-Pol-Nef fusion protein, but because of the instability of rADV constructs expressing Gag-Pol-Nef, the ADV vectors used in this study expressed only Gag-Pol. All HIV or SIV genes used in these vaccine constructs were codon modified as previously described to optimize expression in mammalian cells (4, 8). A modified form of the *env* gene, with mutations in the cleavage site, fusion, and interhelical domains (DCFI), shown to increase antibody responses to Env, was used in all expression vectors. Since these monkeys were eventually challenged with SHIV-89.6P, we refer to the HIV-1 89.6P Env immunogens as "matched" and the HIV-1 HXB2/BaL Env immunogens as "mismatched." To produce the HXB2/BaL Env, the region encoding aa 205 to 361 from the HXB2 Env was replaced with the corresponding region from the BaL strain of HIV-1. In fact, the 89.6P and HXB2/BaL DCFI Env proteins are only 81% identical. The ADV vector contained a deletion in E1 to render the vector replication defective and a partial deletion/substitution in E3 that disrupts the coding sequences for the E3 proteins (5, 15). The rADV expressing either the HXB2/BaL or 89.6P gp140 DCFI was made as described previously (17, 18). The related *gag-pol* or identical *env* cDNA inserts were introduced and matched to the immunogens in the plasmid used for DNA priming as previously described (2, 3). Each plasmid DNA was delivered intramuscularly as a 4-mg inoculum with a needleless Biojector device (Biological; Bioject Medical Technologies, Inc., Beckminister, N.J.) on a schedule of weeks 0, 4, and 8. The levels of in vitro expression of the HXB2/Bal and 89.6P *env* genes were comparable in both the plasmid and rADV vaccine constructs (Fig. 1). A single inoculation of 10^{12} particles of each rADV construct was given intramuscularly to each monkey on week 26.

The immunogenicity of these vaccine constructs was assessed by antibody binding, virus neutralization, and pooled-peptide ELISPOT assays. Plasma obtained 2 weeks after the rADV boost was assessed for BaL and 89.6P gp120 binding and for neutralization of the SHIV-89.6P challenge virus. While the Env-immunized monkeys developed high-titer antibodies to the immunizing BaL or 89.6P gp120, plasma from week 28 of the study, the time of peak ELISA titer antibody responses, failed to neutralize the challenge virus SHIV-89.6P (data not shown).

ELISPOT responses by the PBMCs of all monkeys receiving experimental immunogens were robust (Fig. 2). Cellular immunity to SIV Gag, Pol, and Nef was generated in all groups of vaccinated monkeys, and that to HIV-1 89.6P and HXB2/BaL Env was generated in monkeys receiving these respective Env immunogens. Monkeys injected with the mock Env (empty vectors) did not develop Env-specific cellular immunity. Mean total vaccine-elicited PBMC ELISPOT responses to all viral proteins 2 weeks after the final plasmid DNA inoculations were 942 ± 294 SFC (mean ± standard error) in the matched Env group, 1,588 ± 554 SFC in the mismatched Env group, and 1,255 ± 264 SFC in the mock Env group. Two weeks after boosting with the rADV vectors, total ELISPOT responses were 2,892 ± 1,116, 3,993 ± 1,000, and 3,800 ± 984 SFC in these respective groups, a >2.5-fold increase over the cellular immune responses elicited by DNA priming alone. These responses represented both $CD4^+$ and $CD8^+$ T-lymphocyte responses, as demonstrated in ELISPOT assays performed on unfractionated and $CD8^+$ T-lymphocyte-depleted PBMCs from the monkeys (Fig. 3). While the responses declined in subsequent weeks, high-frequency responses were still detected in PBMCs of the monkeys at the time of viral challenge (1,581 ± 535, 1,908 ± 557, and 1,092 ± 400 SFC, respectively) in these three groups of monkeys. Thus, this vaccine regimen elicited high-frequency $CD4^+$ and $CD8^+$ T-lymphocyte responses to multiple viral proteins. No statistically significant differences in total ELISPOT responses were observed between the three groups of experimentally vaccinated monkeys. The particularly high total SFC responses of the PBMCs of the monkeys in the mock Env group of animals reflected idiosyncratically high responses to the Pol protein (Fig. 3, upper panel). Importantly, there were no significant differences between groups of monkeys in the magnitude of their Gag- and Pol-specific ELISPOT responses as determined by comparison with a Mann-Whitney *t* test.

