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# Dose-dependent inhibition of Gag cellular immunity by Env in SIV/HIV DNA vaccinated macaques

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**Abbreviations:** IM, intramuscular; EP, electroporation; IFN-γ, interferon gamma; bAb, binding antibody

The induction of a balanced immune response targeting the major structural proteins, Gag and Env of HIV, is important for the development of an efficacious vaccine. The use of DNA plasmids expressing different antigens offers the opportunity to test in a controlled manner the influence of different vaccine components on the magnitude and distribution of the vaccine-induced cellular and humoral immune responses. Here, we show that increasing amounts of env DNA results in greatly enhanced Env antibody titers without significantly affecting the levels of anti-Env cellular immune responses. Co-immunization with Env protein further increased antibody levels, indicating that vaccination with DNA only is not sufficient for eliciting maximal humoral responses against Env. In contrast, under high env:gag DNA plasmid ratio, the development of Gag cellular responses was significantly reduced by either SIV or HIV Env, whereas Gag humoral responses were not affected. Our data indicate that a balanced ratio of the 2 key HIV/SIV vaccine components, Gag and Env, is important to avoid immunological interference and to achieve both maximal humoral responses against Env to prevent virus acquisition and maximal cytotoxic T cell responses against Gag to prevent virus spread.

## Introduction

An ideal anti-HIV vaccine should induce effective humoral immunity that disseminate into mucosal sites and is able to prevent infection, and as a second line of defense, multifunctional cytotoxic T cells able to destroy infected cells. We, and others have been working on the development of DNA-based vaccine regimens, which represent an attractive vaccine platform due to its simplicity, versatility and relative ease of manufacturing. To maximize the efficacy of DNA as vaccine platform several components need to be considered including the use of plasmid backbone optimized for efficient replication in bacteria, potent promoters (i.e., human CMV, simian CMV) and polyadenylation signals (i.e., BGH, SV40) and the use of RNA/codon optimized inserts taking into consideration also optimization of the trafficking of the proteins (reviewed Refs. 1-3). Many labs found that inclusion of cytokine or chemokine DNAs as immunological adjuvants augment the vaccine-induced immune responses. In particular, the use of IL-12 was shown to increase the levels of antigen-specific cytotoxic T cells and humoral responses in mice, macaques as well as in the HVTN 080 human immunogenicity trial,[4-14] demonstrating translation from this basic observation from mice to macaques to humans. In addition to optimizing the vaccine components, different DNA delivery systems have been tested including intramuscular (IM) injection with needle and syringe, IM delivery followed by *in vivo* electroporation (IM/EP); skin or intradermal electroporation, gene gun or biojector, liposome delivery with Vaxfectin®, and Dermavir (reviewed Refs. 1-3,15,16 and referenced therein).

In this report, we used the IM/EP delivery of HIV and SIV DNA in rhesus macaques with 6 different vaccine regimens to maximize the induction of humoral and cellular immune



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responses. We show that the ratio of env to gag DNA in the vaccine mixture is critical for the balanced induction of humoral and cellular immune responses.

## Results

### Comparison of SIV and HIV DNA vaccine platforms

We have performed several vaccine studies in macaques using mixtures consisting of SIV gag and SIV or HIV env DNA that also included DNA expressing rhesus macaque IL-12 (rmIL-12) as molecular adjuvant. The DNAs were administered via the intramuscular (IM) injection followed by *in vivo* electroporation. The molecular ratio of env:gag DNAs varied from 1:1 to 3:1, in an effort to enhance the magnitude of the Env-specific humoral responses. In this report, we present a cross-study comparison of immune responses from macaques receiving the same SIV gag DNA combined with either SIV env DNA (groups 1–3) or with HIV env DNA (groups 4–6), as described in **Table 1**.

The animals of group 1 (N = 16) received SIV env and SIV gag DNA at a 1:1 ratio (0.5 mg and 0.5 mg, respectively). The animals of group 2 (N = 4) and group 3 (N = 16) received SIV env and SIV gag DNA at a 3:1 DNA ratio (3 mg and 1 mg, respectively). The animals of group 3 were also co-immunized with purified adjuvanted SIV Env protein at the same site. Similar to these studies, we compared DNA combinations that included HIV env and SIV gag at different molecular ratios. Group 4 animals (N = 6) received HIV env and SIV gag DNA at a 1:1 DNA ratio (1 mg and 1 mg, respectively). Group 5 (N = 9) and group 6 (N = 25) animals received HIV env and SIV gag DNA at a 3:1 DNA ratio (3 mg and 1 mg, respectively). Similar to group 3, the animals of group 6 were co-immunized with purified adjuvanted protein (HIV-1 gp120). We analyzed humoral (**Fig. 1**) and cellular (**Fig. 2**) responses 2 weeks after the 2nd vaccination and compared the immune responses induced by the different vaccine regimens.

