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Comparison of intradermal and intramuscular delivery followed by in vivo electroporation of SIV Env DNA in macaques

Viraj Kulkarni¹, Margherita Rosati², Jenifer Bear¹, Guy R Pilkington¹, Rashmi Jalah¹, Cristina Bergamaschi¹, Ashish K Singh², Candido Alicea¹, Bhabadeb Chowdhury², Gen-Mu Zhang^{1,2}, Eun-Young Kim³, Steven M Wolinsky³, Wensheng Huang⁴, Yongjun Guan⁴, Celia LaBranche⁵, David C Montefiori⁵, Kate E Broderick⁶, Niranjana Y Sardesai⁶, Antonio Valentin², Barbara K Felber^{1,*}, and George N Pavlakis^{2,*}

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Abbreviations: EP, electroporation; ID, intradermal; IM, intramuscular; GzmB, GranzymeB; TM, transitional memory; EM, effector memory

A panel of SIVmac251 transmitted Env sequences were tested for expression, function and immunogenicity in mice and macaques. The immunogenicity of a DNA vaccine cocktail expressing SIVmac239 and three transmitted SIVmac251 Env sequences was evaluated upon intradermal or intramuscular injection followed by in vivo electroporation in macaques using sequential vaccination of gp160, gp120 and gp140 expressing DNAs. Both intradermal and intramuscular vaccination regimens using the gp160 expression plasmids induced robust humoral immune responses, which further improved using the gp120 expressing DNAs. The responses showed durability of binding and neutralizing antibody titers and high avidity for >1 y. The intradermal DNA delivery regimen induced higher cross-reactive responses able to neutralize the heterologous tier 1B-like SIVsmE660_CG7V. Analysis of cellular immune responses showed induction of Env-specific memory responses and cytotoxic granzyme B⁺ T cells in both vaccine groups, although the magnitude of the responses were ~10x higher in the intramuscular/electroporation group. The cellular responses induced by both regimens were long lasting and could be detected ~1 y after the last vaccination. These data show that both DNA delivery methods are able to induce robust and durable immune responses in macaques.

Introduction

DNA is a compelling vaccine vehicle because of its simplicity, scalability, and lack of immunity against the vector. Different DNA delivery methods are being tested, including intramuscular DNA delivery by in vivo electroporation (IM/EP) (reviewed in refs. 1–3); Dermavir,⁴ liposome delivery with Vaxfectin[®],⁵ biojector or intradermal EP (ID/EP)^{6,7} or gene gun.⁸

The development of intramuscular DNA injection followed by in vivo electroporation^{9–14} brought a significant change in the efficiency of DNA delivery, especially to higher mammals like macaques and humans. In the field of HIV/SIV vaccine, several groups showed that IM/EP delivery led to increased immune responses (several fold over those induced by conventional needle and syringe).^{15–18} The magnitude of the DNA induced immune responses could be further augmented by the inclusion of IL-12

DNA as adjuvant in mice and macaques.^{18–25} Importantly, in macaques, the combination of optimized DNA delivery by IM/EP and the inclusion of IL-12 DNA led to robust cellular and humoral immune responses,^{15,18,24–28} which resulted not only in quantitative improvement,^{18,24,25} but also in improved quality of the responses, e.g., increase of antigen-specific cytotoxic T cells and greater breadth of the neutralizing antibody (NAb) responses.²⁴ Similarly, ID/EP delivery of DNA vaccines yielded improved humoral immune responses and led to protection from challenge as judged by lethality, viremia, and morbidity in two independent macaque models testing a multivalent small pox vaccine and a multivalent avian influenza (A/H5N1 vaccine).^{29,30}

DNA as the only vaccine component had been considered poorly immunogenic in humans, until recent results demonstrated that in vivo electroporation is more efficient (more responders, and higher, longer-lasting immunity) than DNA

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delivery by needle/syringe also in man,³¹ corroborating the findings of macaque studies from us and others. Another trial, using HIV gag DNA and IM/EP DNA delivery showed that inclusion of IL-12 DNA is advantageous, resulting in both increased frequency of responders and level of Gag-specific immunity.³² Together, these studies showed that the findings obtained in the macaque model could be well translated to humans, which is an encouraging step in the development of a DNA-based vaccine against AIDS.

This report is a pilot study in macaques to compare a SIVmac251 derived DNA vaccine based on the selection of transmitted Env clones with varying sequences, and delivered by two different routes (intradermal and intramuscular) followed by *in vivo* electroporation. The vaccine candidate and the delivery routes were designed to test whether they might induce broader immunity compared with SIVmac239. Both vaccination regimens induced potent long-lasting immune responses with IM/EP vaccination inducing higher levels of cellular responses, and ID/EP vaccination inducing broader humoral responses.

Results

Identification and characterization of transmitted SIVmac251 Env sequences. The Env sequence from the infectious molecular clone SIVmac239 has been frequently used as antigen to test immunogenicity and potency of SIV vaccines in macaques. We wished to test the hypothesis that a selection of transmitted Env clones with varying sequences might induce broader immunity compared with SIVmac239 Env. Toward this goal, we identified full-length Env sequences present in the plasma early during the peak of primary viremia from macaques infected via the mucosal route with low dose SIVmac251. To obtain a more comprehensive picture of the virus variants present in the SIVmac251 stock and the infected animals, we also interrogated by Single Genome Amplification (SGA) and sequencing the variable regions V1&V2 region (AA 53–260 following the numbering of SIVmac239 containing V1 [AA 113–168] and V2 [AA 168–211] **Fig. 1A and B**), since V1 is known to encompass most of the diversity of the SIV Env. We noted that, despite the diversity of the stock (ref. 33 and our unpublished analysis), viruses sharing a narrow selection of Env sequences were found to replicate in the majority of the analyzed animals. The Env sequences were closely related to those found in the inoculum and represented a single inferred founder virus in 7 of 9 rhesus macaques.

From the collection of sequences, we selected Env sequences with distinct V1-V2 regions for further analysis. Comparison of SIVmac239 to these sequences revealed numerous changes throughout Env in addition to the highly variable V1 region (AA 113–168) (**Fig. 1A**). A cartoon of SIVmac239 indicating the location of the variable (V) and constant (C) regions is shown in **Figure 1B**. In contrast to V1 and the variable V4 (AA 402–432), comparison to mac239 Env showed that the transmitted sequences share several changes to the same AA in V2, V3 (AA 311–344) as well as in C1–C4 and they did not show changes in V5 (AA 459–484) and C5, whereas numerous AA changes are found in gp41 (AA 526–879). Of these sequences, Env 35014_7,

35014 (M766) and mac251_9 share a characteristic V1-V2 region found in several of our infected animals. Of note, the Env sequence 35014 was independently identified by B. Keele et al.³³ (GenBank accession #JQ086004) and also named M766, and is also referred to with this name in this report. Env mac251_15 and mac251_2 contain a different V1-V2 region. In addition, we included the previously reported transmitted Env CR2.RU.3R1³⁴ as a sequence with different V1V2 from a macaque infected with a different SIVmac251 stock.

The phylogenetic tree analysis shows the relation among the selected SIVmac251 Env sequences, which share 4.7–6.8% AA difference with SIVmac239 (**Fig. 1C**). We also include two heterologous Env sequences from SIVsmE660 (CG7V and CG7G)³⁴ which differ by ~20% from the SIVmac251 sequences and are used in neutralization assays (see **Fig. 2**). We generated a collection of vectors producing gp160, the trimeric gp140 and the soluble gp120 of the different Envs from expression-optimized cDNAs, which were cloned into the mammalian expression vector CMVkan (**Table 1**). The Env plasmids were tested for expression upon transient transfection and Western immunoblot assay (**Fig. 3A**). The newly identified SIVmac251 Env proteins were expressed at levels similar to mac239 Env. Two of the Envs (mac251_2 and mac251_9) showed poor cleavage of gp160, with no production of gp41, although no apparent AA changes surrounding the furin cleavage site (AA 525) were noted. In addition to the gp160 Env protein, we also generated plasmids (**Fig. 3B**) expressing the trimeric gp140 (AA 1 to 686) lacking the membrane spanning domain (MSD) and the soluble gp120 (AA 1 to 525). **Figure 3C** shows that uncleaved gp140 and gp120 were efficiently secreted and accumulated in the extracellular compartment. Similar data were obtained for the other Env proteins (not shown) and the plasmids are listed in **Table 1**. The sequences were further tested for function using the pseudotype infection assay (**Fig. 3D**). All sequences produced functional pseudotyped virions, except for the 2 Env with processing defects.