All monkeys were challenged intravenously with 50 50% monkey infective doses SHIV-89.6P on week 38, 12 weeks following the rADV boost, and were monitored for clinical, virologic, and immunologic sequelae of infection. SHIV-89.6P infection causes a precipitous decline in peripheral blood $CD4^+$ T lymphocytes in approximately 75% of immunologically naive rhesus monkeys, and selected vaccine strategies can generate immune responses that blunt this $CD4^+$ T-lymphocyte loss (1, 3, 19, 21). We therefore monitored peripheral blood $CD4^+$ T-lymphocyte counts as an indicator of the clinical status of the monkeys following SHIV-89.6P infection (Fig. 4). A profound loss of $CD4^+$ T lymphocytes was observed in all controls, while substantial blunting of that $CD4^+$ T-lymphocyte depletion was seen in four of the six monkeys receiving the vaccinations with SIV Gag-Pol-Nef plus mock Env. Therefore, as expected based on previous studies (1, 3, 21), vaccine-mediated protection against clinical sequelae of SHIV-89.6P infection was conferred by the Gag-Pol-Nef-containing immunogens. The two groups of vaccinated monkeys that received

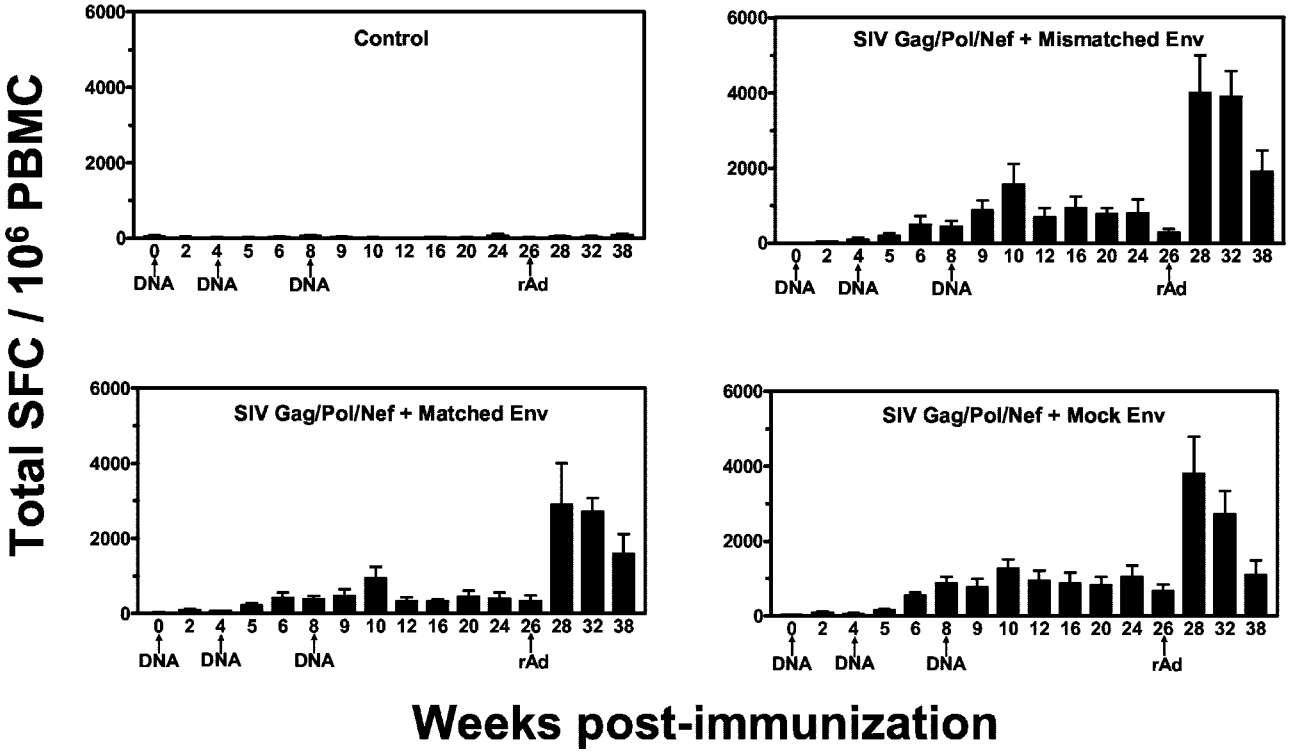

FIG. 2. Vaccine-elicited PBMC IFN-γ ELISPOT responses to SIVmac Gag-Pol-Nef and HIV-1 Env. Freshly isolated PBMCs were assessed for IFN-γ ELISPOT responses after in vitro exposure to peptide pools spanning the SIVmac Gag-Pol-Nef and HIV-1 Env proteins. All Env-specific responses were assessed by using peptides that were matched to the Env immunogen. The terms "matched" and "mismatched" refer to the relationship between the Env immunogen and the challenge virus. Arrows indicate time of inoculation with either DNA or rADV immunogens. Data are presented as the total antigen-specific SFC responses to Gag-Pol-Nef and HIV-1 Env per 10^6 PBMCs and represent the mean values for six monkeys ± standard error.

