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

Viraj Kulkarni, Margherita Rosati, Jenifer Bear, Guy R Pilkington, Rashmi Jalah, Cristina Bergamaschi, Ashish K Singh, Candido Alicea, Bhabadeb Chowdhury, Gen-Mu Zhang, Eun-Young Kim, Steven M Wolinsky, Wensheng Huang, Yongjun Guan, Celia LaBranche, David C Montefiori, Kate E Broderick, Niranjan Y Sardesai, Antonio Valentin, Barbara K Felber & George N Pavlakis

To cite this article: Viraj Kulkarni, Margherita Rosati, Jenifer Bear, Guy R Pilkington, Rashmi Jalah, Cristina Bergamaschi, Ashish K Singh, Candido Alicea, Bhabadeb Chowdhury, Gen-Mu Zhang, Eun-Young Kim, Steven M Wolinsky, Wensheng Huang, Yongjun Guan, Celia LaBranche, David C Montefiori, Kate E Broderick, Niranjan Y Sardesai, Antonio Valentin, Barbara K Felber & George N Pavlakis (2013) Comparison of intradermal and intramuscular delivery followed by in vivo electroporation of SIV Env DNA in macaques, Human Vaccines & Immunotherapeutics, 9:10, 2081-2094, DOI: 10.4161/hv.25473

To link to this article: http://dx.doi.org/10.4161/hv.25473
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 Jalal1, Cristina Bergamaschi1, Ashish K Singh2, Candido Alicea1, Bhabadeb Chowdhury2, Gen-Mu Zhang1,2, Eun-Young Kim3, Steven M Wolinsky1, Wensheng Huang4, Yongjun Guan4, Celia LaBranche5, David C Montefiori5, Kate E Broderick6, Niranjana Y Sardesai6, Antonio Valentini5, Barbara K Felber1,*, and George N Pavlakis2,*

1Human Retrovirus Pathogenesis Section; Vaccine Branch; Center for Cancer Research; National Cancer Institute; Frederick, MD USA; 2Human Retrovirus Section; Vaccine Branch; Center for Cancer Research; National Cancer Institute; Frederick, MD USA; 3Division of Infectious Diseases; The Feinberg School of Medicine; Northwestern University; Chicago, IL USA; 4Institute of Human Virology; Department of Microbiology and Immunology; University of Maryland School of Medicine; Baltimore, MD USA; 5Department of Surgery; Laboratory for AIDS Vaccine Research and Development; Duke University Medical Center; Durham, NC USA; 6Inovio Pharmaceuticals, Inc.; Blue Bell, PA USA

Keywords: intradermal, intramuscular, in vivo electroporation, DNA vaccine, HIV, SIVsmE660, SIVmac239, SIVmac251, transmitted Env, binding antibody, neutralizing antibody, avidity, transitional memory, effector memory

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

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 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...
delivery by needle/syringe also in man, 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. 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. (GenBank accession #JQ080004) 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.3R14 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), 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 Env 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 Env sequences (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
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 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 μl aliquots from the supernatants of daily harvests (20 ml) was performed on gels stained with Coomassie blue.

Figure 1. Amino acid alignment of transmitted SIVmac251 Env sequences. (A) The identified SIVmac251 sequences and the reported SIVmac251 sequence CR2.RU3R1 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 CG7V 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 μl 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

www.landesbioscience.com Human Vaccines & Immunotherapeutics 2083
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. 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...
are difficult to neutralize (which like SIVmac239, appear to constitute a group of Env that macaques showed any neutralizing activity against these Envs, could not be neutralized. None of the plasma samples from the vaccinated and CR2.RU.3R1, included in gp120 and gp140 mixtures) to the ability of another 2 Env (mac251_15 used in all mixtures, 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 pseudotypes. 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