To test the immunogenicity of the identified SIVmac251 Env sequences, groups of BALB/c mice were vaccinated with four different DNAs expressing gp160 from mac239, 251_15, 251_2, and 251_9 by the conventional needle and syringe intramuscular injection method (**Fig. 4A**). All Env vectors induced robust cellular immune responses with similar levels as SIVmac239 Env, including the 2 non-functional Env (mac251_2 and mac251_9) (**Fig. 4B**). Analysis of humoral immunity by ELISA showed the induction of robust bAb titers, except for the 2 Env which showed impaired gp160 processing (**Fig. 4C**). Together, these data led to the selection of 4 newly identified Env for the immunogenicity study in macaques (see below).

Production of SIVmac251 Env proteins from mammalian cells. Another goal of this work has been to select representative sequences able to efficiently produce SIV Env proteins in mammalian cells. We tested this by the generation and screening of stable overproducing HEK293 cells. Stable high producer HEK293 cell clones using *env* DNAs expressing the gp120 form of SIVmac251 35014 (M766) gp120 and the gp140 form of SIVmac251 35014_7 were generated. In addition, we also generated cell lines producing gp140 forms of SIVmac239 and

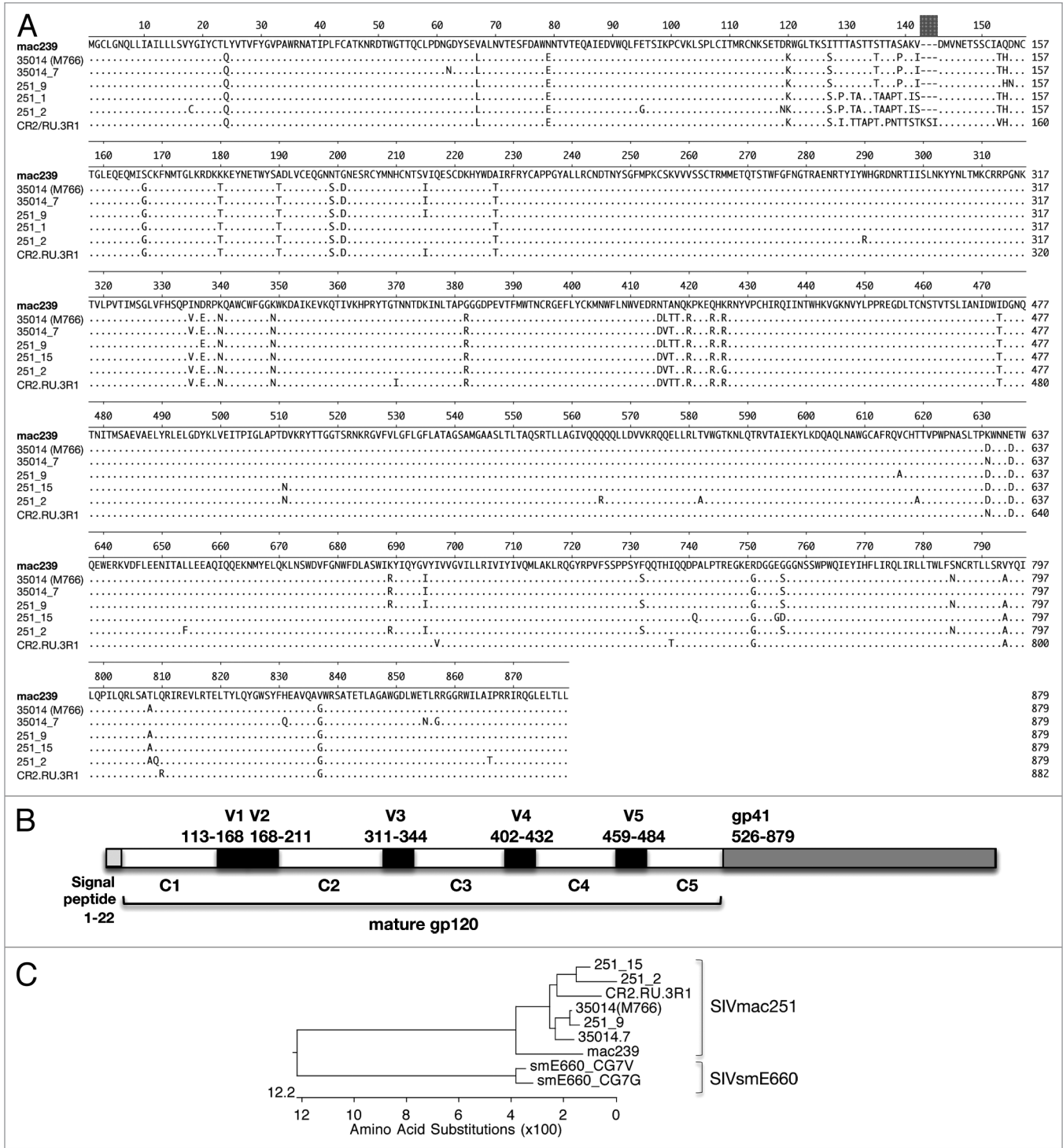


Figure 1. Amino acid alignment of transmitted SIVmac251 Env sequences. **(A)** The identified SIVmac251 sequences and the reported SIVmac251 sequence CR2.RU.3R1 are compared with SIVmac239. **(B)** Cartoon depicts the location of the signal peptide, the variable (V) and conserved (C) regions, and gp41 of SIVmac239. The numbering follows SIVmac239 sequence. **(C)** Phylogenetic tree analysis of the SIVmac239 and the different SIVmac251 sequences. The tree also included 2 SIVsmE660 env CG7G and CG7V that are used in the neutralization assays.

SIVsmE660_CG7V. Selected high producer cell clones were grown in serum-free media in a Hollow Fiber bioreactor that allows high-density growth in defined serum-free medium. Analysis of a 6 μ l aliquots from the supernatants of daily harvests (20 ml) was performed on gels stained with Coomassie

blue and, as a representative example, the production of 35014 (M766) gp120 Env overtime is shown (Fig. 5A). A strong band corresponding to secreted gp120 could be detected in addition to a ~60 kDa band corresponding to albumin, introduced from the seed culture as part of the complete culture medium that