HIV-1 Env in addition to SIV Gag-Pol-Nef immunogens demonstrated even more impressive protection against $CD4^+$ T-lymphocyte loss than the monkeys receiving only the SIV Gag-Pol-Nef immunogens (Fig. 4). The mean peripheral blood $CD4^+$ T-lymphocyte counts on day 168 postchallenge in the groups of experimentally vaccinated monkeys were 363 ± 100 (mean ± standard error) in the mock Env-vaccinated animals, 772 ± 111 in the matched Env-vaccinated animals, and 706 ± 76 in the mismatched Env-vaccinated animals, documenting that statistically significant protection against $CD4^+$ T-lymphocyte loss was afforded by inclusion of an Env component in the vaccine ($P = 0.03$, Kruskal-Wallis test). Importantly, the monkeys that received the mismatched Env immunogens showed comparable protection to those injected with the matched Env immunogens.

Viral replication in the SHIV-89.6P-challenged monkeys was assessed by quantitating viral RNA in their plasma by using a bDNA assay (Fig. 5). Since only 15% of immunologically naïve rhesus monkeys control this virus to undetectable levels following infection, the plasma viral RNA levels at both peak and steady state or set point in experimental animals provide a measure of vaccine-mediated containment of virus. The medians of peak viral loads in the four groups of monkeys were 1×10^8 (control), 6×10^6 (mock Env), 4×10^6 (matched Env), and 1×10^6 (mismatched Env). Thus, the control vaccinees had significantly higher peak viral loads than the vaccinated monkeys (Kruskal-Wallis test, $P = 0.01$). However, the

