

## DNA Vaccines Expressing Different Forms of Simian Immunodeficiency Virus Antigens Decrease Viremia upon SIVmac251 Challenge

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**We have tested the efficacy of DNA immunization as a single vaccination modality for rhesus macaques followed by highly pathogenic SIVmac251 challenge. To further improve immunogenicity of the native proteins, we generated expression vectors producing fusion of the proteins Gag and Env to the secreted chemokine MCP3, targeting the viral proteins to the secretory pathway and to a  $\beta$ -catenin (CATE) peptide, targeting the viral proteins to the intracellular degradation pathway. Macaques immunized with vectors expressing the MCP3-tagged fusion proteins developed stronger antibody responses. Following mucosal challenge with pathogenic SIVmac251, the vaccinated animals showed a statistically significant decrease in viral load ( $P = 0.010$ ). Interestingly, macaques immunized with a combination of vectors expressing three forms of antigens (native protein and MCP3 and CATE fusion proteins) showed the strongest decrease in viral load ( $P = 0.0059$ ). Postchallenge enzyme-linked immunospot values for Gag and Env as well as gag-specific T-helper responses correlated with control of viremia. Our data show that the combinations of DNA vaccines producing native and modified forms of antigens elicit more balanced immune responses able to significantly reduce viremia for a long period (8 months) following pathogenic challenge with SIVmac251.**

The development of effective AIDS vaccines remains a primary challenge in human immunodeficiency virus (HIV) research. Evidence suggests that high titers of neutralizing antibodies and a broad cellular immunity are inversely correlated with disease progression in humans (18, 51, 64), indicating that both humoral and cellular immune responses are important for disease containment. DNA-based vaccines have generated substantial interest due to their ability to elicit immune responses against native proteins with complex structures, as well as their capacity to stimulate both humoral and cellular immunity (13, 40, 65, 67). DNA immunization may provide several advantages over conventional vaccines in terms of proper antigen processing, immunogenicity, safety, and stability (reviewed in references 14, 19, 22, and 35). DNA vectors expressing human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) proteins have been shown to elicit specific humoral and cellular immunity in both mice (38, 53, 69, 71) and macaques (9, 15, 16, 33, 36, 37, 44, 45, 60, 62, 70, 75), although it was observed that antibody development after DNA vaccination was inefficient in macaques. It was also reported that DNA vaccination provides a level of protection in nonhuman primates. Several groups have reported evidence of protection, by measuring reduced levels of chronic viremia, using the simian-human immunodeficiency virus (SHIV) ma-

caque challenge model (reviewed in reference 54). These trials included DNA with interleukin-2 (IL-2) as an adjuvant (5) and DNA priming followed by boosting with recombinant virus, protein, or inactivated virus (2, 15, 16, 61). Other studies, using DNA vaccination followed by SIVmne challenge in cynomolgus macaques, identified some animals capable of controlling viremia (21, 42, 43). However, in other studies, the generated immune responses failed to protect against a highly pathogenic SIV challenge (26, 37, 68). It is important to assess methods able to further improve vaccine protocols using the most appropriate primate models. One important model is SIVmac251 infection of Indian rhesus macaques, which closely parallels HIV-1 infection and AIDS development in humans (12). In this model, little evidence for persistent protection after DNA vaccination has been reported, especially in the absence of recombinant viral boost (17, 26, 37, 44).

Improvement of DNA vaccine vectors expressing HIV and SIV proteins is an important objective in our laboratory. We have demonstrated that the inefficient expression of HIV structural proteins in absence of the essential regulatory protein Rev is due to the presence of instability sequences scattered throughout the messenger RNAs (59). In fact, removal of such sequences by point mutations enhanced the expression of viral proteins in the absence of Rev (46, 57, 58). Such constructs are highly immunogenic in mice and macaques, whereas previous vectors showed low immunogenicity. A DNA-prime/attenuated poxvirus-booster vaccine study with this first generation of optimized SIV constructs (RNA or codon optimized) demon-

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strated increased cytotoxic T lymphocyte and lymphoproliferative responses (LPR) as well as a reduction of viremia after mucosal challenge with highly pathogenic SIVmac251 (23, 24).

The present study was performed to evaluate the ability of DNA-based vaccines expressing combinations of native and modified SIV antigens (Gag and Env) to elicit specific immune responses and to provide protection against a pathogenic SIVmac251 challenge. Reduction in plasma levels of the challenge virus was observed in the vaccinated animals. Hence, a combination of DNA vectors expressing native and modified forms of viral antigens provides protection against a highly pathogenic virus challenge. These results demonstrate long-lasting protective immunity and reduced viremia after vaccination with DNA only and provide evidence for the use of such DNA vectors as part of AIDS vaccine strategies.

#### MATERIALS AND METHODS

**DNA vectors.** The plasmids used for DNA vaccination contain the cytomegalovirus promoter, the bovine growth hormone polyadenylation site, and the kanamycin-resistant gene. The RNA optimized expression vectors for *gag* and *env* were generated by removal of the inhibitory sequences by multiple silent point mutations not affecting the sequence of the encoded proteins, as previously described for HIV-1 *gag* and *env* (46, 57–59), using synthetic DNAs. The secreted and intracellularly degraded variants of the SIV antigens were generated by fusion of either IP10-MCP3 (7) or of a beta-catenin (CATE)-derived peptide (amino acids [aa] 18 to 47) (1) at the N terminus of Gag and Env, replacing the myristoylation signal of Gag or the signal peptide of Env, respectively. Plasmids *gagDX* (1S) and *p39gag* (71S) are fully optimized and produce *p57gag* and *p39gag*, respectively. Gag fusion proteins contain five amino acids (ASAGA) linking the respective signal peptide to the second amino acid of *gag*, generating *pCATE-gagDX* (2S), *pCATE-p39gag* (3S), and *pMCP3-p39gag* (4S). For all vaccinations except the last *pCATE-p39gag* (3S) was used, which was then replaced by *pCATE-gagDX* (2S). *pEnv-CTE* (56S) is partially optimized and contains the constitutive transport element (CTE) of SRV-1 located between *env* and the polyadenylation signal, while *pEnv* (61S) is fully optimized, producing higher levels of Env. The latter variant was used in the last vaccination. The Env fusion proteins contain a 3-aa linker (ICS) between the signal and the 25th aa of Env, generating *pMCP3-env* (60S) and *pCATE-env* (59S).

**Transient transfections and protein analysis.** Human 293 cells were transfected by the calcium phosphate coprecipitation technique. The supernatants and cells were harvested after 48 h and were analyzed by Western immunoblot and SIVp27gag antigen capture assays as described (20).

**Immunization, challenge, and sample collection.** All animals in the study were colony-bred Indian rhesus macaques (*Macaca mulatta*) and were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International. Screening for 10 major histocompatibility complex (MHC) class I alleles (A01, A02, A08, A11, B01, B03, B04, B17, w201, 0401/06) was performed by PCR (D. Watkins, Wisconsin Regional Primate Center). Highly purified, endotoxin-free DNA plasmid preparations were produced using a QIAGEN kit (Hilden, Germany) and were adjusted to 1 mg/ml in phosphate-buffered saline (PBS). The animals were immunized via the intramuscular route with a total of 6 mg of DNA (3 mg each of *gag*- and *env*-expressing plasmids) (see Fig. 2A). Each DNA was injected separately at four different sites. For the last boost, all animals received the fully optimized *env* expression plasmids and CATE-p57gag (2S), while for the previous vaccinations *env* was expressed from partially optimized mRNAs containing the RNA export signal CTE.