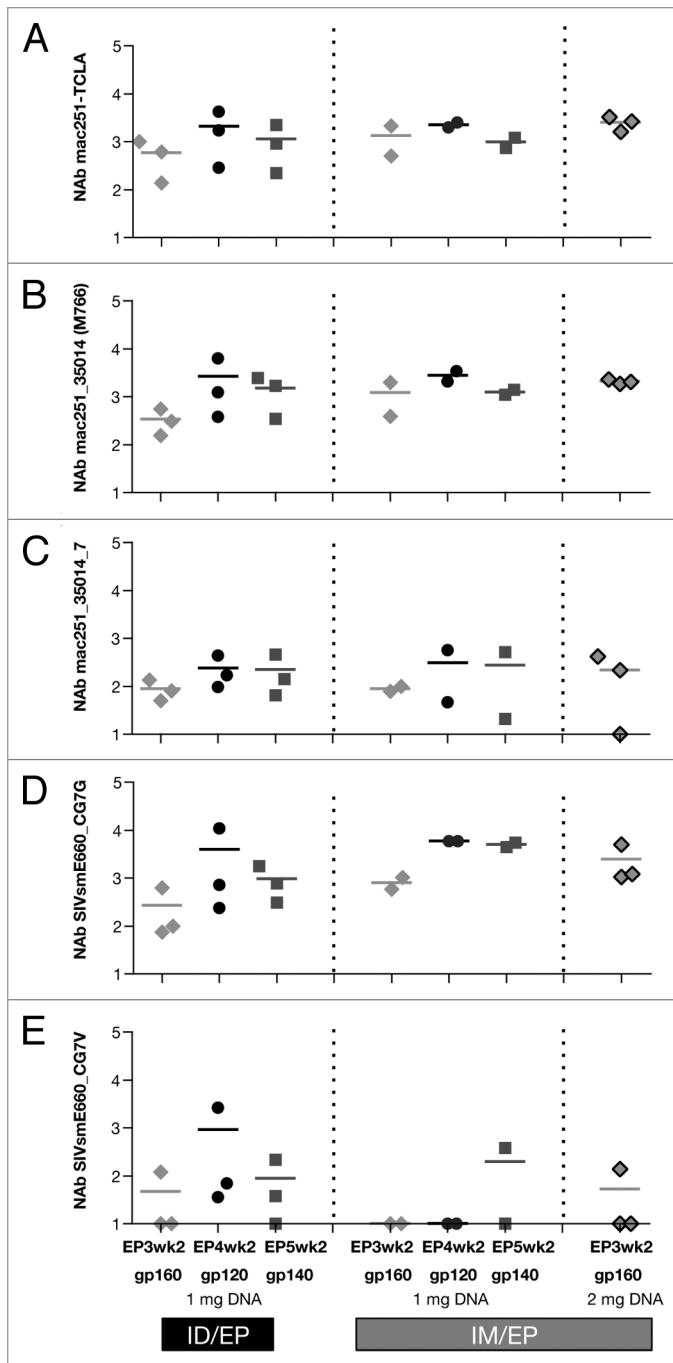


Figure 2. Induction of neutralizing antibody in macaques immunized via the intradermal (left panel) and the intramuscular (middle and right panels) route followed by EP. Neutralizing antibody titers were determined against SIVmac251-TCLA measured in M7-Luc cells (A) and against pseudotyped viruses containing the following Env: SIVmac251 35014 (M766) (B) and 35014_7 (C), the heterologous SIVsmE660 Env CG7G (D) and CG7V (E) were measured using the TZM-bl assay. The NAb measurements of a group of macaques which received 3 vaccinations of the same DNAs (total 2 mg) expressing the gp160 forms of Env via the IM/EP delivery is included (right panel). Neutralization titers shown are the log of the reciprocal dilution of sample that reduced the signal by 50% compared with virus in the absence of sample.

contained fetal calf serum. The culture medium was changed daily and typically after 7–10 d, albumin was no longer detectable. High level of Env production persisted for > 15 weeks of continuous cell growth. The estimated Env production is ~3 mg/20 ml/day using purified SIV gp120 Env protein as standard. Supernatants collected after the 1st week of culturing the cells in the Hollow Fiber bioreactor were enriched for Env and were used for protein isolation using standard lectin column purification. Similar stable daily production was obtained for the other Env proteins (not shown). After purification, the proteins were analyzed on non-denaturing gels and stained with Coomassie blue, which revealed high quality of the produced monomeric SIVmac 35014 (M766) gp120 as well as stable trimeric forms of SIVmac 35014_7 and SIVsmE660_CG7V gp140 Env (Fig. 5B).

Vaccination via intradermal/EP and intramuscular/EP route induces robust humoral immune responses. We compared the immunogenicity of a combination of SIV Env DNA in rhesus macaques using intradermal (ID) and intramuscular (IM) DNA delivery followed by in vivo electroporation. As vaccine, we followed a sequential immunization scheme (outlined in Fig. 6A) using a mixture of Env DNAs expressing gp160 (EP1–3), gp120 (EP4) or gp140 (EP5). Macaques received the same DNA vaccine (1 mg/animal) via intradermal/EP ($n = 3$) or intramuscular/EP ($n = 2$). Similar levels (reciprocal endpoint titer log 4.5–5) of SIVmac251 binding Ab (bAb) were found in the plasma (Fig. 6B) using the ID/EP or the IM/EP delivery method. The bAb showed similar longevity during the 11 weeks of follow-up (EP3wk2 to EP4) with a decline of < 0.5 log. Vaccination with DNAs expressing the secreted gp120 forms of Env increased the bAb endpoint titers in both groups (EP4wk2) to levels slightly (~0.5 log) higher than the levels obtained upon vaccination with DNAs expressing the gp160 forms of Env. This is likely due to the more efficient production of gp120 from these plasmids compared with the gp160 vectors (see Fig. 3C). Subsequent vaccination with plasmids expressing the trimeric gp140 showed similar bAb levels, likely due to the fact that maximal levels were obtained using the gp120 DNAs mixture. Together, these data show that both DNA delivery systems efficiently induced robust systemic binding Ab levels in the vaccinated macaques.

We added 3 animals to the IM/EP group, which were vaccinated with a 2 mg dose using the gp160 forms only of the same four Env DNAs administered separately (using a slightly different schedule with vaccinations at 0, 4, 12 weeks). These animals showed similar reciprocal endpoint bAb titers of ~log 5 (not shown) and allowed us to include the data from this group (after gp160 DNA vaccination) in this study. The responses from the 1 and 2 mg dose IM/EP groups trended to be higher (~1.5 log) compared those we reported from macaques ($n = 8$) which received 0.5 mg gp160 *env* DNA administered via IM/EP.²⁴ These data indicated that higher humoral responses could be obtained using the IM/EP delivery by increasing the *env* DNA dose, with 1 mg reflecting saturating levels in the rhesus macaque.

Intradermal DNA delivery induced more robust broadly neutralizing antibodies. We next tested the neutralization capability of the vaccine-induced Env antibodies (Fig. 2) induced upon ID/EP (Fig. 2, left panel) and IM/EP (Fig. 2, middle

Table 1. SIV Env and functional characteristics

SIV	Plasmid name	GenBank accession #	Optimized <i>env</i> DNA expression plasmids (plasmid code)			gp120/gp41 production from gp160 DNA	Function in pseudotype assay	Ability of Env to be neutralized (TZM-bl assay)	Evaluation of neutralizability of Env (TZM-bl assay)
			gp160	gp140	gp120				
mac251	mac239	M33262	99S	237S	173S	yes	yes	no	difficult to neutralize
	35014 (M766 ³⁴)	KF003433 (JQ086004 ³⁴)	221S	241S	246S	yes	yes	yes	tier 1A-like
	35014_7	KF003434	220S	234S	230S	yes	yes	yes	tier 1B-like
	251_9	KF003437	219S			poor gp41 cleavage	no		
	251_15	KF003436	217S	240S	229S	yes	yes	no	difficult to neutralize
	251_2	KF003435	218S			poor gp41 cleavage	no		
	CR2.RU.3R1	FJ578044 ³⁴	231S	242S	223S	yes	ND	no	difficult to neutralize
smE660	CG7G	FJ578381 ³⁴	251S	254S		yes	ND	yes	tier 1A-like
	CG7V	FJ578428 ³⁴	252S	255S		yes	ND	Yes (ID/EP group)	tier 1B-like

ND, not determined.

panel). We also added the results from the group that received IM/EP vaccination with the 2 mg DNA dose (Fig. 2, right panel) which shows similar data as the 1 mg group (Fig. 2, middle panel, compare gp160 DNA vaccination only). The neutralization capability was measured against a panel of SIV Env variants including the homologous T cell-adapted (TCLA) SIVmac251 (Fig. 2A), two transmitted SIVmac251 Env 35014 (M766) (Fig. 2B), 35014_7 (Fig. 2C) as well as mac251_15 (not shown) and CR2.RU.3R1 (not shown). Plasma samples collected 2 weeks after the gp160 (EP3wk2), gp120 (EP4wk2) and gp140 (EP5wk2) DNA vaccinations were analyzed. Overall, similar NAb titers were detected using ID/EP (left panel) and the IM/EP vaccination regimens (Fig. 2A–C) upon vaccination with different forms of Env (gp160, gp120, gp140).

We noted a distinct difference in neutralization of the transmitted Env 35014_7 (Fig. 2C) compared with Env_35014 (M766) (Fig. 2B), with the latter being more easy to neutralize (see also Table 1) as shown by the higher NAb titers measured in both vaccine groups. We noted this difference also upon analysis of macaques vaccinated with SIVmac239 Env only (unpublished observation). Thus, inclusion of 35014_7 Env in the vaccine mixture did not improve the ability to neutralize 35014_7 Env pseudotyped virions. It is noteworthy that the mature gp120 forms of 35014_7 and M766 differ by only 3 AA (1 AA lies in the N-terminal portion [C1], 2 AA lie in V4, while the remaining 8 AA changes lie in gp41; Fig. 1A). Interestingly, these 3 AA changes in gp120 generated an Env protein with profoundly different behavior in the TZM-bl neutralization assay. We tested the ability of another 2 Env (mac251_15 used in all mixtures and CR2.RU.3R1, included in gp120 and gp140 mixtures) to be neutralized. None of the plasma samples from the vaccinated macaques showed any neutralizing activity against these Envs, which like SIVmac239, appear to constitute a group of Env that are difficult to neutralize (Table 1).