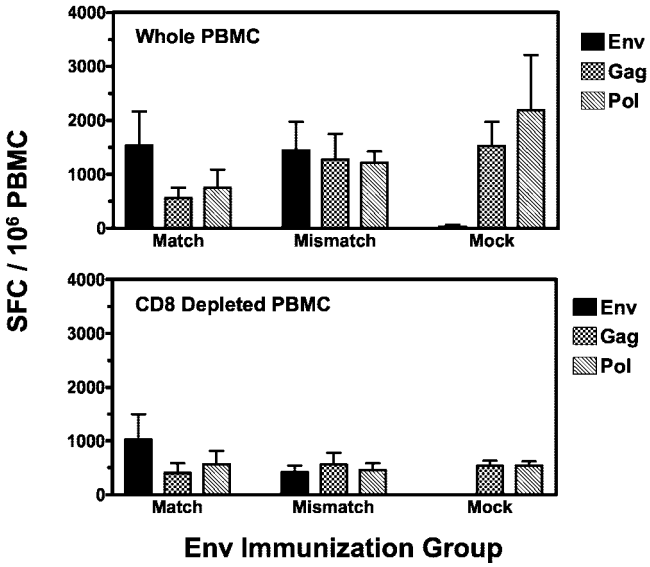

FIG. 3. Vaccine-elicited PBMC IFN-γ ELISPOT responses to individual viral proteins assessed 2 weeks following rADV boosting. ELISPOT responses to SIVmac239 Gag and Pol and HIV-1 Env antigens were assessed. Env-specific responses were assessed with peptides that were matched to the Env immunogen, and mock Env-vaccinated monkeys were assayed with 89.6P peptide pools. ELISPOT assays were performed on whole PBMCs or PBMCs depleted of $CD8^+$ T lymphocytes. Data are presented as the mean SFC responses to individual viral proteins per 10^6 PBMCs and represent the mean values for six experimentally vaccinated monkeys ± standard error.

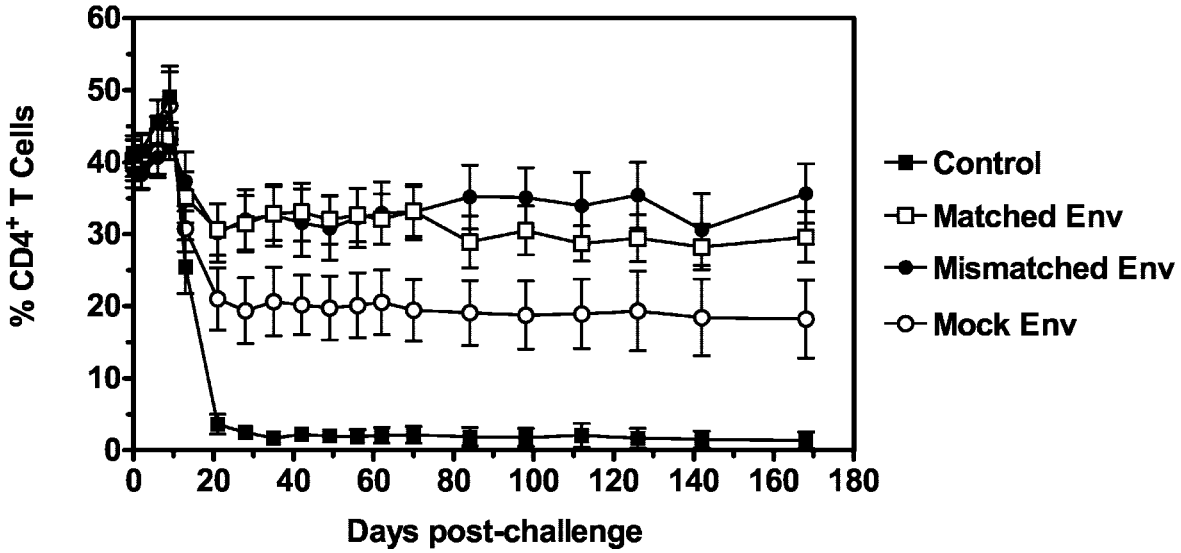

FIG. 4. Postchallenge peripheral blood $CD4^+$ T-lymphocyte counts. These values represent the mean percentage of $CD3^+$ $CD4^+$ lymphocytes assessed prospectively on all experimental monkeys through day 168 postchallenge.

three groups of experimentally vaccinated monkeys did not differ significantly in their peak viral loads ($P = 0.28$, Kruskal-Wallis test).