The SIVmac251 challenge stock (prepared by Ranajit Pal, Advanced Bio-Science Laboratory) was derived from the SIVmac251 stock originally prepared by Ronald Desrosiers. Animals were challenged intrarectally with a 1:10 dilution of the virus stock, containing approximately 10 animal-infectious units (52).

Blood samples were obtained in acid citrate dextrose tubes. After centrifugation, plasma was collected and stored at  $-80^{\circ}\text{C}$ . Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation over Histopaque (Sigma) and were used fresh for lymphocyte proliferation assays. Aliquots were viably frozen in fetal bovine serum and 10% dimethyl sulfoxide and used for enzyme-linked immunospot (ELISPOT) assays.

**Lymphocyte proliferation assay.** Fresh PBMCs ( $3 \times 10^6$  cells/ml) were resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated human

AB serum (Sigma-Aldrich, St. Louis, MO), seeded in 96-well plates (density,  $3 \times 10^5$  cells/well) in triplicate, and cultured for 3 days in the absence or presence of 1  $\mu\text{g/ml}$  of native high-pressure liquid chromatography-purified SIV p27gag or gp120env proteins (Advanced BioScience Laboratories, Rockville, MD) per well. Phytohemagglutinin was used as a positive control. The cells were then pulsed overnight with 1  $\mu\text{Ci}$  of  $^3\text{H}$ -labeled thymidine before harvesting. The stimulation index was calculated as the ratio between the thymidine incorporated by PBMCs in the presence of viral proteins and the thymidine incorporated by PBMCs cultured in medium only.

**ELISPOT assay.** ELISPOT assays were performed according to a modified version of the method of Newberg et al. (47). Ethanol-treated 96-well Multi-screen-IP (Millipore, Bedford, MA) plates were coated overnight with 100  $\mu\text{l}$  of 7.5  $\mu\text{g/ml}$  anti-human gamma interferon (IFN- $\gamma$ ) (B27 clone; BD Biosciences, San Diego, CA) per well, washed with PBS containing 0.01% Tween 20, blocked for 2 h with PBS containing 3% human serum at room temperature, and washed. PBMCs at  $3 \times 10^5$ /well were assayed in triplicate in RPMI 1640 containing 3% human serum and SIV-specific peptides (1  $\mu\text{g/ml}$  for each peptide) using pools of 15-mer peptides with an overlap of 11 amino acids (Infinity Inc. Biotech Research and Resource, Aston, PA) spanning the complete *gag* gene and the N-terminal half of the *env* gene. The plates were incubated for 18 h at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ , treated with ice cold water, and washed. Biotinylated rabbit polyclonal anti-IFN- $\gamma$  (Biosource, Camarillo, CA.) at 0.5  $\mu\text{g/ml}$  in PBS containing 0.5% human sera (filtered with Steriflip-GP; Millipore, Bedford, MA) was added to the plates and incubated for 2 h at room temperature, washed, and incubated for 1 h at room temperature with streptavidin-alkaline phosphatase (Southern Biotechnology, Birmingham, AL) in PBS containing 3% human sera and 0.005% Tween. Following washes, the plates were developed with one-step Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Pierce, Rockford, IL). Spots were counted on a C.T.L. ELISPOT reader (Cellular Technology Ltd., BD Biosciences, San Diego, CA) and analyzed by using ImmunoSpot software version 2.06. The cutoff was defined as the average spots in the negative control plus 2 standard deviations. Cells treated with medium alone were used as the negative control. Specific spots to a given peptide pool were calculated by subtracting the cutoff value and adjusted to the number of spot-forming cells per million PBMCs.

**Antibody assays.** Serial dilutions of plasma were incubated with purified p27gag or gp120 Env protein of SIVmac251 bound to microtiter plates, and optical absorbance at 450 nm was determined. The binding titers are reported as the reciprocal of the highest dilution scoring positive (having a value at least two times higher than the average values obtained with negative-control sera). Neutralizing antibodies against primary SIVmac251 (PBMC-grown) were measured in M7-Luc cells as described (41). The primary isolate neutralizing antibody titer is the reciprocal plasma dilution at which the relative luciferase units were reduced 80% compared to virus control cells (primary-SIVmac251).

**Measurement of SIV RNA copy number.** SIV RNA copy numbers were determined by a nucleic acid sequence-based isothermal amplification assay using SIVmac251-specific primers (55). The assay has a sensitivity threshold of 2,000 copies/input volume of 100  $\mu\text{l}$ . Plasma samples having values below the assay threshold were assigned a value of 10,000 copies/ml.

**Statistical analysis.** For statistical analysis median viremia levels during acute-phase infection (weeks 1 to 4) and chronic phase infection (weeks 5 to 25) were compared using the Wei-Johnson test (72). Correlations of immune responses with levels of viremia were determined using the Spearman rank correlation method. The *P* values for all analyses were corrected by the method of Hochberg (25) for multiple comparisons between pairs of vaccine groups, over phases of infection or over assays of immune response.

#### RESULTS

**Expression vectors for SIV *gag* and *env* antigens.** High levels of SIVmac239 *gag* and *env* expression have been achieved through RNA optimization (46, 57–59) (Fig. 1B, lane 1, for Gag and Fig. 1E, lane 1, for Env). Such vectors, expressing the native forms of Gag and Env, were previously shown to induce SIV-specific immune responses in immunized macaques (24). Their use in a DNA prime/poxvirus-based NYVAC SIV boost further showed great benefit of the DNA priming resulting in control of the SIVmac251 challenge virus (23). In an effort to further improve antigen presentation and immunogenicity, two types of modifications were made. First, a signal sequence and