### Augmentation of Env antibody titers by increasing env DNA dose

To compare the humoral responses (**Fig. 1**), we measured binding antibody (bAb) titers to SIVmac251 Env and to SIV $p27^{gag}$ by standard ELISA (**Fig. 1A, B**, respectively). We found that increasing the amount of SIV env DNA in the vaccine led to a significant augmentation of the bAb titers to SIV mac251 Env (group 1, mean titer 2.5 log and group 2, mean titer 4.4 log; **Fig. 1A**). These data demonstrate that the 0.5 mg dose did not maximize bAb responses and that increasing the env DNA dose achieved significantly higher bAb levels. We also compared the responses to those obtained after inclusion of Env protein in the vaccine (group 3). In this co-immunization protocol the adjuvanted protein is administered into the same muscle following the DNA delivery. Inclusion of gp120 Env protein led to a further significant increase in humoral responses (group 3, mean titer ~6 log). These data show that the env DNA-only vaccine, even including a higher dose of env DNA, did not induce maximal bAb responses. Inclusion of gp120 protein was necessary to maximize the development of humoral responses. We previously reported that inclusion of protein using a molecular env:gag DNA ratio of 1:1 resulted in great increase of Env humoral responses.[17-19]

We also compared the SIV $p27^{gag}$ humoral responses and found no differences in bAb titers among the different vaccine regimens (range of titers ~5–6 log among the 3 groups; **Fig. 1B**) and showed that the development of Gag bAb was not affected by the increased amounts of env DNA or the inclusion of Env protein in the vaccine.

Similar observations were made using a vaccine that included SIV gag DNA and HIV env DNA (**Fig. 1C and D**). Increasing the molecular ratio of env:gag DNA to 3:1 led to significant augmentation of HIV IIIB Env bAb responses (group 4 mean titer ~3.5 log; group 5 mean titer 5.3 log; **Fig. 1C**). Thus, like the SIV env plus SIV gag DNA vaccine, we found significant augmentation of the Env bAb titers upon increasing the env DNA amount in the vaccine. Inclusion of purified SIV Env protein in the vaccine further elevated the Env bAb titer significantly (group 6; mean titer ~6.5 log) (**Fig. 1C**). Thus, for both the SIV and HIV DNA-only vaccine regimens the magnitude of the Env humoral responses could be increased using higher amounts of env DNA. Even under these conditions, both vaccines failed to reach maximal bAb levels, which were achieved however upon inclusion of Env protein in the co-immunization protocol. As noted for the SIV env-SIV gag vaccine groups, similar levels of Gag humoral responses were induced by these vaccine regimens (mean titer of ~5 log among the 6 groups) (**Fig. 1D**).

In summary, we concluded that the Env-specific humoral responses induced by the DNA vaccine platform can be improved by increasing the env DNA dose for both SIV and

**Table 1.** Vaccination overview

| | | Molecular ratio | DNA dose (in mg) | | | | Env protein | |
|---|---|---|---|---|---|---|---|---|
| Group | N | env:gag DNA | SIV gag | SIV env | HIV env | Env in DNA vaccine | SIV | HIV |
| 1 | 16 | 1:1 | 0.5 | 0.5 | | gp160 | none | |
| 2 | 4 | 3:1 | 1 | 3 | | gp160 | none | |
| 3 | 16 | 3:1 | 1 | 3 | | gp160 (N = 4) gp160 and gp120 (N = 12) | 100 μg | |
| 4 | 6 | 1:1 | 1 | | 1 | gp160 and gp140 | | none |
| 5 | 9 | 3:1 | 1 | | 3 | gp160, gp140 and gp120 | | none |
| 6 | 25 | 3:1 | 1 | | 3 | gp160 and gp120 (N = 9)gp120 (N = 16) | | 100 μg |

HIV resulting in significantly higher Env humoral responses without affecting the humoral responses to Gag.