The group of monkeys that received SIV Gag-Pol-Nef plus mismatched Env immunogens also demonstrated better containment of virus at set point than the monkeys receiving SIV Gag-Pol-Nef plus mock Env immunogens. The log copies of plasma viral RNA on day 168 postchallenge in the groups of experimentally vaccinated monkeys were 3.70 ± 0.52 (mean ± standard error) in the mock Env-vaccinated animals, 3.61 ± 0.35 in the matched Env-vaccinated animals, and 2.38 ± 0.18 in the mismatched Env-vaccinated animals, with statistically significant lower plasma viral RNA levels afforded by inclusion of a mismatched Env component in the vaccine ($P = 0.04$, Kruskal-Wallis test). A trend toward an association between total SFC responses both pre- and postchallenge and postchallenge viral load was observed. The absence of a significant difference in plasma viral RNA levels between the groups of experimentally vaccinated monkeys receiving the matched Env immunogens and those receiving the mock Env immunogens may reflect unusually low T-cell responses to the Gag immunogens in the matched Env-vaccinated animals (Fig. 3).

To analyze the mechanism mediating improved protection against $CD4^+$ T-lymphocyte loss in the Env-immunized monkeys, the antiviral humoral immune response was evaluated. Anti-Env antibody could potentially contribute to protection by neutralizing infectious virus at the time of challenge. Alternatively, a rapidly evolving anamnestic neutralizing antibody response after infection could contribute to the control of viral spread. None of the vaccinated monkeys had detectable plasma neutralizing antibodies at the time of challenge, suggesting that vaccine-elicited preexisting neutralizing antibody

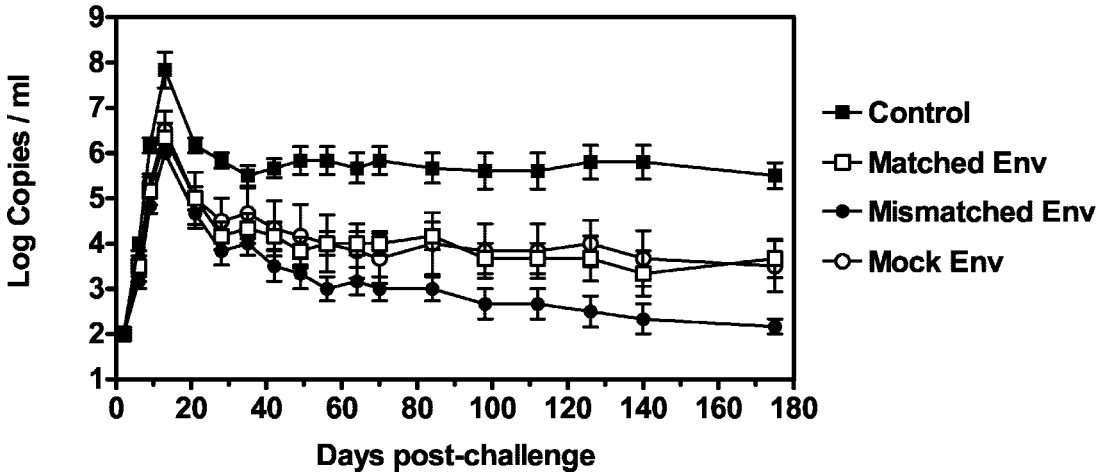

FIG. 5. Postchallenge plasma viral RNA levels. These values were determined by an ultrasensitive bDNA amplification assay with a detection limit of 50 copies/ml. The values plotted represent the geometric mean ± standard error at each sampling time for each experimental group of monkeys.