The plasma samples were also tested for their ability to neutralize the heterologous SIVsmE660 Env (Fig. 2D and E) CG7G and CG7V which differ by ~20% from the SIVmac251 Envs present in the vaccine mixture (see also Fig. 1C). The Ab from both the ID/EP and the IM/EP groups were able to neutralize the tier 1A-like SIVsmE660_CG7G (Fig. 2D) to similar extent. Interestingly, we noted a more robust neutralization capability to the tier 1B-like SIVsmE660_CG7V by the antibodies induced by the ID/EP protocol, especially after vaccination with gp120 forms of Env (EP4wk2) (Fig. 2E, left panel) with more responders and higher titers. Of note, CG7G and CG7V Env are 99% identical and differ by 4 AA in gp120 (1AA lies in the N-terminal portion [C1], 2 AA lie in V1, 1 AA lies in C5) and 5 AA lie in gp41. Despite this high homology in gp120, we noted a distinct neutralization pattern by these 2 Env in the pseudotype assay. This observation is reminiscent to the finding using the different highly homologous gp120 proteins from SIVmac251, which we described above. Thus, it is interesting to note that the gp120 proteins differing by very few AA (3 AA in the SIVmac251 Envs or 4 AA in the E660 Envs) can have such a profound distinct characteristics in the NAb assay. This finding is agreement with a previous observations of a single AA change in V2 of HIV Env³⁵ and of truncation of SIV gp41³⁶ that also altered neutralizability.

To understand whether the inclusion of several Env in the DNA vaccine cocktail induced Ab with broader crossreactivity, we compared the results shown in Figure 2 to our previously reported data of macaques vaccinated with mac239 gp160 DNA.²⁴ Using the same panel of Env, we did not find broader crossreactivity in macaques vaccinated with a mixture of different SIVmac251 Env vs. only SIVmac239 Env. More importantly however, we noted a trend that the ID/EP delivery is more potent, inducing more consistent and higher tier NAb to the difficult to neutralize E660_CG7V. Thus, the delivery method to the skin

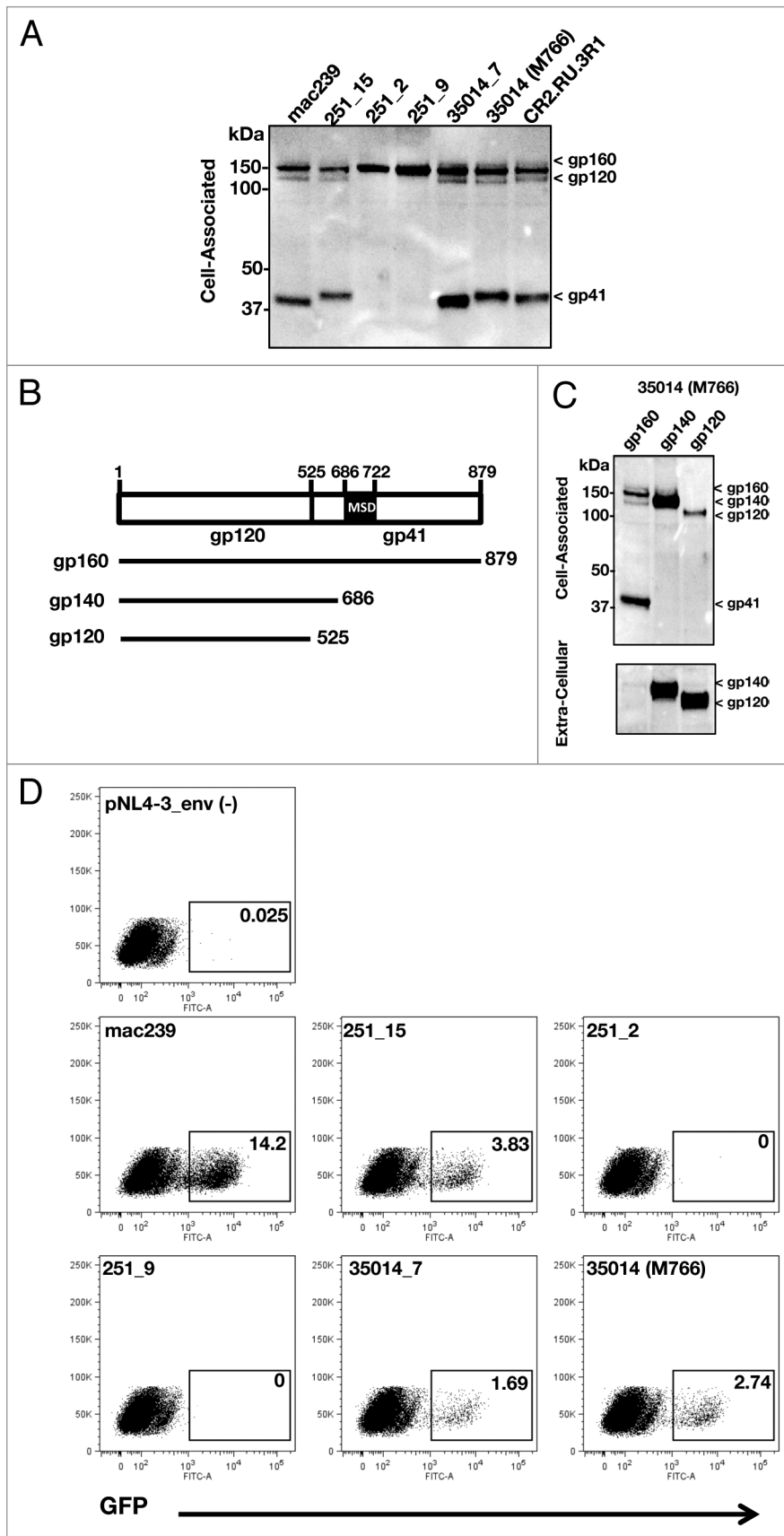


Figure 3. Characterization of different SIV mac251 Env proteins. **(A)** Western immunoblot analysis of the expression of SIVmac239 Env and the different SIVmac251 Envs upon transient transfection of HEK293 cells. **(B)** Cartoon depicting gp160, gp140 and gp120 Env. **(C)** Western immunoblot analysis the gp160, gp140 and gp120 Env of SIVmac251_35014 (M766) upon transient transfection of HEK293 cells. Cell-associated and extra-cellular fractions are shown. **(D)** Pseudotyped recombinant HIV-luc virus reporter (NL4-3.LucR⁻) viruses were generated using the SIVmac239 Env and the indicated SIVmac251 Env. Infectivity was tested in modified CEM-X174 cells that contain the HIV-LTR promoter linked to GFP stably integrated. GFP expression, which serves as read-out for infection, was measured by FACS analysis.

appeared to have an important feature to induce antibodies with better breadth.

Longevity of humoral immune responses. To address the persistence of the humoral immune responses, the animals were monitored for 14–20 mo after the last vaccination (Fig. 7). The bAb titers to SIVmac239 showed an initial decline during the first ~2 mo after the last vaccination, which was followed by similar persistence in the intradermal/EP and intramuscular/EP groups (Fig. 7A). We also monitored the avidity of the SIVmac239 bAb (Table 2). The avidity index of sham DNA injected animals or pre-samples were below detection limit (assigned to 0.1%). The mac239 bAb showed an avidity index with a range of 15–47% at 2 weeks after the last vaccination, and these values did not change significantly overtime, indicating that the quality of the vaccine-induced antibody responses was maintained over time. In addition, we also found persistence of NAbs to SIVmac_35014 (M766) (Fig. 7B) and the heterologous SIVsmE660_CG7G (Fig. 7C) using both of the vaccination methods. Together, long-term follow-up showed that both ID/EP and the IM/EP DNA delivery methods induced Env-specific antibodies with similar potent durability.