**High molecular ratio of env to gag DNA impaired the development of SIV Gag-specific T cell responses**

Next, we analyzed the cellular responses in these vaccine groups (**Fig. 2**). We found that increasing the dose of SIV or HIV Env DNA did not affect the levels of Env-specific IFN-$\gamma^+$ T cell immune responses (**Fig. 2A, C**, respectively). The groups that received SIV Env DNA showed a median response of 0.3–0.43% IFN-$\gamma^+$ T cells (**Fig. 2A**), while the HIV env group showed a median response of 0.1–0.19 % IFN-$\gamma^+$ T cells. These data demonstrate that the 0.5 mg env DNA dose induced the highest cellular responses, albeit this dose was suboptimal to inducing maximal humoral responses (**Fig. 1A, C**, respectively).

Unexpectedly, we found that using a higher dose of SIV env DNA led to significant, ~4-fold, reduction in Gag-specific IFN-$\gamma^+$ T cell levels (**Fig. 2B**; group 1, mean ~0.4%; group 2, ~0.1% of Gag-specific T cells). We noted that the response measured from animals of group 3 was further slightly reduced upon inclusion of adjuvanted protein (compare group 2 and group 3). The animals enrolled in these studies have diverse MHC haplotypes and there was no apparent association found between MHC and the observed vaccine responses. Thus, these data indicated that SIV Env potently suppressed the development of Gag-specific T cell immunity while not affecting the Gag humoral responses.

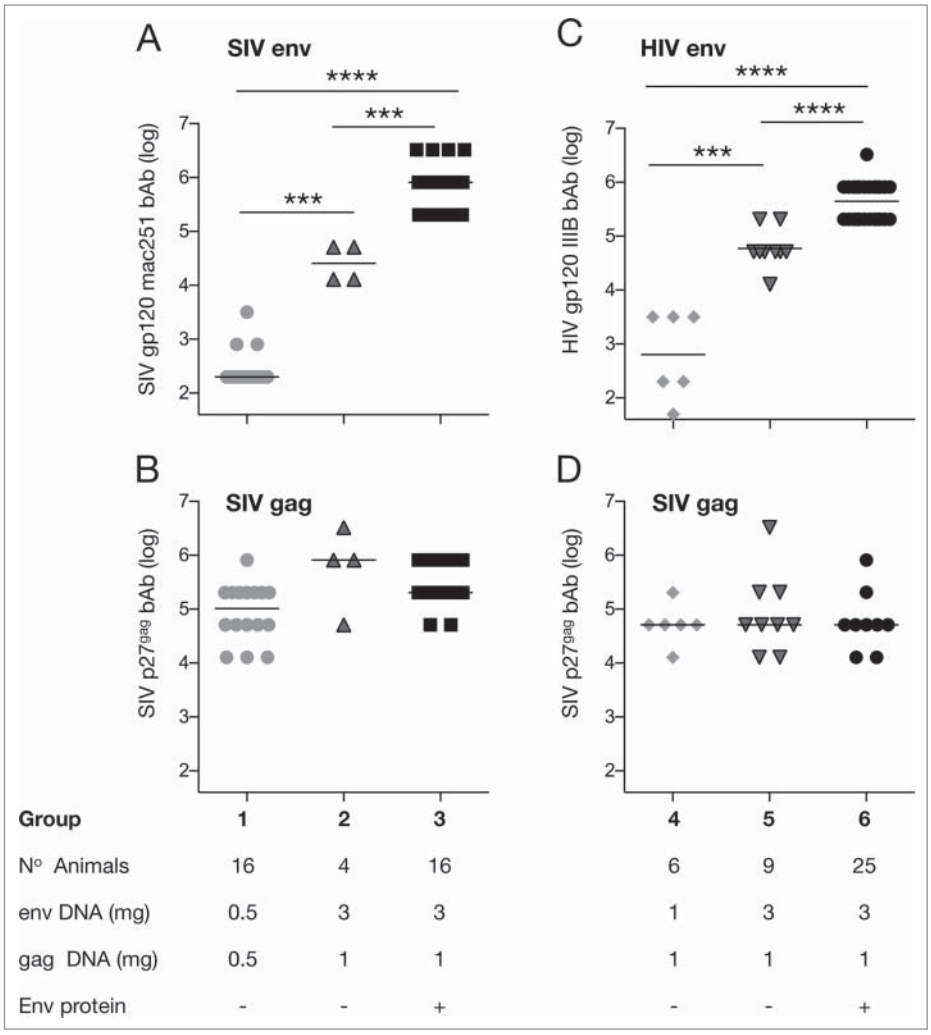


**Figure 1.** Binding antibody titers to SIV Gag and SIV or HIV Env among the different vaccine groups; (**A-D**). Endpoint bAb titers (log) to SIVmac251 gp120 Env (**A**), $p27^{gag}$ (**B and D**) and HIV IIIB gp120 Env (**C**) were measured 2 weeks after the 2nd vaccination. Asterisks designate statistically significant differences between groups (*** $P < 0.001$ and **** $P < 0.0001$) using the non-parametric 2-tailed t-test (Mann-Whitney). Median values are indicated.