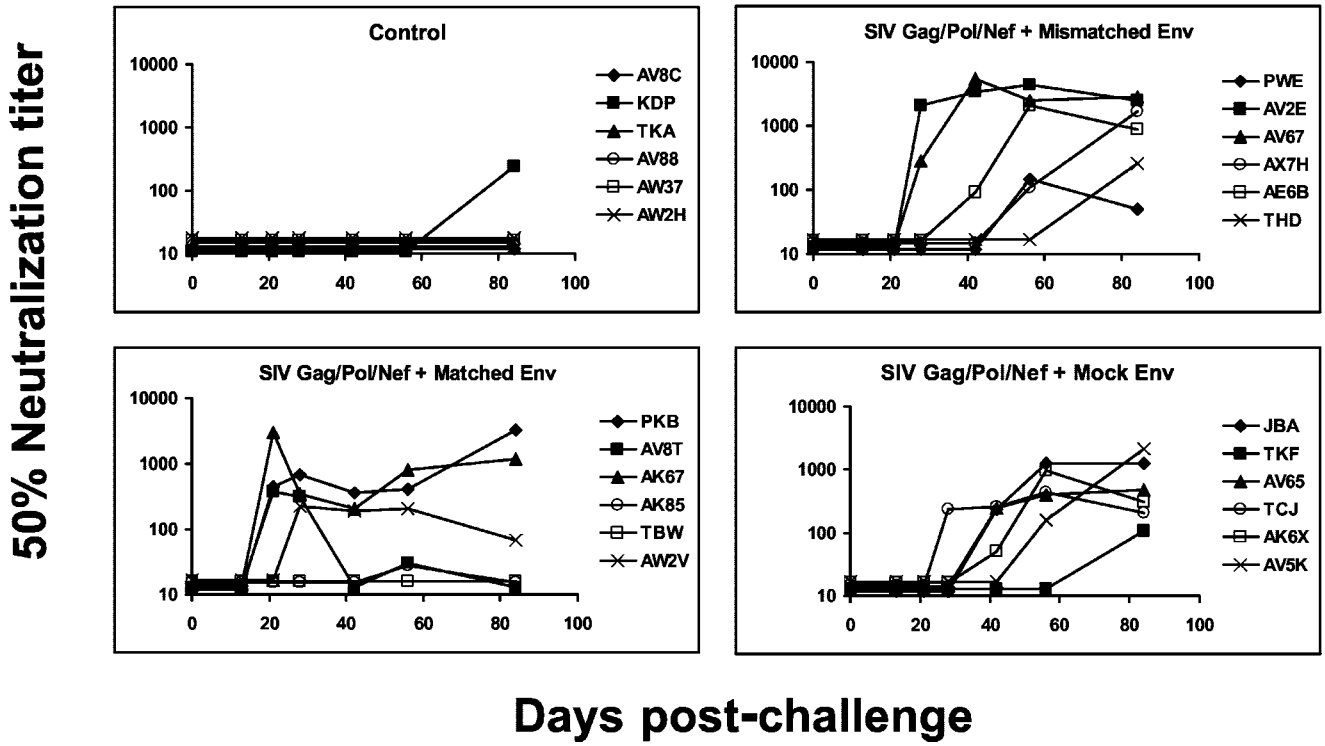

FIG. 6. Plasma SHIV-89.6P neutralization titers determined from plasma samples obtained from the monkeys following SHIV-89.6P challenge. Neutralization was determined with an MT-2 dye exclusion assay.

did not contribute to viral containment. The evolution of an antibody response that neutralized the challenge virus SHIV-89.6P was monitored on a weekly basis in vaccinated monkeys after viral challenge (Fig. 6). At 3 weeks postchallenge, three animals in the matched Env group, but none in the mock or mismatched Env groups, showed an anamnestic response to the challenge virus. However, there was no statistically significant difference between the three groups of experimentally vaccinated monkeys in time to the detection of neutralizing antibody or number of animals developing detectable neutralizing antibody responses. Of note, the statistical tests applied to these data have very little power to detect differences among groups because of the small number of monkeys in each experimental group. Thus, it remains possible that neutralizing antibodies had an effect that we were unable to detect.