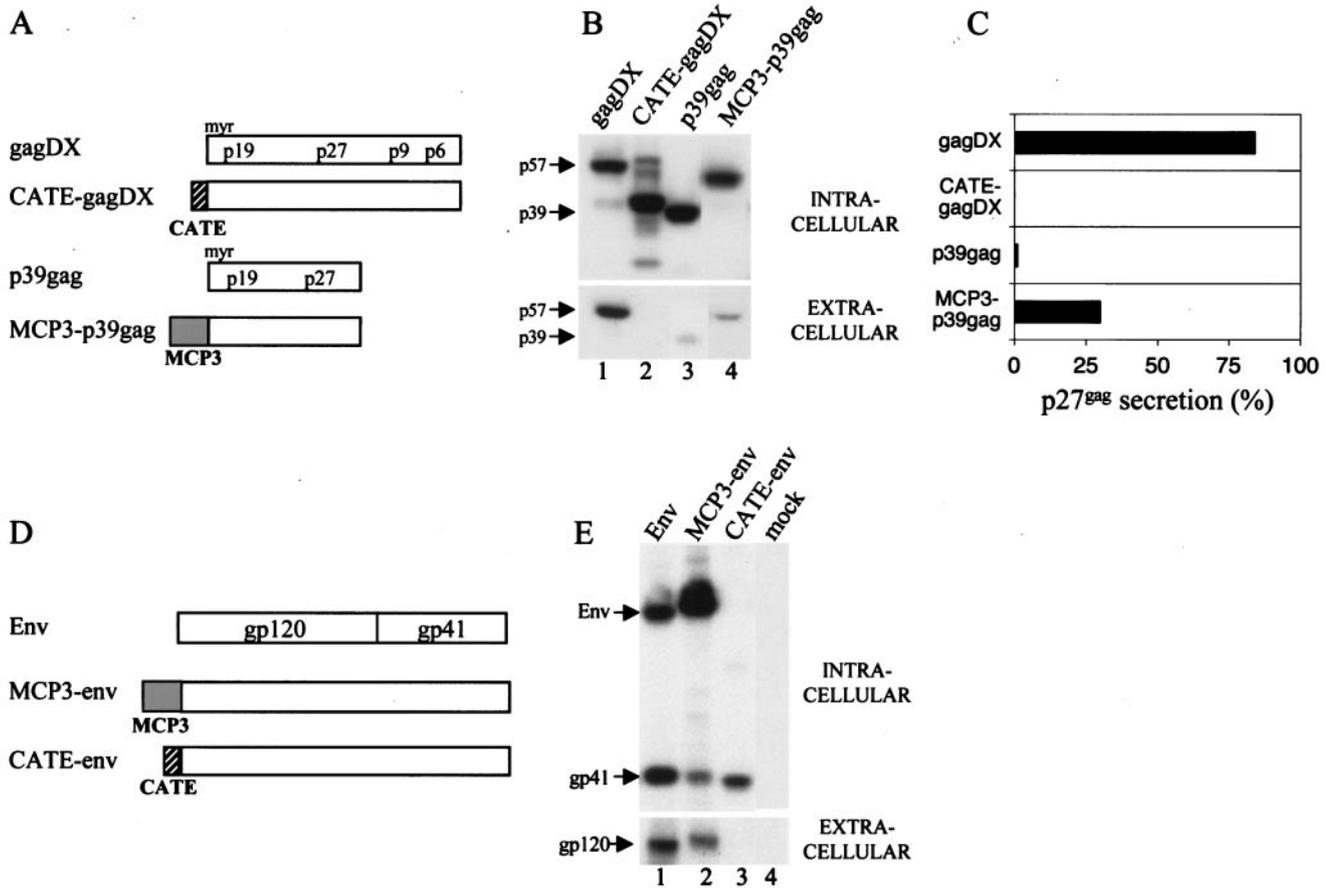


FIG. 1. SIV protein expression analysis in 293 cells. (A) Schematic representation of RNA-optimized SIV *gag* expression vectors encoding the p57gag (gagDX) and p39gag proteins. Sequences fused to *gag* are shown in boxes, striped for the catenin peptide (CATE) and grey for MCP-3 chemokine (MCP3). (B) Expression and localization of the different *gag* constructs. Human 293 cells were transfected with the indicated plasmids, and protein production was analyzed by Western immunoblotting using sera from SIV-infected macaques. For this analysis 1/50 of intracellular protein extract and 1/150 of the supernatant was used. (C) Quantitation of Gag levels in the intracellular and extracellular compartments of the experiment shown in Fig. 1B using p27gag antigen capture assay. The percentage of secreted Gag is shown. (D) Schematic representation of RNA-optimized SIV *env* expression vectors. Symbols are as described for Fig. 1A. (E) Expression and localization of the different *env* constructs. Human 293 cells were transfected with the indicated plasmids, and protein production was analyzed by Western immunoblotting in both intra- and extracellular compartments as described for Fig. 1B.

the entire chemokine MCP-3 coding region, previously shown to enhance the ability of some antigens to trigger strong immune response (7), were linked to the N termini of Gag and Env (Fig. 1A and D). Alternatively, a peptide derived from beta-catenin providing a ubiquitination signal was linked to the N terminus of Gag and Env. The resulting modified SIV antigens have different properties as shown in Fig. 1. Whereas the native p57gag protein expressed from pGagDX formed particles and was secreted (Fig. 1B), CATE-gagDX was rapidly degraded in the cells and did not appear in the medium. In contrast, MCP3-p39gag fusion was stable and was secreted more efficiently than p39gag (Fig. 1B [compare lane 3 containing unmodified p39gag to lane 4] and Fig. 1C). In similarity to Gag results, the MCP3-env chimera was secreted (Fig. 1E, lane 2), whereas the CATE-env fusion was unstable and did not appear extracellularly (Fig. 1E, lane 3). The gp120/gp160 form of the CATE-env was not visualized because of its instability, and only a cell-associated Env fragment of approximately 40 kDa was visible. Thus, both Gag and Env are degraded upon

fusion to the CATE signal. Env degradation appears to be more rapid, indicating that Gag may be more resistant to degradation. These results indicate that the introduced modifications altered significantly the stability and trafficking of the antigens, justifying the analysis of immunogenicity of such forms. Vaccination studies using mice indicated that the combination of three different plasmids (unmodified, MCP-3 fusion, and CATE fusion) showed better immunogenicity (M. Rosati, A. Von Gegerfelt, C. Alicea, B. K. Felber, and G. N. Pavlakis, unpublished data). In addition, we found that the fusions to MCP-3 provided the best antibody response. This was verified in preliminary DNA vaccination experiments using two macaques, which did not develop antibodies after repeated vaccination with an optimized expression vector producing native Gag but developed a strong antibody response after vaccination with MCP3-gag (Rosati et al., unpublished).

**DNA-based vaccination decreases viremia following mucosal challenge.** To test the ability of the antigens to protect against SIV challenge, we immunized Indian rhesus macaques