To further investigate this dichotomy in humoral versus cellular responses to Gag and Env and to understand whether this is restricted to SIV Env, we also analyzed the cellular immune responses in groups 4 to 6, which received a combination of the same SIV gag DNA and HIV env DNA. A higher dose of HIV env DNA also led to ~2-fold reduction in Gag-specific T cell levels (**Fig. 2D**) comparing groups 4 and 5, although similar levels of Gag humoral responses were found (**Fig. 1D**). Upon co-immunization with HIV Env protein (group 6) a further reduction in Gag-specific T cells was noticed (**Fig. 2D**).

In conclusion, a vaccine consisting of high molecular ratio of HIV or SIV env DNA to gag DNA is responsible for the potent negative effect on the development of Gag-specific cellular immune responses. These data suggest a dominant immunological interference of the SIV as well as the HIV Env epitopes resulting in the suppression of the development of robust Gag-specific cellular responses, while not affecting the development of humoral responses.

## Discussion

In this report, we compared SIV/HIV DNA vaccine mixtures containing different molecular ratios of env and gag DNA with the goal to maximize the induction of humoral and cellular immunity to Env and Gag in rhesus macaques. In this cross-study comparison, we found that increasing the env DNA dose significantly contributed toward maximizing Env humoral responses. We also noted that a vaccine combining high dose of env DNA and Env protein, for both SIV and HIV, led to further increase in Env antibody titers, suggesting that DNA only does