Cellular immune responses induced upon intradermal and intramuscular delivery of Env DNA plasmids. The induction of Env-specific cellular immune responses was monitored upon stimulation of PBMC with SIVmac239 Env peptide pool (15-mer overlapping by 11 AA) and measured by intracellular cytokine staining and flow cytometry. The two

IM/EP immunized animals showed robust responses with a frequency of 0.1–0.6% of Env-specific IFN- γ T cells (Fig. 8A). Similar responses were measured in the 3 macaques that were vaccinated only with gp160 DNAs (Fig. 8D). The cellular responses induced against SIV Env were mediated typically by both CD4⁺ and CD8⁺ T cells and, importantly, these responses were ~10-fold higher in the IM/EP vaccinated macaques (Fig. 8A and D) compared with the ID/EP immunized animals (Fig. 8F). Detailed analysis of the Env-specific responses of both groups of macaques showed induction of transitional (CD28⁺CD95⁺CCR7⁻) and effector (CD28⁻CD95⁺CCR7⁻) memory T cells (Fig. 8B, E and G) containing the cytotoxic marker granzyme B (GzmB) (Fig. 8C and H). Importantly, the T cell responses were also long-lasting and could be detected for >1 y after the last vaccination. Together these data demonstrate that both vaccination protocols induced durable cytotoxic cellular immune responses although the IM/EP delivery route induced higher levels of Env-specific T cell responses.

Discussion

We have a long-standing interest in optimizing DNA-based vaccine approaches, and thus, we have taken sequential steps to improve efficacy of this vaccine vehicle including RNA or codon optimization of the DNA expressing the immunogen, optimization of expression vectors, antigen design, choice of DNA adjuvants such as IL-12 and IL-15, and DNA delivery methods.^{15,20,24-28,37-46} Our recent data on the combination of SIV DNA and inactivated SIV particles, used as protein source in the same vaccine regimen, revealed great potency to augment humoral responses.²⁸ To improve Env immunogenicity, we describe herein the testing of a panel of transmitted SIV *env* DNA and establishment of HEK293 cell lines stably producing trimeric gp140 and soluble gp120 Env proteins, which will allow us to further expand on this study by using purified Env proteins instead of virus particles.

In this report, we tested different DNA-only delivery methods and compared the immunogenicity of the same cocktail of expression-optimized SIV *env* DNAs using 2 different routes of vaccine delivery in macaques: intradermal/EP and intramuscular/EP. We did not find significant differences in magnitude of the humoral responses using these two DNA delivery methods. Importantly, both vaccine regimens induced potent long-lasting humoral responses, detectable for > 1 y. The IM/EP DNA delivery method was previously reported to induce long-lasting systemic responses to HIV/SIV immunogens in macaques.^{24,26-28,47}

To test the breadth of the responses, we used a panel of SIVmac and SIVsm Env sequences in pseudotype neutralization assays. The ability to neutralize the tier 1B-like SIVsmE660_CG7V Env served as a measure of neutralization strength. Interestingly, we found more potent NAb to the heterologous, more difficult to neutralize SIVsm E660_CG7V in macaques vaccinated via the ID/EP route. These data are reminiscent of our previous report where we found the presence of NAb to E660_CG7V only upon inclusion of IL-12 DNA as vaccine adjuvant in the SIVmac239

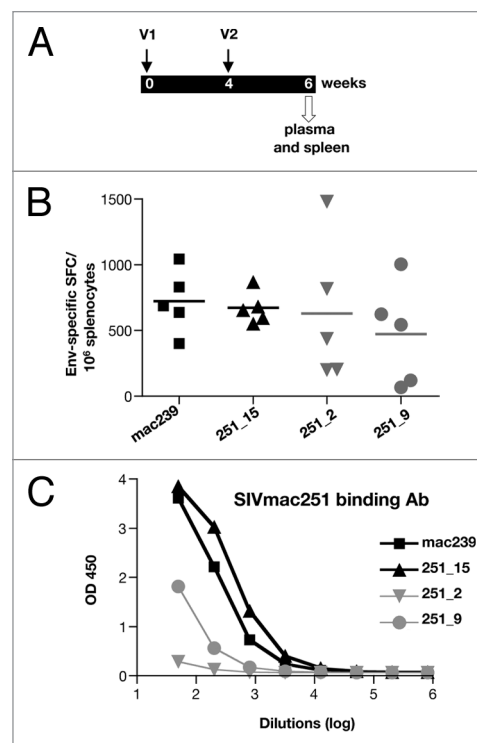


Figure 4. Immunogenicity of Env DNAs in mice. (A) Outline of vaccination study of BALB/c mice. Mice (n = 5/group) were vaccinated via the IM route (needle and syringe) at 0 and 4 weeks with SIV Env DNA formulated in PBS, and were sacrificed 2 weeks after the 2nd vaccination. Spleen and blood were analyzed. (B) Splenocytes were stimulated with an Env peptide pool (15-mer, 11 AA overlap) and the IFN- γ producing T cells were measured by ELISPOT assay. (C) The Env-specific binding antibodies titers were measured by ELISA using serial dilutions of pooled plasma samples.

IM/EP vaccinated macaques.²⁴ Thus, the delivery route (e.g., ID/EP) or inclusion of IL-12 cytokine DNA contributed significantly to the induction of humoral responses with improved breadth.

In contrast to the humoral responses, we found a significant difference in the quantity of the cellular immune responses, with the ID/EP delivery inducing lower responses. Despite this, both vaccine regimens induced Env-specific transitional and effector memory T cells and the cells contained the cytotoxic marker granzyme B, a desired vaccine-induced T-cell response. Thus, the cellular responses although different in magnitude, showed the necessary potency as T-cell vaccine. As a matter of fact, the potency of the vaccine responses induced upon IM/EP DNA delivery has been tested upon subsequent challenge of macaques with pathogenic SIV^{25,26,28,48,49} and with SHIV⁵⁰ and showed significant reduction of viremia.

Different EP delivery methods developed by Inovio have been reported which showed improved immunity including HIV/SIV DNAs in macaques using intradermal low-current EP,⁵¹ or using intradermal/subcutaneous EP,⁵² as well as devices that deliver DNA simultaneously to both skin and muscle of mice and guinea pigs⁵³ and intradermal route in mice.⁵⁴ Here, we directly compared the immune responses induced upon IM

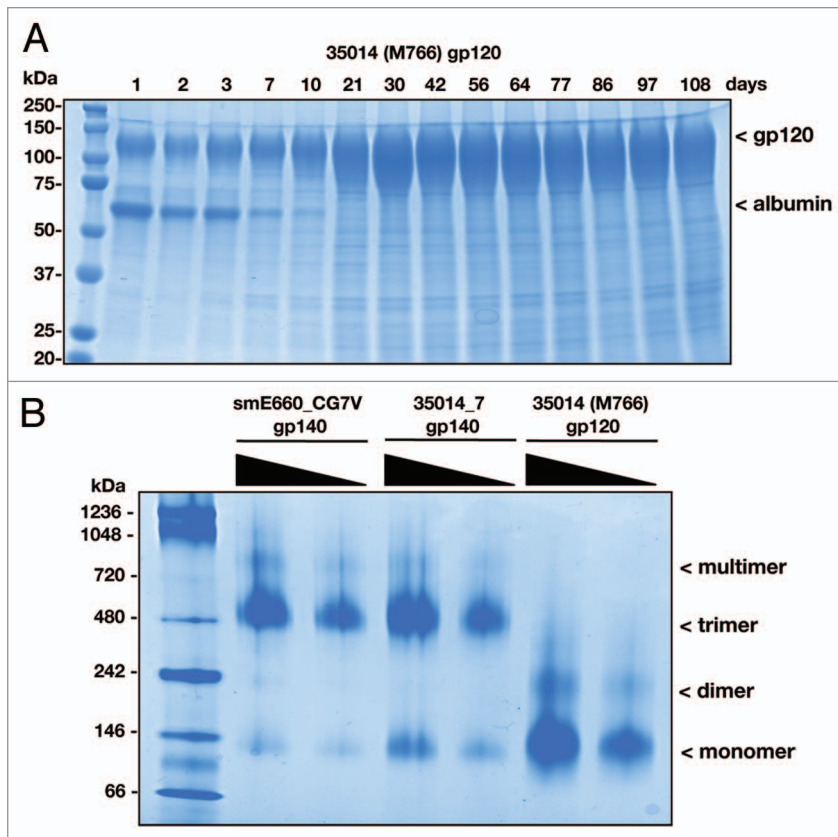


Figure 5. Production and purification of SIV Env protein from stable HEK293 cells. (A) Samples of culture media from Fibercell grown HEK293 cells expressing SIV env protein were assessed for purity on Coomassie blue stained SDS PAGE gels (10% TGX gel, Bio-Rad). (B) Purified SIVsmE660_CG7V gp140, SIVmac gp140 (35014_7) and gp120 (35014/M766) proteins were assessed for purity and quality on non-denaturing gel (10% TGX gel) and visualized by Coomassie blue staining.

vs. ID delivery followed by EP of the same DNA mixtures into macaques. Our data suggest that a combination of ID and IM delivery is most effective in eliciting high humoral and cellular responses. Indeed, such a delivery device has been developed and shown superior results than either of the routes⁵³ and should be tested in the macaque model.