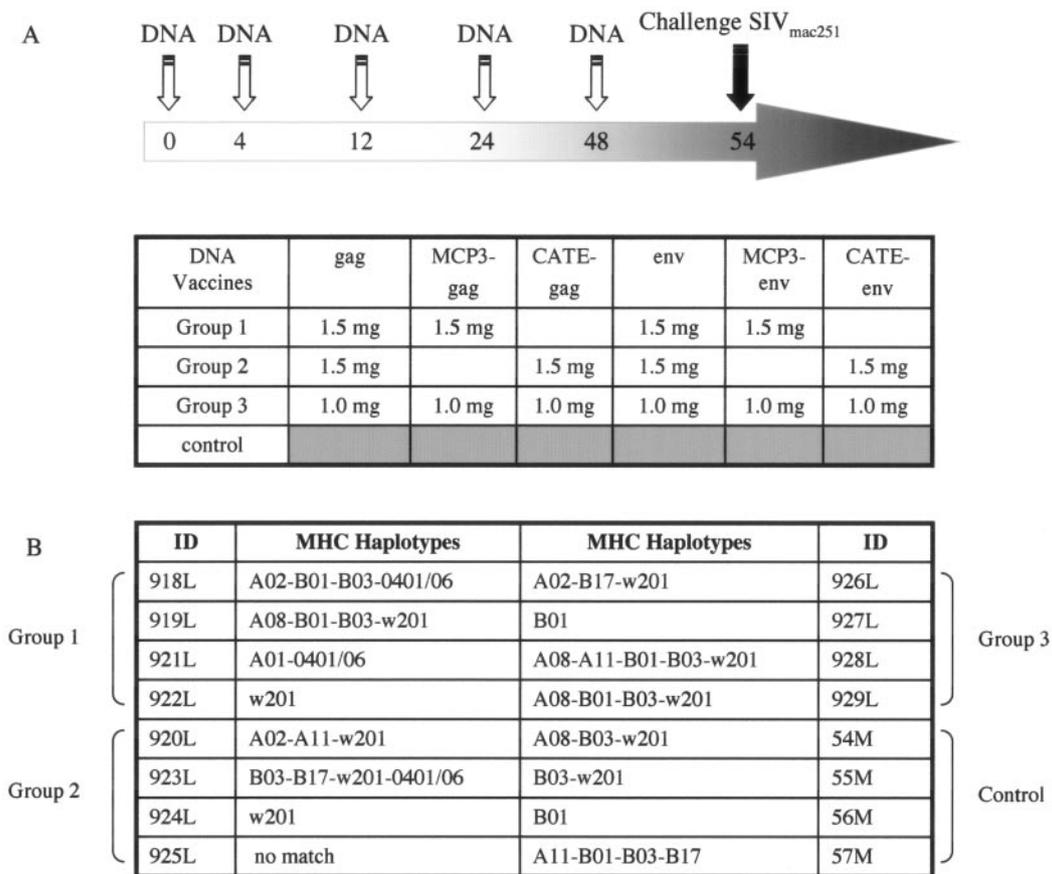


FIG. 2. Study design. (A) Four groups of four Indian rhesus macaques were enrolled. Group 4 remained unvaccinated and served as the control group. All other animals were vaccinated five times at 0, 4, 12, 24, and 48 weeks with a total of 6 mg of plasmid DNAs (3 mg for Gag and 3 mg for Env), followed by mucosal challenge with SIVmac251. All animals received the vectors expressing the native SIV Gag and Env proteins. In addition, all animals received vectors expressing modified Gag and Env proteins. Group 1 received p57gag (gagDX) and gp160 (env) together with MCP-3 fusion proteins pMCP3-p39gag and pMCP3-env; group 2 received the expression vectors for the native antigens together with CATE fusion proteins pCATE-p39gag and pCATE-Env; group 3 received the combination of all six plasmids. Different plasmids expressing identical Env fusions as well as pCATE-p57gag were used for the last vaccination, as detailed in Materials and Methods. (B) MHC haplotypes of the individual animals using a set of 10 MHC class I alleles. Only one animal had the MamuA\*01 haplotype. Ten additional control animals were used in the comparisons (see text). Of these, two (39F, 44F) were MamuA\*01 positive.

with plasmids expressing the native and modified Gag and Env proteins in different combinations (Fig. 2A) and examined virus propagation as well as cellular and humoral immune responses before and after pathogenic challenge by SIVmac251. Prior to vaccination, all animals were analyzed for a set of 10 MHC class I alleles (Fig. 2B). The individual animals showed a diverse array of MHC class I alleles, and only animal 921L (group 1) had the MamuA\*01 allele. Three groups of four animals were immunized via the intramuscular route using a total of 6 mg plasmid DNA per vaccination (3 mg of gag and 3 mg of Env-expressing DNA vectors, respectively) injected at different sites.

On the basis of the preliminary immunogenicity analysis in mice and macaques, three combinations of antigens were tested in macaques in an effort to improve the outcome of DNA vaccination. All three groups received the vectors expressing native Gag and Env (Fig. 2A). Group 1 received native and MCP3-tagged antigens (1.5 mg each of pSIVp57gag, pMCP3-p39, p239env, and pMCP3-239env). Group 2 received a combination of native and intracellularly

degraded antigens (1.5 mg each of p57gag, pCATEp57p239env, and pCATE-239env). Group 3 received a combination of all six molecules (1 mg each). The vaccination schedule included a total of 6 mg plasmid DNA per immunization and a total of five vaccinations given at weeks 0, 4, 12, 24, and 48.

Six weeks after the last vaccination the immunized and four naive macaques were intrarectally challenged with a highly pathogenic stock of SIVmac251. All animals became infected, although we observed significant differences among the vaccinated and the control animals (Fig. 3). We also included in the analysis 10 additional control animals, which were infected with the same dilution of the virus stock in the same animal facility within a few weeks of our challenge and which were analyzed with the same plasma virus load assay method. Results obtained with eight of these animals (8 M, 12 M, 13 M, 27F, 34 M, 39F, 44F, 46F) were published elsewhere (52), and two animals (538L and 539L) were from a separate study. The median peak virus load during the acute phase for the 14 control animals was  $10^{8.2}$  RNA copies/ml, and the median virus

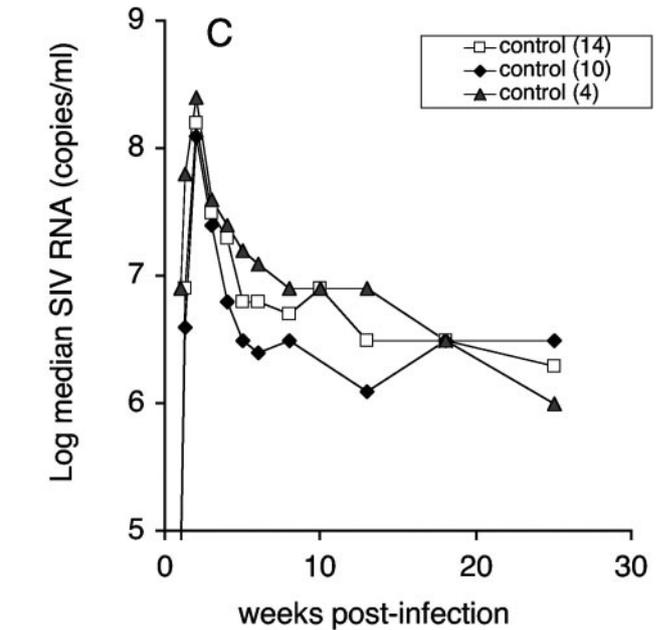
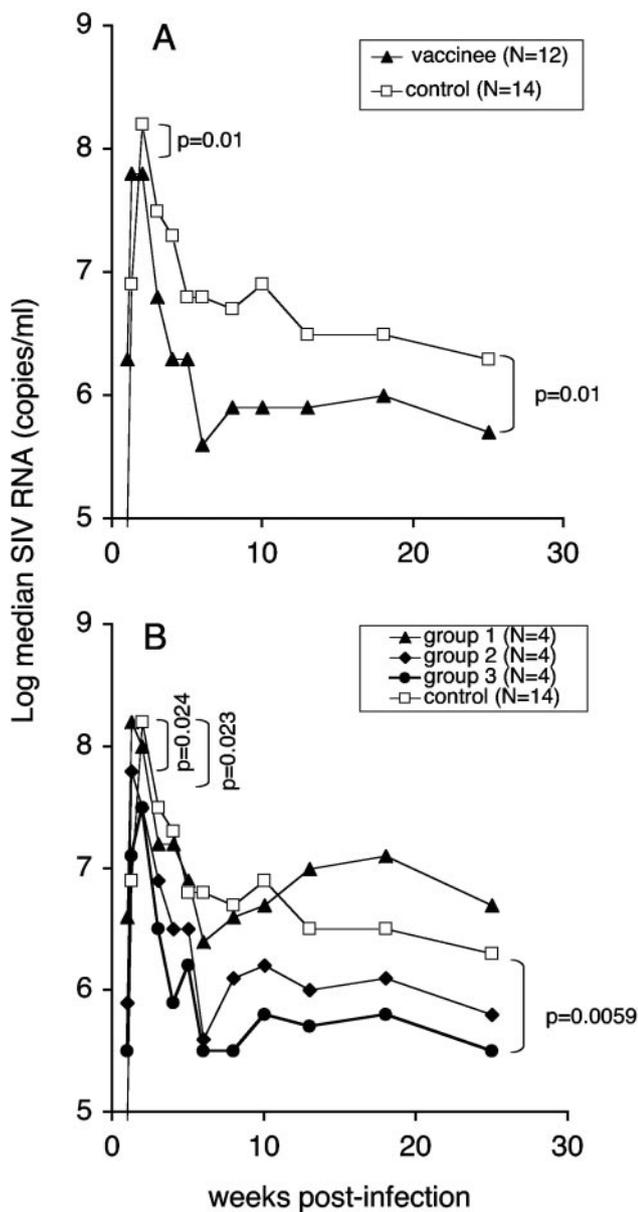


FIG. 3. Viral loads of DNA-immunized and control animals after mucosal challenge with SIVmac251. (A) The log median SIV RNA loads for all vaccinees ( $n = 12$ ) and controls ( $n = 14$ ) are shown. The  $P$  value (0.01) indicates significant difference during both the acute and chronic phase. Controls include 10 additional macaques challenged with the same virus stock as described in Materials and Methods. (B) The log median SIV RNA loads are shown for each individual group and the 14 control animals. The  $P$  value indicates significant difference between group 3 and control ( $P = 0.0059$ ). In addition, virus loads in groups 2 and 3 were significantly lower than in the control groups during acute viremia ( $P = 0.024$  and  $P = 0.023$ , respectively). (C) Log median virus loads of the two control groups used for the comparisons ( $n = 4$ ,  $n = 10$ ) and the combined control group ( $n = 14$ ). (D) Sequential viral load determinations for individual animals in the four groups, shown as log SIV RNA copies per ml of plasma. Filled symbols indicate individual animals with viral loads less than the control animal loads.

load during the entire chronic phase was  $10^{6.7}$  RNA copies/ml, confirming the robustness of the viral stock.