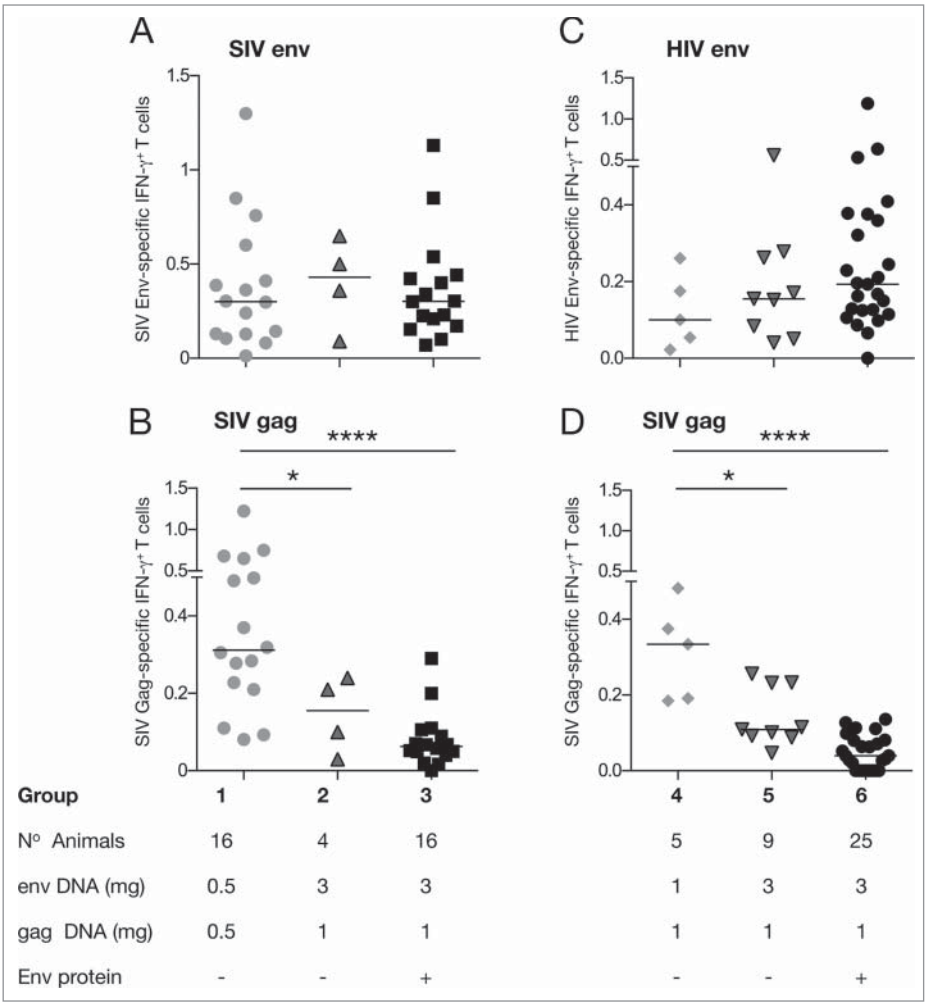


**Figure 2.** Cellular immune responses in the different vaccine groups. Antigen-specific T cell responses were measured in PBMC 2 weeks after the 2nd vaccination. PBMC were stimulated with peptide pools covering SIV gp160 mac239 (**A**), SIV p39gag (**B and D**) or HIV gp120 PTE (**C**). The frequency of the antigen-specific T cells producing IFN-γ is shown. Asterisks designate statistically significant differences between groups (*$P < 0.05$, ***$P < 0.001$ and ****$P < 0.0001$) using the non-parametric 2-tailed t-test (Mann-Whitney). Median values are indicated. Note that only 5 animals from group 4 (panels **C and D**) were analyzed for cellular responses.

not fully maximize such responses. We previously reported that inclusion of protein in the vaccine also had the advantage of better mucosal dissemination of the humoral responses,[17,19] which could also be the result of higher antibody responses. On the other hand, no changes in the levels of Env-specific cellular responses were noted under these conditions, suggesting that the responses reached with the 0.5 mg DNA dose could not be further increased using a 3 mg dose. Unexpectedly, we found that a higher Env DNA dose led to a significant reduction in Gag-specific cellular responses, while not affecting the levels of the Gag humoral responses. These findings could also explain the failure to detect Gag cellular responses in a clinical trial, where a 3:1 ratio of env:gag DNA was used as vaccine prime[20,21] and it is possible that this contributed to the efficacy failure of the phase 2 trial.[22] The great loss of cellular responses to Gag is of concern because several lines of evidence support an important role of these responses in control of viremia. We had reported that Gag cellular responses significantly contributed to the control of SIVsmE660 upon challenge of vaccinated macaques.[19] Similarly, several studies in long-term nonprogressors demonstrated unequivocally an association of Gag cellular immune responses and control of HIV.[23-31] Immune interference of Env and Gag has been previously reported in DNA vaccinated mice. Toapanta et al[32] reported that HIV Env but not SIV or EIAV Env interfered with the development of HIV Gag responses. On the other hand, Bockl and Wagner[33] reported that co-delivery of HIV env with HIV gag DNA led to largely abrogated Gag-specific T cell responses and that this effect was linked to the H-2(d) T cell epitope V11V in Env. Both studies share the overall conclusion that there is an immunological interference between Env and Gag in Balb/c mice. Bockl and Wagner[33] further observed that changing the ratio of the gag and env DNA in the vaccine mixture and the spatial or temporal separation of the Gag and Env antigens in the vaccination induced more balanced T cell responses. Overall, this study in mice is in good agreement with our data from DNA vaccinated macaques. Together these findings show that there is strong immunological interference between Env and Gag epitopes, probably linked to the needed association of peptide and MHC class I, which may explain why the interference does not affect humoral responses.

Similar to the Env interference in the development of Gag cellular immune responses, we have previously reported that there is also immunological interference among epitopes within Gag, with the less conserved epitopes suppressing the responses to the conserved epitopes.[34-36] A vaccine strategy priming with conserved epitopes followed by full-length Gag boost was shown to induce robust cellular and humoral responses to the conserved epitopes. This approach altered the immunodominance hierarchy, magnitude and breadth of cellular responses targeting Gag epitopes. In addition to playing a role in controlling viremia in SIV infected macaques and in HIV infected persons,[19,23-31] GagPol-specific $CD4^+$ T-cells were also shown to increase the development of Env-specific humoral response in mice, suggesting a cross-talk between immune responses and intrastructural help for Env-specific B cells responses.[37] Taken together these results indicate that the balanced ratio of the vaccine components is critical for the induction of

optimal cellular and humoral immune responses to Gag and Env. The use of DNA as vaccine platform offers the flexibility to adjust vaccine components to maximize their efficacy.