Mucosal dissemination is critical for an HIV/SIV vaccine, since this is the route of natural infection. Although it has not been tested yet for the intradermal/EP delivery method, the use of intramuscular/EP elicited robust, long-lasting systemic immune responses that disseminate into mucosal sites in vaccinated macaques.^{27,28} It was further shown that IM/EP did not increase the activation state of SIV-specific CD4⁺ T cells in the gastrointestinal mucosa, thus no increase in target cells for HIV infection was induced by this delivery method.⁵⁵ Overall, our study demonstrates that both routes of immunization followed by EP induce robust and durable immune responses in macaques. Thus, a combination of immune responses induced via ID and IM delivery routes may be able to further improve immunity, and to reduce virus acquisition. Further studies will be needed to evaluate the efficacy of these two delivery systems upon challenge with pathogenic SIV.

Materials and Methods

Identification of transmitted SIVmac251 Env sequences. The SIVmac251 stocks were generated by propagating the original SIVmac251 isolate in monkey peripheral blood mononuclear cells.⁵⁶ Two stocks were used to infect rhesus macaques (stock 2000: animals 35003, 35014, 33390; and stock 06: animals M761, M766, M752, M757, M764, M741) using low dose inocula. Plasma samples collected at the peak of infection were used for RNA extraction by PureLink viral RNA isolation kit (Invitrogen). The full-length *env* (2.86 kb) or the V1-V2 region (586 bp) of the *env* gene were reverse transcribed with ThermoScript (Invitrogen) using reverse outer primer and amplified by nested PCR using High Fidelity Platinum Taq Polymerase (Invitrogen) and performed single genome sequencing assay (SGA) as described.⁵⁷ Approximately 10,000 copies of genomic RNA were amplified for each sample at SGA. Thermal cycling conditions for full-length *env* genes were as follows: an initial 1 min denaturation at 94 °C, followed by 35 cycles of 94 °C for 15 sec, 55 °C for 30 sec, and 68 °C for 3 min; and 68 °C for 20 min extension. Thermal cycling conditions for *env* V1-V2 were as follows: an initial 1 min denaturation at 94 °C, followed by 35 cycles of 94 °C for 30 sec, 54 °C for 30 sec, and 68 °C for 80 sec; and 68 °C for 3 min extension. The primer position (corresponding to SIVmac251 GenBank Accession number M19499) and sequence for full-length *env* genes were: external sense primer: SIV251_REV_outF 5'-CACATGCTAT TGTA AAAAGT GTTGCTA-3' (6433–6459) and external antisense primer: SIV251_NEF_outR, 5'-TGTAATAAAT CCCTTCCAGT CC-3' (9440–9461), internal sense primer: SIV251_REV_inF, 5'-ACCATTGCCA GTTTTGT TTTT CTTA-3' (6459–6482), and internal antisense primer: SIV251_NEF_innerR, 5'-ATACCCCTAC CAAGTCATCA TCT-3' (9337–9359). The PCR products were sequenced with six sequencing primers as follows: SIV251_6532F 5'-TCCGAAAAAG GCTAAGGCTA ATAC-3', SIV251_6928F 5'-TGAGACAGATAGATGGGGATTGACA-3', SIV251_8079R 5'-TTTATAATCT CCCAACTCCA ATCG-3', SIV251_7888F 5'-AGACAAATAA TCAACTTGT GCATAAA-3', SIV251_8404F 5'-AATGCTTGGG GATGTGCGTT TAG-3', and SIV251_9359R 5'-ATACCCCTAC CAAGTCATCA TCT-3'. The primer position and sequence for V1-V2 *env* were: external sense primer: SIV251_V1–2_outF 5'-CTGCTTATCG CCATCTTGCT TTTA-3' (6601–6624) and external antisense primer: SIV251_V1–2_outR, 5'-CGTAATTCCT TCTATGCCGT TCC-3' (7851–7873), internal sense primer: T7-SIV251V1–2_inF, 5'-TAATACGACT CACTATA-GGGGAACAAC TCAG-3' (T7 + 6733–6755), and internal antisense primer: M13R-SIV251V1–2_inR, 5'-CAGGAAACAG

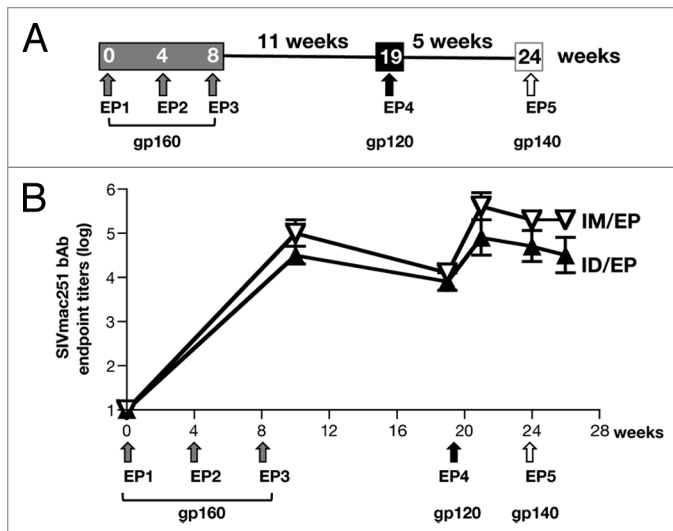


Figure 6. Analysis of humoral immune responses upon vaccination of macaques with *env* DNA. (A) Outline of vaccination study of macaques indicating the sequential immunization with DNA mixtures expressing a cocktail of multivalent Env gp160 (EP1-EP3), gp120 (EP4) and gp140 (EP5). (B) Endpoint SIVmac251 Env binding Ab titers are shown for the animals vaccinated ID/EP and the IM/EP DNA delivery regimen using the same DNA mixtures.

CTAT-GACCACCACC TTAGAAC-3' (7349–7365+M13R). Internal sense and antisense primers were flanking with T7 and M13R sequence at the 5' end of the primers, respectively, so that the sequencing was performed with T7 and M13R primers. PCR products were amplified for sequencing using Applied Biosystem's BigDye Terminator v1.1 Ready Reaction mix (Applied Biosystems). Sequencing products were detected using Applied Biosystems 3730xl DNA analyzer. Sequencing products were detected using Applied Biosystems 3730xl DNA analyzer. Sequence alignments and phylogenetic analysis were performed with the CUSTALW method using the Lasergene software package (DNASTAR).

DNA vectors and in vitro expression. Selected Env sequences were RNA-optimized and cloned into the CMVkan vector comprising the CMV promoter, the bovine growth hormone polyadenylation signal and the kanamycin gene in the plasmid backbone.³⁷ All *env* sequences are submitted to GenBank and listed in Table 1. GenBank submission for 35014 and 251_2 represent optimized consensus sequences. All plasmid DNAs were produced in *E. coli* DH10B (Invitrogen) grown at 32 °C and endotoxin-free DNA was purified (Qiagen). The DNAs were resuspended in sterile water (Hospira, Inc.). Env production was measured in supernatant and cell-associated fractions (1/200 of fraction) from transfected HEK293 cells using Western immunoblot (1:5000 dilution of pooled sera from SIVmac251 infected macaques, followed by 1:10,000 dilution of HRP-labeled anti-monkey antiserum (Fitzgerald Industries International Inc.). The bands were visualized using the enhanced chemiluminescence (ECL) plus western blotting detection system (GE HealthCare).