The log-transformed median virus loads of all the vaccinated animals as a group (a combination of groups 1, 2, and 3;  $n = 12$ ) were compared to the virus load of the 14 control animals (Fig. 3A). This comparison showed a statistically significant difference between the vaccinee ( $n = 12$ ) and the control ( $n = 14$ ) in both the acute phase ( $P = 0.010$ ) and the chronic phase ( $P = 0.010$ ) of infection (Wei-Johnson test). Therefore, DNA vaccination alone was effective and protected the animals from development of high viremia for a long period during chronic infection.

We next compared the individual vaccinated groups to the controls, to evaluate the efficacy of the three tested vector combinations. The comparison of the log-transformed median virus loads of the individual groups (group 1, 2, and 3;  $n = 4$  for

each group) and the combination of all controls ( $n = 14$ ) is shown in Fig. 3B. Of note is group 3, which received the combination of all antigens and shows strongest control of viremia (1 to 2 logs less than control) up to the end of the study at week 33. Analysis using the Wei-Johnson test showed that group 3 differed significantly from the control group during both the acute phase (days 7 to 28,  $P = 0.023$ ) and the chronic phase (days 35 to 171,  $P = 0.0059$ ). The difference between group 2 and controls was statistically significant only during the acute phase at the  $P = 0.024$  level, whereas no significant difference was found for group 1.

We note that analysis including all 14 control animals leads to a conservative evaluation of differences between vaccine and control groups. Figure 3C shows the median virus loads of the 4 controls and of the additional 10 controls compared to the total control group of  $n = 14$ . Inclusion of the 10 additional

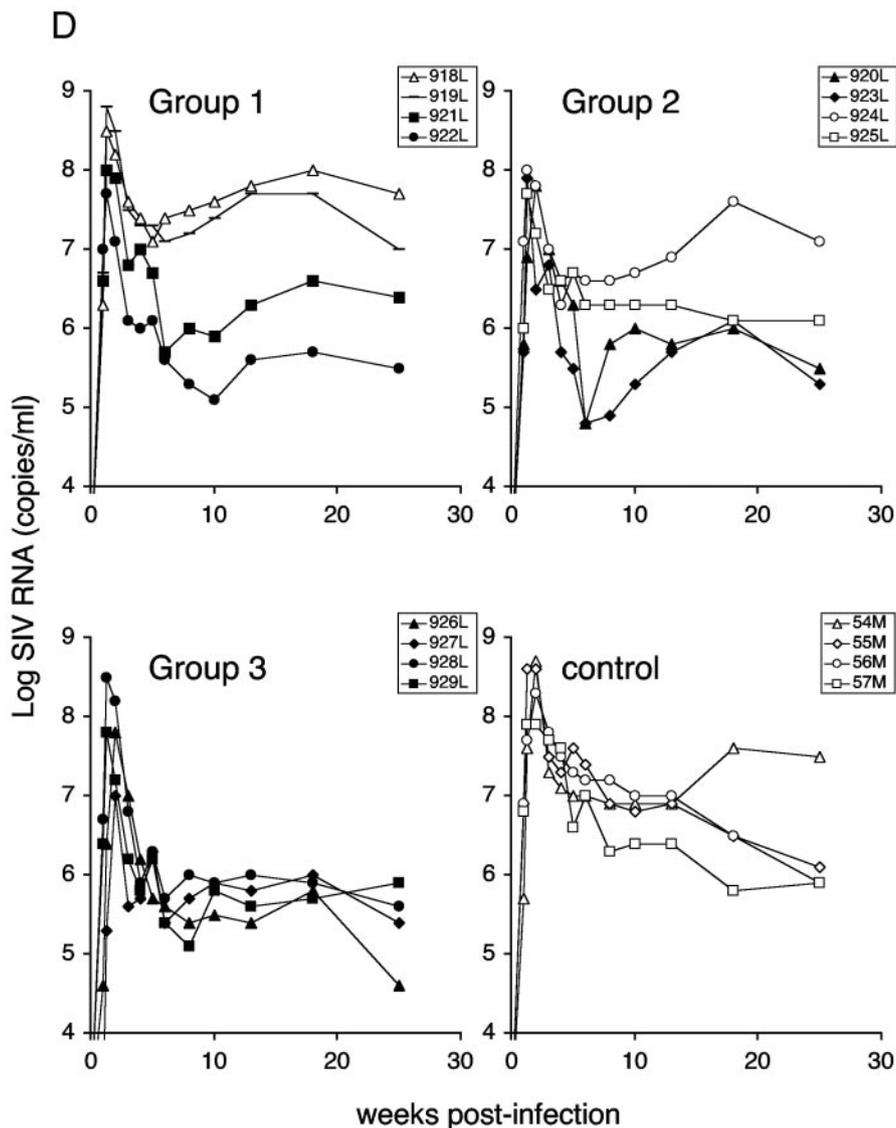


FIG. 3—Continued.

controls leads to more-conservative estimates of the difference between control and vaccinee groups, because the 10 additional controls had lower viral loads than the group of 4 controls at almost every time point. Analysis by the Wei-Johnson method showed that the two groups of controls were not different during the chronic period of infection. The log-transformed virus loads of the individual animals in each group are shown in Fig. 3D. Interestingly, all animals in group 3 showed a consistent control of viremia with little animal-to-animal variation, despite the genetic differences among the monkeys (Fig. 1B). In contrast, in groups 1 and 2 half of the animals (indicated with filled symbols) showed decreased levels of viremia during the chronic phase similar to those seen with group 3.

The analysis by the Wei-Johnson nonparametric test does not make assumptions about the form of the distributions in the groups. Alternative parametric analyses were also performed using repeated-measure analysis of variance

(ANOVA) to compare each group to the group of the four controls. The *P* values derived from the repeated-measure ANOVA were adjusted by Dunnett's method in analyses where multiple groups were compared to the same controls. The difference between group 3 and the four control animals is significant also using repeated-measure ANOVA ( $P = 0.035$ ), even when the greatest estimate of variance across all four groups was applied, in agreement with the results of the non-parametric Wei-Johnson test. The difference between group 1 or 2 and the four controls did not reach statistical significance. Therefore, DNA vaccination alone elicited immune responses capable of reducing viremia during both the acute and chronic phases of SIV infection. This long-lasting effect on viremia against a highly pathogenic stock of SIVmac251 was more prominent when the different forms of DNA antigens were combined (group 3). Analysis of immune responses also indicated that group 3 showed strong postchallenge immune responses to SIV antigens (see below). These results suggest that

TABLE 1. Binding antibodies to SIV Gag (left panel) and Env (right panel) in the plasma of the immunized animals at indicated time points<sup>a</sup>