To evaluate further whether the emergence of a neutralizing antibody response was associated with either clinical or virologic events following SHIV-89.6P challenge, a linear regression analysis was performed to evaluate the association of detectable neutralizing antibodies with either plasma viral RNA levels or peripheral blood $CD4^+$ T-lymphocyte counts. In fact, these variables showed no significant association with the development of neutralizing antibody, whether assessed on the basis of its emergence over time or its detection at a single time during the first 6 weeks following challenge. Therefore, we were unable to demonstrate that neutralizing antibodies directed against SHIV-89.6P contributed to viral containment after challenge. Finally, the fact that the mismatched Env group appeared to control plasma viremia more effectively than the matched Env group further suggests that neutralizing antibodies did not substantially contribute to viral containment.

To examine the possible contribution of Env-specific T-cell responses to protective immunity in these monkeys, PBMC cellular immune responses from the four groups of experimental monkeys were assessed 1 week following rADV boosting for cellular immunity to a pool of HIV-1 89.6P Env peptides in an ELISPOT assay (Fig. 7, top panel). The mean responses were 449 ± 122 SFC (mean ± standard error) in the matched Env group, 730 ± 306 SFC in the mismatched Env group, and 13 ± 8 SFC in the mock Env group. The apparent higher PBMC SFC response in the HXB2/Bal Env-vaccinated monkeys to 89.6P Env than to HXB2/BaL Env does not achieve statistical significance. Thus, impressive cellular immunity was seen in PBMCs of the HXB2/Bal Env-immunized monkeys that reacted with the Env of the challenge virus.

Since cellular immune responses that develop following initial infection contribute to containment of AIDS virus spread (3), we reasoned that differences in these responses to Env between the groups of vaccinated monkeys may explain the differences in their clinical outcomes. Therefore, we assessed HIV-1 89.6P Env-specific T-cell responses in these monkeys 3 and 10 weeks following SHIV-89.6P challenge (Fig. 7). Strikingly, PBMCs of the monkeys that received either matched or mismatched Env immunogens developed dramatically higher ELISPOT responses to these Env peptides than did the monkeys that received the mock Env immunizations ($P = 0.002$, Wilcoxon rank sum test). Therefore, a strong association was seen between the generation of Env-specific T-cell responses postchallenge and the inclusion of either matched or mis-

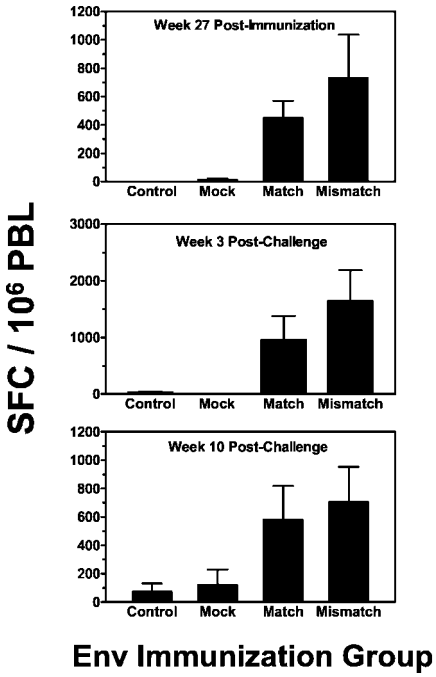

FIG. 7. 89.6P Env-specific PBMC IFN-γ ELISPOT responses assessed 1 week following rADV boost and both 3 and 10 weeks following SHIV-89.6P challenge. ELISPOT responses were determined after in vitro exposure of PBMCs (peripheral blood lymphocytes [PBL]) to peptide pools spanning the HIV-1 89.6P Env protein. The bars represent the mean values for six monkeys with the standard error shown.

matched Env immunogens in the vaccine regimens of these monkeys.