Env protein production from stable HEK293 cells. Linearized Env expression plasmids, purified using Nucleotide Removal Kit

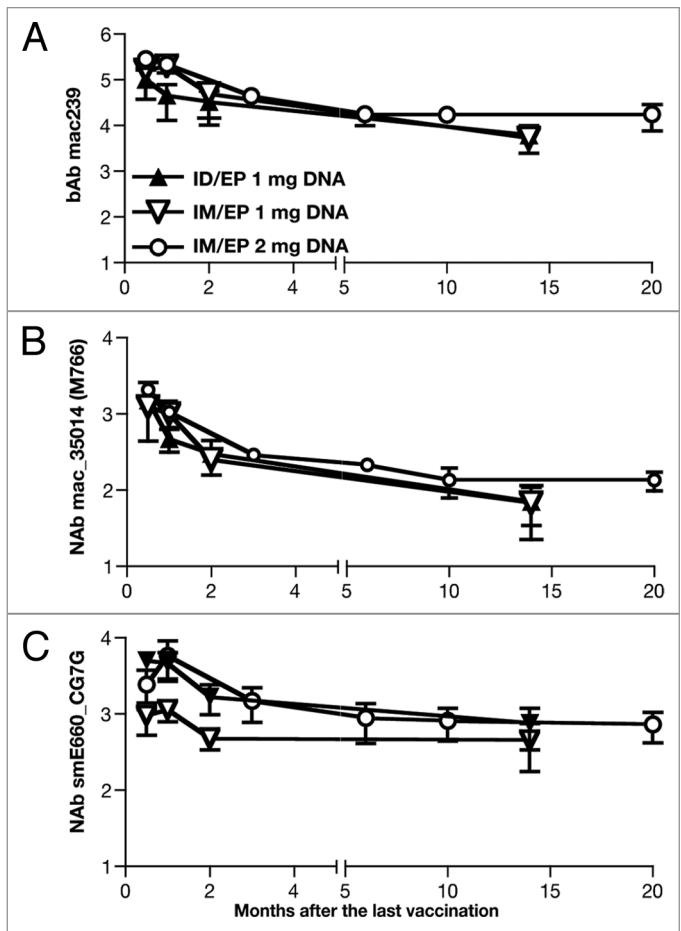


Figure 7. Persistence of humoral responses in macaques vaccinated via the intradermal and the intramuscular route. The animals were monitored for 14–20 mo after the last vaccination. Humoral immune responses were measured in the macaques that received DNA via the intradermal ($n = 3$) and the intramuscular route ($n = 5$, with 3 macaques receiving 1 mg DNA and 2 macaques receiving 2 mg DNA). (A) Endpoint binding Ab titers to SIVmac239, (B) NAb titers to SIVmac_35014 (M766) and (C) NAb to the heterologous SIVsmE660_CG7G (tier 1A-like) are shown as log of reciprocal dilution of plasma that produces a 50% reduction in signal compared with wells receiving no sample.

Table 2. Avidity of SIVmac239 Env binding antibody over time

		Avidity measurements after last Env DNA vaccination (in month)						
		0.5	1	3	6	10	14	20
ID/EP	M694	23.3	30.0					49.6
	M710	30.3	31.8					21.3
	M084	21.1	16.8					ND
IM/EP	M092	21.1	32.7					60.7
	M095	47.4	31.2					62.6
	M705	25.9	20.4	20.3	30.8	35.7		32.5
	M781	15.3	14.2	12.8	26.2	29.1		28.8
	M782	37.0	39.9	42.2	43.7	52.3		41.1

ND, not determined.

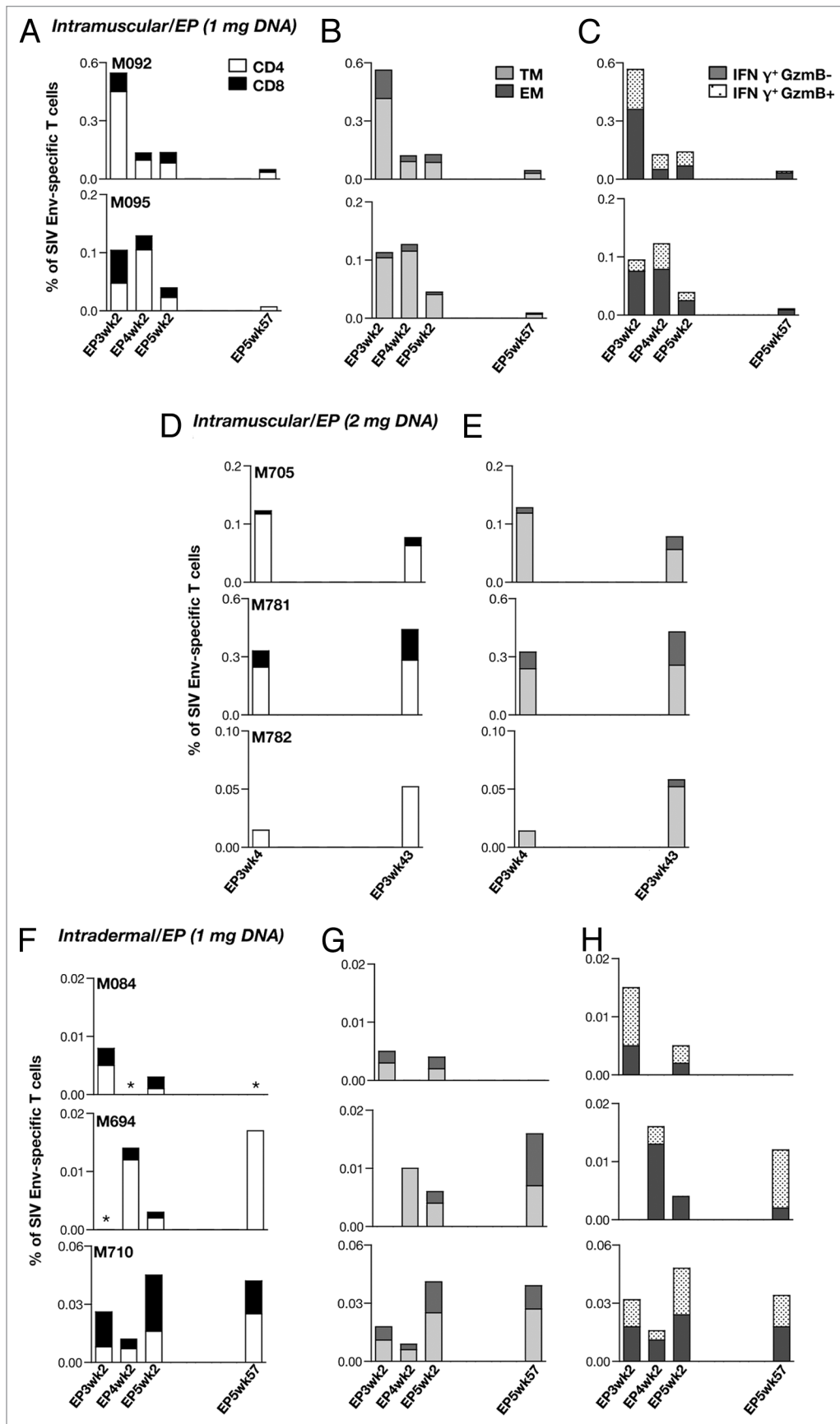


Figure 8. For figure legend, see page 2091.

Figure 8 (See opposite page). Comparison of magnitude and longevity of cellular responses in macaques vaccinated via the intramuscular (A–C, D–E) and the intradermal (F–H) route. Cellular immune responses were measured in PBMC upon stimulation with a SIVmac239-specific peptide pool (15-mer overlapping by 11 AA) at 2 weeks after vaccination with gp160 (EP3wk2), gp120 (EP4wk2) and gp140 (EP5wk2) (A–C, F–H). Animals in D–E were monitored after the 3rd gp160 DNA vaccinations. All animals were monitored for ~1 y and the cellular responses were measured at EP5wk57 and EP3wk43, respectively. The responses are shown as % Env-specific IFN- γ ⁺ T cells among CD4⁺ and CD8⁺ (A, D, F); transitional (TM; CD28⁺CD95⁺CCR7⁻) and effector memory (EM; CD28⁻CD95⁺CCR7⁻) (B, E, G) T cells. The frequency of the Env-specific IFN- γ ⁺ granzyme B⁺ T cells is shown (C, H). The macaques of panels B and E were not analyzed for this function. Asterisk indicates that the sample scored negative for the assay.