Group	Animal	SIV Gag antibody titer in plasma at indicated time point (wk)							SIV Env antibody titer in plasma at indicated time point (wk)				
		0-12	14 <sup>b</sup>	24	26 <sup>c</sup>	48	50 <sup>d</sup>	54 <sup>e</sup>	0-24	26 <sup>e</sup>	48	50 <sup>d</sup>	54 <sup>e</sup>
1	918L	–	800	50	3,200	200	3,200	200	–	–	–	–	–
	919L	–	200	–	200	–	3,200	50	–	–	–	50	200
	921L	–	–	–	200	–	800	50	–	–	–	50	–
	922L	–	800	50	800	50	3,200	200	–	50	10	50	800
2	920L	–	200	–	50	–	–	50	–	–	–	10	–
	923L	–	50	50	800	800	3,200	800	–	10	10	10	–
	924L	–	–	–	–	–	200	–	–	10	–	–	10
	925L	–	–	–	–	–	–	–	–	10	–	–	–
3	926L	–	50	50	800	800	3,200	200	–	10	10	50	200
	927L	–	–	–	50	50	200	50	–	–	10	10	–
	928L	–	200	50	200	–	800	50	–	10	10	50	200
	929L	–	50	50	3,200	200	800	50	–	10	10	10	–

<sup>a</sup> Reciprocal titers are given. –, negative values.

<sup>b</sup> Two weeks after third immunization.

<sup>c</sup> Two weeks after fourth immunization.

<sup>d</sup> Two weeks after fifth immunization.

<sup>e</sup> Four weeks after fifth immunization.

the combination of three different forms of antigens used in group 3 may have resulted in a better control of viremia.

**Enhancement of humoral immunity.** We measured the presence of binding antibodies to both p27gag and gp120 by enzyme-linked immunosorbent assay in the vaccinated animals both before and after challenge (Table 1 and Fig. 4). Table 1 shows the development of anti-Gag and anti-Env antibodies during DNA vaccination, and Fig. 4A shows the reciprocal titers at week 50, 2 weeks after the last immunization.

After three DNA inoculations, 8 of 12 animals developed specific humoral immune responses to Gag. The number of animals responding was further increased to 10 of 12 animals after the fourth immunization. The humoral immune response to Env was in general lower and appeared later compared to Gag results. At week 50 (Fig. 4A), anti-Env antibodies were detected in 9 of 12 animals (titers of 10 to 50). Despite the differences in the magnitude of Gag and Env humoral immune responses, all the animals in groups 1 and 3, which received native and MCP-3 tagged antigens, developed antibodies. In contrast, only two of four animals in group 2, which received native and CATE-fusion antigens, showed persistent Gag and Env humoral immune responses (Table 1). Therefore, the MCP-3 fused forms of the antigens (common in groups 1 and 3) were associated with stronger humoral immune responses. Following challenge (Fig. 4B), we observed rapid increases (detectable by days 3 to 14 postchallenge) of humoral immune responses, predominantly to Gag, demonstrating an anamnestic immune response in all the vaccinated groups compared to the challenged control animals.

Group 3 animals also developed neutralizing antibodies against SIVmac251 grown in primary cells faster than the other groups, as early as weeks 4 to 8 after challenge (Table 2). No neutralizing antibodies were detected prior to challenge by this assay. Therefore, the development of neutralizing antibodies was more rapid after challenge in group 3 compared to the other vaccinated groups and the controls. However, no statis-

tically significant differences in binding antibody titers or in neutralizing activity were observed between the DNA-immunized animals and the control group when comparing the magnitude of the response. Therefore, the contribution of humoral immune responses to the control of viremia is unclear.

**Correlation of SIV-specific postchallenge cellular immune responses to the control of viremia.** We measured lymphoproliferative responses (LPR) to Gag and Env proteins at different times both before and after challenge (Fig. 5). Before challenge, all vaccinated animals developed specific LPR to Gag and Env, with peak stimulation indices ranging between 4 and 47 and between 4 and 27, respectively. Following SIV challenge, we observed a decline in LPR to gp120env in all groups. LPR to p27gag were similar postchallenge except for group 1, which had reduced LPR. Control animals had low LPR after challenge.

We also used ELISPOT assays to measure the frequency of antigen-specific IFN- $\gamma$ -producing PBMCs after overnight incubation with pools of overlapping SIV Gag or Env peptides (Fig. 6). Similar peak ELISPOT values for SIV Gag were measured in all three vaccine groups before challenge (Fig. 6A). The ELISPOT values for Env were significantly lower (Fig. 6B). Whereas all animals in group 1 showed Env-specific ELISPOT values, only 1 and 3 animals responded in groups 2 and 3, respectively. Following challenge, we observed an enhancement in the frequencies of ELISPOTs for both viral antigens in vaccinated animals. The control animals had low ELISPOT values compared to the other groups. Animals in group 3 showed the strongest responses to Gag and Env.

Figure 7 shows the correlation between the log-transformed median viral load measures and the postchallenge immune responses during the chronic phase using the Spearman rank correlation method. We found a strong negative correlation between chronic viremia and SIV-specific ELISPOT values for both Gag ( $P = 0.013$ ,  $r = -0.70$ ) (Fig. 7A) and Env ( $P = 0.0085$ ,  $r = -0.73$ ) (Fig. 7B). Therefore, protection from high

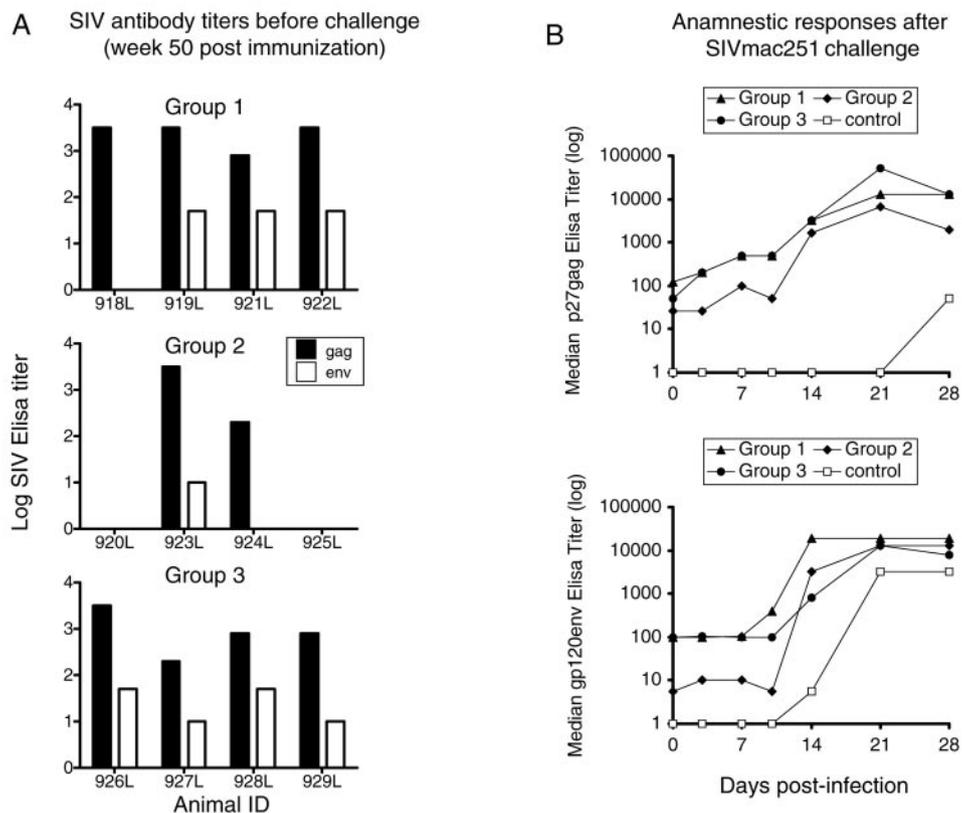


FIG. 4. Humoral immune responses. (A) Binding antibodies to SIV Gag (solid bar) and Env (open bar) in the plasma of vaccinated animals, determined at week 50 (4 weeks prior to challenge). The log reciprocal titers are shown. The control animals were negative prior to challenge (see also panel B). (B) The median reciprocal antibody titers to SIV Gag (top panel) and Env (bottom panel) in the plasma of vaccinated (groups 1 through 3) and control animals were determined at the indicated days after SIVmac251 challenge.