DISCUSSION

The present study demonstrates that HIV Env contributes to immune protection in a simian lentivirus challenge model. Importantly, protection was observed when the Env immunogen was matched or mismatched relative to the challenge viral strain. These findings suggest that it is advisable to include this gene product in vaccine candidates, even if such vaccines do not elicit a broadly neutralizing antibody response.

This study also provides an important opportunity to evaluate the contribution of an Env immunogen to both the immunity and protection elicited by a multicomponent immunodeficiency virus vaccine. It is interesting that the total SFC response of PBMCs of the vaccinated monkeys was no greater in the monkeys receiving Env as well as Gag-Pol-Nef immunogens than in those receiving only the Gag-Pol-Nef immunogens. This may reflect a ceiling to the cellular immune response that can be generated following immunization with a particular vaccine modality. This finding is also consistent with the possibility that the addition of an Env immunogen to the Gag/Pol immunogens may have decreased the immune responses to Gag and Pol.

Also surprising is the observation of better immune containment of SHIV-89.6P in the monkeys receiving the mismatched Env than in those receiving the matched Env immunogen. Cross-reactive T-cell epitopes in conserved regions of Env and a particularly poor immune response to Gag in the matched Env-immunized monkeys could certainly have contributed to this phenomenon (Fig. 3 and 7). There was no evidence in the present study that neutralizing antibodies to the challenge virus contributed to viral containment. However, it remains possible that the number of monkeys in each experiment arm was insufficient to provide the power needed to detect an effect of neutralizing antibodies or that Fc-mediated antibody effects played a role in viral control that we did not measure. In fact, data from two recent studies have suggested an association between anti-Env binding antibodies and viral containment in the SHIV-89.6P challenge model (2, 6).

The discordance between the peripheral blood $CD4^+$ T-lymphocyte counts and the plasma viral RNA levels in the SHIV-89.6P-infected monkeys that received the matched Env immunogens was unexpected. The presumption has been that viral load and $CD4^+$ T-lymphocyte counts are closely tied in SHIV-89.6P-infected monkeys. However, it has recently been shown that the ability of the HIV-1 envelope glycoproteins to fuse membranes, which has been implicated in the induction of viral cytopathic effects in vitro, contributes to the capacity of a pathogenic SHIV to deplete $CD4^+$ T lymphocytes in vivo (7). It is therefore possible that a vaccine-associated prevention of $CD4^+$ T-lymphocyte loss may occur irrespective of the level of viral replication.

Shiver et al. demonstrated that *Mamu-A*01*$^+$ rhesus monkeys that were vaccinated with rADV-SIV *gag* had undetectable plasma viral RNA at a set point following SHIV-89.6P challenge (21). This degree of viral containment was much more impressive than that seen in the present study, despite the fact that the monkeys in that earlier study received a less complex immunization regimen than the monkeys in the present study. The higher plasma viral RNA levels in all of the SHIV-89.6P-infected monkeys in the present study is consistent with the observation that the *Mamu-A*01* allele contributes to improved cellular immune response-mediated containment of SHIV-89.6P replication (13, 16, 24). Nevertheless, the clinical protection observed in the present study in *Mamu-A*01*$^-$ rhesus monkeys indicates that vaccine-associated protective immunity is not only seen in *Mamu-A*01*$^+$ animals.

The present study provides a strong rationale for including Env antigens in HIV vaccines that advance into human efficacy trials. The current focus of effort on Env immunogen design continues to center on modifications that will induce broadly neutralizing antibodies (12). Such modifications will no doubt further enhance vaccine efficacy if successful. However, this study indicates that the inclusion of Env as a vaccine immunogen, even if it does not induce a broadly neutralizing antibody response, contributes to virus containment and immune preservation. The enhanced breadth of cellular immunity appears sufficient to improve the clinical protection conferred by vaccination.

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