(Qiagen), were used to transfect HEK293 cells together with pRSVhygro as selection marker. Stable high producer HEK293 cell clones were generated using Env plasmids expressing SIVmac251_35014 (M766) gp120 (plasmid 246S), 35014_7 gp140 (plasmid 234S) and SIVsmE660_CG7V gp120 (plasmid 261S). The following clones were among the highest producers of 35014 (M766) gp120 (clone 36), 35014_7 gp140 (clone 47), SIVsmE660_CG7V gp120 (clone X). Env proteins were produced from selected stable clones grown in serum-free media in a Hollow Fiber bioreactor that allows high-density growth without any animal components (Fibercell®) as described.⁵⁸ Supernatants (6 μ l of daily harvests of 20 ml) were monitored on gels stained with Coomassie blue. Serial dilutions of the samples were analyzed, together with purified SIV gp120 protein (a gift from E. Chertova) to calibrate, after staining of the gels with Emerald Green.

Virus infectivity assay. Pseudovirus preparations were generated upon cotransfection of HEK293 cells with 5 μ g pNL4–3. LucR-E- (NIH AIDS Research and Reference reagent program, Division of AIDS, NIAID;⁵⁹) and 50 ng of the different SIV Env expressing plasmids using the calcium phosphate method. Eight hours following transfection, the culture medium was replaced with fresh complete DMEM containing 10% FBS. Twenty-four hours later, the cell supernatants were collected and 1 ml of cell-free supernatant was added to 3 to 5 million of CEMx174-GFP, an indicator cell line that contains the HIV-LTR promoter linked to GFP stably integrated.⁶⁰ After a 2-h incubation, 1 ml of medium was added and the cells were incubated for 48 h. GFP expression in infected cells was monitored by flow cytometry.

DNA vaccination of mice. Female BALB/c (6 to 8 weeks old) were obtained from Charles River Laboratories, Inc. and were housed at the National Cancer Institute in a temperature-controlled, light-cycled facility. The mice were immunized by intramuscular injection (week 0 and 4) with 100 μ g plasmids expressing the indicated Env gp160 plasmids. Two weeks after the last vaccination, spleens and plasma were collected to measure cellular and humoral immune responses as described below.

DNA vaccination of macaques. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. 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., and were approved by the Institutional Animal Care and Use Committee (OLAW assurance number A3467-01 and USDA Certificate number 51-R-0059). Macaques received a mixture of 4 *env* plasmids DNA (1 mg) via intradermal route (ID) or via intramuscular route (IM) route followed by in vivo electroporation (Inovio Pharmaceuticals, Inc.). The ID EP was performed using the surface EP device (SEP) which allows for

shallow ID DNA delivery using a novel 4 \times 4 minimally invasive needle array.^{51,61} This ID device differs from other invasive EP devices in that the electrodes are minimally invasive (make contact with the skin surface but do not penetrate) and also operate at significantly lower voltages (15–25 V). The electrode configuration is designed to achieve threshold electric fields over a wider area at lower applied voltages. Specifically, the EP applicator consists of gold-plated stainless steel needle electrodes with trocar grinds at 1.5 mm spacing in a 4 \times 4 array pattern. The surface device was built with attachment cord for linkage to the ELGEN 1000 pulse generator (Inovio Pharmaceuticals). The following Env DNA were used: SIVmac239 and SIVmac 251_15 DNAs as gp160, gp140, gp120; SIVmac 35014 (M766) was included in the gp160 and gp140 mixtures; SIVmac 35014_7 was included in gp160 and gp120 mixtures; and SIVmac CR2.RU.3R1 was included in the gp120 and gp140 mixtures. For ID vaccination, macaques M084, M694 and M710 received a mixture of 1 mg DNA (0.25 mg of each plasmid) resuspended in total of 0.25 ml of sterile water, injected as 5 \times 50 μ l blebs on 3 places (2 \times 50 μ l closely spaced [2 \times]; 1 \times 50 μ l injected separately) on the back of the animal, followed by EP. For the IM vaccination (macaques M092 and M095) 1 mg of the *env* DNA mixture was resuspended in 0.6 ml water and injected at 2 sites (0.3 ml each, left and right internal thighs) followed by EP. The IM/EP was performed using the ELGEN Twin Injector (Inovio Pharmaceuticals) which consists of an outer housing with an inner wagon carrying two standard 1 ml syringes with 21 g needles, 4 mm apart.⁶² A gearing system presses the piston of the syringes when the wagon slides forward in order to inject DNA during the insertion. The needles subsequently serve as electrodes. The needles penetrate the full depth of the targeted muscle, distributing the DNA in a columnar fashion throughout the muscle and co-locate the electrical field with the delivered plasmid. The device operates at an applied voltage of 60 and pulses twice, both of 60 ms duration. The ELGEN Twin Injector is directly linked to the ELGEN 1000 pulse generator (Inovio Pharmaceuticals). Macaques (M705, M781, M782) received 3 vaccinations with 0.5 mg DNA each of the same 4 gp160 DNAs resuspended separately in 0.3 ml each and injected at 4 different sites (arms and thighs). Blood samples were collected at each vaccination and at various time points throughout the course of vaccination to measure cellular and humoral immune responses.

Humoral immune responses. The end-point binding antibody titers to SIVmac251 gp120 were measured by ELISA (Advanced BioScience Laboratory, Inc.) and titers greater than the mean +3SD of normal plasma at OD450 were considered positive. Antibody titers to SIVmac239 and antibody avidity upon treatment with 1.5 M sodium thiocyanate (NaSCN; Sigma-Aldrich) were measured as described.^{63,64} Neutralizing

antibody titers were determined using the M7-luc assay for the TCLA-SIVmac251/H9 and the TZM-bl assay for the transmitted SIVmac251 35014_7 and 35014 (M766), and the heterologous SIVsmE660_CG7G and SIVsmE660_CG7V.⁶⁵

Cellular immune responses. Mouse splenocytes were incubated with a pool of overlapping 15-mer peptides (1 µg/ml final concentration) spanning the entire gp160 region of SIVmac239 (Infinity Inc. Biotech Research and Resource) and ELISpot assays were performed as described.³⁸ Env-specific spots were calculated by subtracting the cut-off value and adjusted to the number of spot-forming cells per million splenocytes.

The frequency of antigen-specific T cells from vaccinated macaques was determined upon stimulation with the Env peptide pool followed by intracellular cytokine staining and multiparametric flow cytometry as described.²⁸ The antibody panel for surface staining consisted of: CD3-APCCy7, CD4-AmCyan, CD95-FITC (BD PharMingen), CD8-AF-405 (Invitrogen), CD28-PerCP Cy5.5 (BioLegend), CCR7-APC (R&D Systems Inc.) and CD45RA-AF700 (ABD Serotec). The intracellular staining was performed using IFN-γ-PE Cy7 (BD PharMingen) and granzyme B-PE (Invitrogen) antibodies. PBMC were cultured in medium without peptide pools as negative control or stimulated with phorbol myristate acetate (PMA) and calcium ionophore (Sigma) as positive control. Positive samples had a frequency of IFN-γ positivity in the peptide-stimulated sample that was > 2 fold higher than the frequency obtained in unstimulated (minus peptide) medium only control sample. At least 10⁵ T cells from each sample were acquired on an LSR II flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (Tree Star, Inc.).

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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 that have been licensed to several companies. There are no further patents, products in development or marketed products to declare. KEB and NS are employed by Inovio Pharmaceuticals, Inc. as such receive salary, and bonuses and stock options as compensation.

BKF, GNP, AV: designed, coordinated the study, analyzed the data, and wrote the paper. VK, MR, JB, GRP, RJ, CB, AKS, CA, BC, G-MZ: performed experiments and analyzed the data. E-YK, SMW: performed Env identification study. WH, YG: performed binding Ab and avidity assays. CL, DCM: performed and analyzed NAb assays. KEB, NYS: contributed electroporation delivery methods and electroporation devices to access IM and ID tissues for immunization.

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