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ORIGINAL ARTICLE

DNA vaccination by intradermal electroporation induces long-lasting immune responses in rhesus macaques

Viraj Kulkarni[1], Margherita Rosati[2], Rashmi Jalah[1], Brunda Ganneru[2], Candido Alicea[1], Lei Yu[3], Yongjun Guan[3], Celia LaBranche[4], David C. Montefiori[4], Alan D. King[5,†], Antonio Valentin[2], George N. Pavlakis[2] & Barbara K. Felber[1]

1 Human Retrovirus Pathogenesis Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA
2 Human Retrovirus Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA
3 Department of Microbiology & Immunology, Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, USA
4 Duke Human Vaccine Institute, Durham, NC, USA
5 Cyto Pulse Sciences, Inc., Glen Burnie, MD, USA
† Syndecion, LLC, 1450 S. Rolling Road, Baltimore, MD, USA

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Correspondence
Barbara K. Felber, PhD, Human Retrovirus Pathogenesis Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute, P.O. Box B, Building 535, Room 209, Frederick, MD 21702, USA.
Tel.: 301 846 5159;
fax: 301 846 7146;
e-mail: barbara.felber@nih.gov

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Abstract

Background A desirable HIV vaccine should induce protective long-lasting humoral and cellular immune responses.
Methods Macaques were immunized by *env* DNA, selected from a panel of recently transmitted SIVmac251 Env using intradermal electroporation as vaccine delivery method and magnitude, breadth and longevity of humoral and cellular immune responses.
Results The macaques developed high, long-lasting humoral immune responses with neutralizing capacity against homologous and heterologous Env. The avidity of the antibody responses was also preserved over 1-year follow-up. Analysis of cellular immune responses demonstrated induction of Env-specific memory T cells harboring granzyme B, albeit their overall levels were low. Similar to the humoral responses, the cellular immunity was persistent over the ~1-year follow-up.
Conclusion These data show that vaccination by this intradermal DNA delivery regimen is able to induce potent and durable immune responses in macaques.

Introduction

The development of an effective vaccine able to address the AIDS pandemic remains a major challenge. The RV144 clinical trial in Thailand, in which a combination of recombinant canarypox ALVAC®-HIV together with gp120 Env protein (AIDSVAX® B/E) was used, achieved a modest success in protection from HIV-1 infection [41], but no evidence of vaccine-induced virus control was found in vaccinated individuals who became HIV-1 infected, and the protective ability of the vaccine waned rapidly over time. Thus, a vaccine regimen capable of inducing protective and durable immunity is sought. Among the different HIV/SIV vaccine methodologies that are currently being studied, the use of DNA is a compelling vaccine vehicle because of its simplicity, scalability, lack of immunity against the vector, and the induction of long-lasting immune responses [23, 39, 40]. To improve the immunogenicity of DNA-based anti-HIV/SIV vaccines, different DNA delivery methods are being tested, including intramuscular DNA delivery by *in vivo* electroporation (IM/EP) (reviewed in [6, 20, 47]), skin or intradermal electroporation [9, 16, 17, 19, 21, 27, 32, 42], DermaVir [33], liposome delivery with Vaxfectin® [30, 48], gene gun [13], or biojector [1, 22, 49]. We and others have previously reported that macaques immunized with SIV/HIV DNA alone administered by needle and syringe via the intramuscular (IM)

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route developed immune responses against the virus able to potently reduce viremia upon infection [2–5, 7, 8, 10, 12, 15, 35, 38, 45, 46, 51]. Although the magnitude of the responses was relatively low, these studies demonstrated the importance of cell-mediated immunity in the control of viremia. A significant improvement in the vaccine immunogenicity was observed using IM injection followed by *in vivo* electroporation (IM/EP) as a DNA delivery method (reviewed in [20, 47]), resulting in robust and durable cellular and humoral immune responses [5, 11, 18, 24, 28, 31, 34, 39, 40, 44, 45, 50, 51] detected for >5 years after the last vaccination [23, 39, 40], which also indicated remarkable durability. The efficacy of this vaccine-induced immunity was demonstrated by a significant reduction in viremia in SIV-infected macaques [11, 34, 39, 44, 45, 50, 51]. The results from a recent phase I clinical trial, in which an HIV DNA vaccine delivered via IM/EP together with IL-12 DNA as adjuvant resulted in higher frequency of responders and higher longer-lasting immunity compared with needle/syringe delivery [25], indicate that results obtained from the macaque model can predict the outcome in humans.

In this report, we have evaluated the immunogenicity of an SIV Env DNA vaccine delivered via the intradermal (ID) route followed by *in vivo* electroporation (ID/EP) in mice and macaques, and we demonstrate induction of robust immunity in both animal models. The vaccine elicited persistent humoral and cellular responses in macaques which were detectable ~1 year after the last vaccination. Thus, ID/EP is a promising DNA vaccine delivery method able to induce durable immunity in non-human primates.