viremia in this model was associated with high levels in ELISPOT assays for the structural proteins Gag and Env. A moderate negative correlation was found between T-helper responses to gag (LPR) and virus loads ( $P = 0.026, r = -0.65$ ) (Fig. 7C). Similar significant negative correlations were found when comparing the viral load during acute infection to the postchallenge immune responses. There were no significant correlations with prechallenge immune responses. In conclusion, we found a significant negative correlation between virus load and cellular immune responses, since the animals having high responses in all groups had the lowest virus loads.

**DISCUSSION**

In the present study we established that DNA vaccine vectors producing a combination of native and modified forms of SIV antigens enhanced the ability of both arms of the immune system to mount an immune response against the virus. The combination of DNA vectors led to the development of a more balanced immune response than previously observed in DNA-vaccinated macaques. We demonstrated that DNA-immunized monkeys developed Gag and Env binding antibodies (Table 1 and Fig. 4). An anamnestic antibody response was observed after challenge, and the animals in group 3 also developed neutralizing antibody earlier than all other groups and controls. In addition, the DNA-immunized animals showed cellu-

lar immune responses as determined by LPR and ELISPOT assays during the immunization period (Fig. 5 and 6). ELISPOT values were increased after challenge and correlated with protection from high viremia (Fig. 7).

Although all animals were infected by the SIVmac251 challenge, immunized animals were protected from high viremia compared to unvaccinated controls in both the acute and the chronic phase of infection. Animals in group 3 receiving the combination of the three different forms of antigens achieved the greatest reduction of viremia, which was statistically significant. Although the results for the other two groups did not achieve statistical significance in the chronic phase, we note that 2/4 animals in each of these groups (in total 8/12 vaccinated animals in this study) had lower level of viremia compared to the controls. Comparison of all 12 vaccinated animals to the controls also showed a statistically significant difference in chronic viremia, which is a predictor of disease development. The DNA-immunized monkeys showed reduced viremia for 8 months following SIVmac251 challenge. This finding suggests that the combinations of vectors used in this study are useful for the generation of protective immunity and should be also studied in prime-boost experiments. The levels of viremia in the vaccinated animals, although reduced, remain substantial. This may be a consequence of the highly pathogenic virus stock used, which resulted in peak viremia values higher than  $10^8$ . Although DNA alone is not sufficient to provide complete

TABLE 2. Postchallenge neutralizing antibody titer in plasma to primary SIV<sub>mac251</sub> (in M7-luc cells)

Group	Animal	Titer at indicated time point (wk) <sup>a</sup>			
		0	4	8	13
1	918L	–	–	–	20
	919L	–	–	–	73
	921L	–	–	–	–
	922L	–	–	–	–
2	920L	–	–	–	70
	923L	–	–	–	180
	924L	–	–	–	68
	925L	–	–	–	>540
3	926L	–	94	59	86
	927L	–	–	32	130
	928L	–	–	61	119
	929L	–	–	–	85
Control	54M	–	–	–	102
	55M	–	–	–	47
	56M	–	–	–	106
	57M	–	–	39	27

<sup>a</sup> The neutralizing antibody titer is the reciprocal plasma dilution at which the relative luminescence unit values were reduced 80% compared to virus control wells (no serum sample present). –, titer <20.

virus control in this model, our results indicate the utility of DNA vaccination using multiple optimized vectors as part of vaccine strategies against HIV.

The animal model used here is considered one of the closest to HIV-1 infection in humans, since SIV<sub>mac251</sub>-infected macaques develop high primary viremia and a chronic active infection with high plasma virus loads, which leads to immunodeficiency and death within a half year to 3 years in the

majority of the animals. As in human populations, rapid and slow disease progressors are observed. This model has not been used extensively for efficacy trials of DNA-only vaccination, and some reported trials showed little or no evidence of long-lasting vaccine protection. Many studies involving DNA vaccination have been performed using the SHIV challenge model, especially challenge with the pathogenic SHIV89.6P. Infection by this virus leads to a rapid disease progression and severe depletion of CD4<sup>+</sup> cells. This is in contrast to SIV<sub>mac251</sub> in rhesus macaques and HIV-1 in humans, where disease progression and lymphocyte depletion is a much slower process. SHIV89.6P infects different subset of cells due to its CXCR4 tropism, compared to primarily CCR5-tropic HIV-1 in humans (48). It can be argued that SIV<sub>mac251</sub> challenge reflects more accurately the chronic active disease caused by CCR5-tropic HIV-1 in humans.

Several groups conducting DNA vaccine studies using SIV challenge stocks have concluded that there is minimal or no long-lasting benefit of DNA-only vaccination (5, 17, 26, 37, 44). In contrast, our study provides evidence for control of viremia after challenge with highly pathogenic SIV<sub>mac251</sub>. In agreement with other reports showing that the containment of viremia was associated with an increase in T-cell-specific responses (2, 5, 24), we find a strong correlation between the Gag-specific or Env-specific IFN- $\gamma$  immune responses and the ability to control viremia in the vaccinated animals.

Recently, the immunogenicity of the DNA vaccine regimens has been improved by different kinds of adjuvants including genetic adjuvants and different delivery methods (50, 56, 61, 63). Although the efficacy of several novel techniques in controlling a pathogenic virus challenge is yet to be proven, Barouch and coworkers reported that seven of eight animals receiving DNA together with IL-2 immunoglobulin fusion pro-

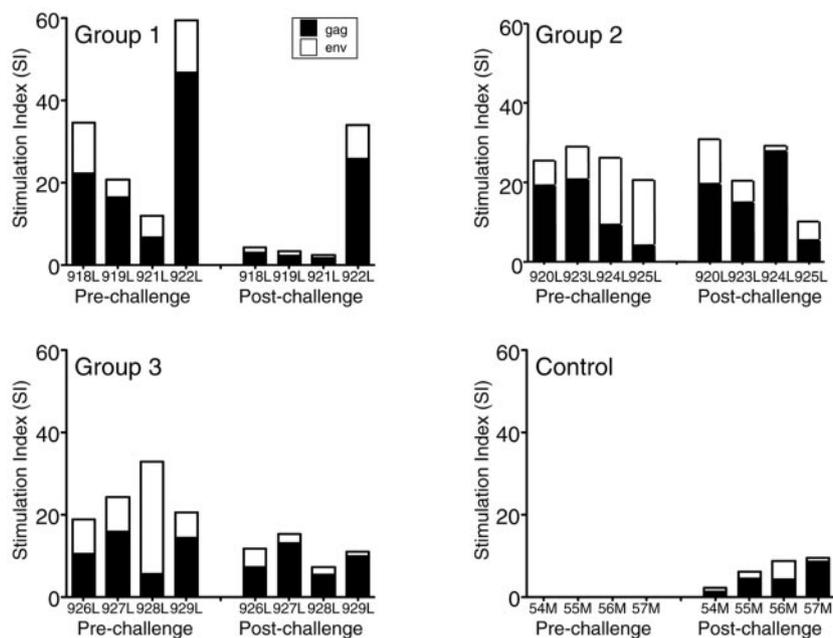


FIG. 5. SIV-specific T-helper responses. Pre- and postchallenge peak lymphoproliferative responses to Gag and Env of the individual macaques in the four groups, after stimulation with purified proteins as detailed in Materials and Methods. Solid bars, Gag severalfold stimulation over control; open bars, Env severalfold stimulation.