### Table 1. SIV Env and functional characteristics

<table>
<thead>
<tr>
<th>SIV</th>
<th>Plasmid name</th>
<th>GenBank accession #</th>
<th>Optimized env DNA expression plasmids (plasmid code)</th>
<th>gp120/gp41 production from gp160 DNA</th>
<th>Function in pseudotype assay</th>
<th>Ability of Env to be neutralized (TZM-bl assay)</th>
<th>Evaluation of neutralizability of Env (TZM-bl assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mac239</td>
<td>M33262</td>
<td></td>
<td>gp160 gp140 gp120</td>
<td>yes yes no</td>
<td></td>
<td></td>
<td>difficult to neutralize</td>
</tr>
<tr>
<td>35014 (M766)</td>
<td>KF003433 (JQ086004)</td>
<td>2195 gp120 gp140 gp120</td>
<td>yes yes yes</td>
<td>yes yes yes</td>
<td>tier 1A-like</td>
<td></td>
<td></td>
</tr>
<tr>
<td>251_9</td>
<td>KF003434</td>
<td></td>
<td>242S 241S 246S</td>
<td>yes yes yes</td>
<td>yes yes yes</td>
<td>tier 1B-like</td>
<td></td>
</tr>
<tr>
<td>251_15</td>
<td>KF003436</td>
<td></td>
<td>240S 245S 245S</td>
<td>yes yes yes</td>
<td>yes yes yes</td>
<td>difficult to neutralize</td>
<td></td>
</tr>
<tr>
<td>251_2</td>
<td>KF003435</td>
<td></td>
<td>245S 245S 245S</td>
<td>yes yes yes</td>
<td>yes yes yes</td>
<td>difficult to neutralize</td>
<td></td>
</tr>
<tr>
<td>CR2, RU.3R1</td>
<td>FJ578044</td>
<td></td>
<td>231S 232S 223S</td>
<td>yes ND no</td>
<td>yes</td>
<td>tier 1A-like</td>
<td></td>
</tr>
<tr>
<td>smE660</td>
<td></td>
<td></td>
<td>gp160 gp140 gp120</td>
<td>yes yes yes</td>
<td>yes</td>
<td>tier 1B-like</td>
<td></td>
</tr>
<tr>
<td>CG7G</td>
<td>FJ578381</td>
<td></td>
<td>gp120 gp140 gp120</td>
<td>yes yes yes</td>
<td>yes</td>
<td>tier 1A-like</td>
<td></td>
</tr>
<tr>
<td>CG7V</td>
<td>FJ578428</td>
<td></td>
<td>gp120 gp140 gp120</td>
<td>yes yes yes</td>
<td>yes</td>
<td>tier 1B-like</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.
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.24 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.24,26-29,47

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 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 SIV25,26,28,48,49 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.24 Here, we directly compared the immune responses induced upon IM
Identification of transmitted SIVmac251 Env sequences. The SIVmac251 stocks were generated by propagating the original SIVmac251 isolate in monkey peripheral blood mononuclear cells. 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. 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 TGTAAAAAGT GTTGCTA-3’ (6433 → 6459) and external antisense primer: SIV251 NEF_outR, 5’-TGTAATAAAT CCCTTCCAGT CC-3’ (9440 ← 9461), internal sense primer: SIV251_REVinF, 5’-ACCATTGCCAGTTTTGTTT CTTA-3’ (6459 → 6482), and internal antisense primer: SIV251NEF_innerR, 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’-TGTAATAAAT CCCTTCCAGT CC-3’ (9440 ← 9461), internal sense primer: SIV251_V1–2_inF, 5’-TAATACGACT CACTATAAC TCAG-3’ (T7 + 6733 → 6755), and internal antisense primer: M13R-SIV251V1–2inR, 5’-CAGGAAACAG
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. 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 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/IP and the IM/IP DNA delivery regimen using the same DNA mixtures.

**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.
Figure 8. For figure legend, see page 2091.
(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. Supernatants (6 μl 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 μg pNL4–3. Luciferase– (NIH AIDS Research and Reference reagent program, Division of AIDS, NIAID;59) and 50 ng of the different SIV Env linked to GFP stably integrated.60 After a 2-h incubation, 1 ml of GFP, an indicator cell line that contains the HIV-LTR promoter was added upon cotransfection of HEK293 cells with 5 μg pNL4–3. Eight hours following transfection, the culture medium was replaced with fresh complete DMEM containing 10% FBS. Twenty-four hours following transfection, 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 μg 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 × 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 × 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 gp140 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 × 50 μl blebs on 3 places (2 × 50 μl closely spaced [2×]; 1 × 50 μl 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. A gear 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.53,64 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 5 and 35014 (M766), and the heterologous SIVmE660 CC7G and SIVmE660 CC7V.

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.28 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), CD8-4F4-5 (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 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.

References
5. Sullivan SM, Doukas J, Harikka J, Smith L, Rolland CA, BC, G-MZ: performed experiments and analyzed the data. 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.

Acknowledgments
We are grateful to D Weiss, J Treece, I Kalisz, R Pal and staff at Advanced BioScience Laboratories, Inc., Rockville, for their expert help. We thank A von Gegerfelt, B Keele, JJS Cadwell, and E Chertova for discussions, R Desrosiers, R Pal, and NIAID for SIVmac251 virus and macaque plasma samples, and T Jones for editorial assistance. pNL4-3.LucR-E- was provided by NIH AIDS Research and Reference reagent program, Division of AIDS, NIAID. This work was supported by the Intramural Research Program of the National Cancer Institute, National Institutes of Health (NCI/NIH) and by NIH HHSN 27201100016C.

Disclosure of Potential Conflicts of Interest
GPN 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.


www.landesbioscience.com Human Vaccines & Immunotherapeutics 2093