## Materials and Methods

### Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Advanced BioScience Laboratories, Inc.. Institutional Animal Care and Use Committee (OLAW assurance number A3467-01 and USDA Certificate number 51-R-0059) approved the animal protocol. Indian rhesus macaques were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International at the Advanced BioScience Laboratories, Inc.. (Rockville, MD). Some of the animals were housed at the NIH facility (Bldg 14). The animals were vaccinated as part of other studies (AUP417,[19] AUP490,[38,39] AUP522, and AUP469). The data presented in this work were not previously reported.

### Vaccine

SIV gag DNA vectors included p57$^{gag}$ (plasmid 206S) and the MCP3p39$^{gag}$ (plasmid 209S). The SIV env DNAs expressed gp160 [group 1, 2 and 4 animals of group 3[19,38,39]] or gp160 and gp120 (12 animals in group 3). The HIV env DNAs expressed clade B and clade C gp160 and gp140 (group 4 and 5) and clade B gp120 (group 5). For group 6, the plasmids expressed gp160 and gp120 (N = 9) or only gp120 (N = 16) and no difference in gp120 PTE-specific T cell responses was found among these animals

All HIV and SIV plasmids express RNA/codon-optimized SIV and HIV genes from the CMV promoter using the vector CMVkan.[40] The SIV gag and SIV env expressing plasmids were described elsewhere.[38,41,42] Optimized rhesus IL-12 DNA[6] was included as immunological vaccine adjuvant. Endotoxin-free DNAs (Qiagen) were prepared according to the manufacturer's protocol. HEK293 grown SIV or HIV Env proteins were lectin-column purified and 100 μg Env protein adjuvanted with 10 μg EM-005[17,18] was included in some of the vaccines (groups 3 and 6).

### Vaccination

The macaques received SIV gag DNA and SIV or HIV env DNA as indicated in **Table 1**. All DNA vaccine mixtures contained 0.1 μg of macaque IL-12 DNA and were formulated in 0.6 ml of sterile water (Hospira, Inc.., Lake Forest, IL). The DNA vaccine mixtures were delivered via intramuscular (IM) injection at 2 different sites (0.3 ml each site) followed by *in vivo* electroporation using the Elgen 1000 device (Inovio, Pharmaceuticals, Inc., Blue Bell, PA) as reported previously.[43] Blood was collected 2 weeks after the 2$^{nd}$ vaccination. SIV/HIV-specific cellular immune responses were measured in PBMC and humoral responses were measured in the plasma.

### Cellular responses

Cellular immune responses were measured by intracellular cytokine staining assay using cryopreserved PBMCs stimulated with peptide pools spanning SIV mac239 (gp160), SIVmac239 gag (p39$^{gag}$) and using the most frequent potential HIV T cell epitopes (PTE) Env pool (Cat #11551, AIDS Research and Reference Reagent Program, Germantown, MD) using 15-mer peptides overlapping by 11 AA (final concentration of 1 μg/ml). Immunostaining and flow cytometric analysis was performed as described.[19,39] For all the analyzed vaccinated animals a PBMC sample cultured without peptide stimulation was included as negative control. The frequency of IFN-$\gamma^+$ T cells is reported after subtracting the background value obtained in the absence of peptides from that of peptide-stimulated samples. Samples were considered positive when the frequency of IFN-$\gamma^+$ T cells in the peptide-stimulated samples was at least 2-fold higher than the frequency obtained in the unstimulated medium-only control sample.

### Humoral responses

Binding antibodies to SIVmac251 p27$^{gag}$, SIVmac251 Env and HIV IIIB Env were monitored on 4-fold serial dilutions of plasma samples by standard ELISAs (Advanced Bioscience Laboratory, Rockville, MD). The mean optical absorbance ($A_{450}$) plus 3 standard deviations obtained from non-immune rhesus macaque plasma samples were applied as cut-off for the assays.

### Disclosure of Potential Conflicts of Interest

GNP and BKF are inventors on US Government-owned patents and patent applications related to DNA vaccines and gene expression optimization. SR is a founder and shareholder in Immune Design Corp., which has certain rights to the adjuvant used in this study under license agreement with IDRI. NYS is a full time employee of Inovio Pharmaceuticals and as such receives compensation in the form of salary and stock options. There are no further patents, products in development or marketed products to declare.


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