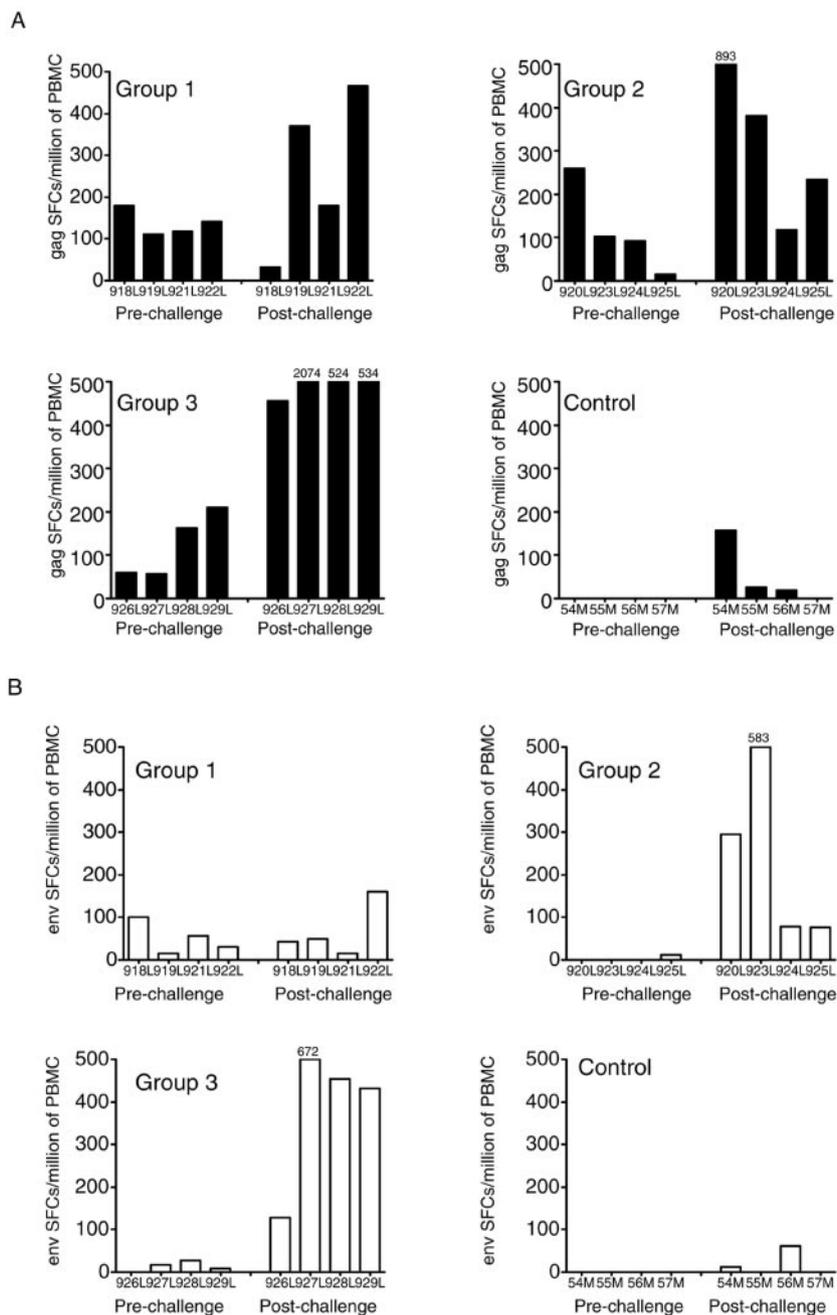


FIG. 6. CD8 immune responses. (A) Peak ELISPOT responses to SIV Gag peptides (spot-forming cells [SFCs] per 10<sup>6</sup> PBMCs). Prechallenge and postchallenge peak values are shown for the individual animals. Numbers indicate the ELISPOT values for samples higher than 500 SFC/million PBMCs. (B) Peak ELISPOT responses to SIV Env peptides. Numbers indicate the ELISPOT values for samples higher than 500 SFC/million PBMCs.

tein controlled SHIV challenge to levels below detection (5). Similarly, Shiver and coworkers established that three of three monkeys immunized with DNA and CRL1005 as the adjuvant controlled SHIV to levels below detection (61). In both studies, the frequencies of prechallenge antiviral T cells in animals receiving DNA plus adjuvant were higher than in those receiving DNA alone.

Studies using HIV DNA vectors in human clinical trials include both prophylactic and therapeutic vaccines (10). Vac-

cine safety has been achieved in phase I studies, and no significant adverse effects were reported (39, 66). If safe and effective, DNA vectors for immunization may show several advantages, including the flexibility to combine many different antigens and different forms of the same antigen, as shown herein. They can also be combined with cytokines and many other molecular adjuvants, which have been shown to further enhance DNA vaccination, such as IL-2, IL-12, and IL-15 (3, 4, 6, 8, 11, 27–32, 34, 49, 73, 74). In addition, they can be deliv-

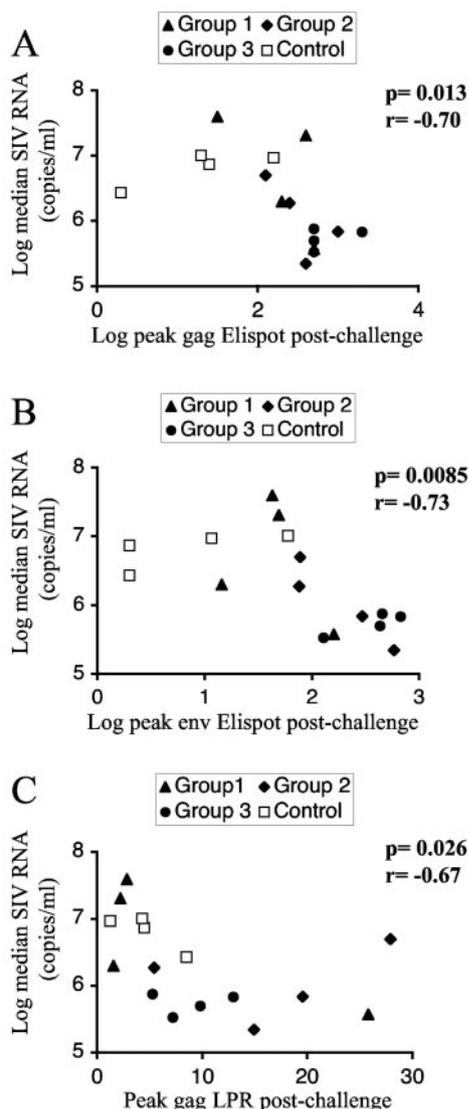


FIG. 7. Correlation of immune responses to viremia. Correlations between chronic virus loads expressed as log median SIV RNA (copies per ml of plasma) and log peak gag ELISPOT (A), log peak Env ELISPOT (B), and peak gag LPR (C) results. Animals in the different groups are indicated by different symbols. *P* values and Spearman *r* correlation coefficients are given.

ered many times in anamnestic immunizations without the limitations of the viral vectors, which elicit strong immune responses to the vector.

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