Materials and methods

DNA vectors

SIV Env sequences were RNA-optimized and cloned into a CMVkan vector comprising the CMV promoter, the bovine growth hormone polyadenylation signal, and the kanamycin gene in the plasmid backbone [46]. The following forms of SIV Env were used as plasmid DNA: mac239 gp160, gp140, and gp120 (plasmids 99S, 237S, and 173S, respectively [29]); mac251_15 gp160, gp140, and gp120 (plasmids 217S, 240S, and 229S, [29]); mac251_35014 (also referred to as macM766) gp160 and gp140 (plasmids 221S and 241S [29]); mac 35014_7 gp160 and gp120 (plasmids 220S and 230S [29]); mac CR2.RU.3R1 [26] gp140 and gp120 (plasmids 242S and 223S, [29]) (see also [29] for GenBank entries of our SIV Env). The SIVmac Env sequences 35014 (M766), 35014_7, and CR2.RU.3R1 are from the recently transmitted SIVmac251 viruses [26, 29]. All plasmid DNAs were produced in *Escherichia coli* DH10B (Invitrogen, Carlsbad, CA, USA) grown at 32°C, and the purified endotoxin-free DNAs (Qiagen, Valencia, CA, USA) were resuspended in sterile water (Gibco, Grand Island, NY, USA).

DNA vaccination of mice

Female BALB/c mice (6–8 weeks old) were obtained from Charles River Laboratories, Inc. (Frederick, MD, USA) and were housed at the National Cancer Institute, Frederick, MD, in a temperature-controlled, light-cycled facility. The mice were immunized by intradermal injection followed by *in vivo* electroporation using the DermaVax EP device (Cellectics, Paris, France, formerly CytoPulse Sciences, Glen Burnie, MD, USA) at weeks 0 and 4 using a dose of 2, 10, or 50 μg of plasmid DNA expressing the SIVmac239 Env gp160. Two weeks after the last vaccination, spleen and plasma were collected to measure cellular and humoral immune responses as described below.

DNA vaccination of macaques

This study was carried out 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., MD, and were approved by the Institutional Animal Care and Use Committee (OLAW assurance number A3467-01 and USDA Certificate number 51-R-0059). The macaques were recycled from a previous study where they were infected 3.3–3.5 years prior by SHIV SF162 and controlled virus to undetectable levels, and macaque M078, infected 5.5 years prior by SHIV89.6, that had a very low viremia of 317 RNA copies/ml. The animals did not have detectable humoral or cellular responses to SIV Env at the onset of the study. The four Indian rhesus macaques (M511, M687, M693, and M078) received mixtures of 4 *env* plasmids DNA (total 1 mg) via ID route followed by *in vivo* electroporation using the DermaVax EP device (Cellectics, formerly CytoPulse Sciences) as detailed in Table 1. Macaques 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 three places (2 × 50 μl closely spaced in two places each; 1 × 50 μl injected at a separate place) on the back of the animal, followed by EP. Pulse parameters and intradermal electrode used were as described

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Table 1 SIV Env DNA used in the sequential vaccine

Vaccinations Env sequences	V1–V3 gp160	V4 gp120	V5 gp140
SIVmac239	+	+	+
SIVmac251_15	+	+	+
SIVmac_35014 (M766)	+		+
SIVmac35014_7	+	+	
SIVmacCR2.RU.3R		+	+

[43]. Blood samples were collected at each vaccination and at various time points throughout the course of vaccination to measure cellular and humoral immune responses. Macaque M693 developed acute intestinal obstruction (trichobezoar) and secondary intussusception, not SHIV related, and was electively euthanized before the end of the study (9 weeks post-V5).

Humoral immune responses

The binding antibody end-point titers to HIV-1 IIIb and SIVmac251 gp120 were determined by ELISA (Advanced BioScience Laboratory, Inc. Rockville, MD, USA), and values greater than the mean +3 SD 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, St. Louis, MO, USA) were measured as described [14, 37]. 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 SIVmac251_35014 (M766), SIVmac251_15 (217S), and CR2.RU.3R1 (231S), and the heterologous SIVsmE660_CG7G and SIVsmE660_CG7V as described [24, 28, 29, 36].

Cellular immune responses

Pooled mouse splenocytes (n = 5) 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, Aston, PA, USA), and intracellular cytokine staining was performed as described [28]. The antibody panel for surface staining consisted of CD3-APCCy7 (clone 145-2C11), CD4-PerCP (clone RM4-5), and CD8-Pacific Blue (clone 53-6.7) (BD Pharmingen, San Diego, CA, USA). Splenocytes were washed, and after permeabilization with Cytofix/Cytoperm (BD Pharmingen), intracellular staining was performed using a FITC-conjugated anti-IFN-γ (clone XMG1.2) antibody (BD Pharmingen). PBMC were isolated from the vaccinated macaques at the indicated time points. The frequency of antigen-specific T cells was determined upon stimulation with the SIVmac239 Env peptide pool followed by intracellular cytokine staining and multiparametric flow cytometry as described [24, 39, 40]. The antibody panel for surface staining consisted of CD3-APCCy7 (clone SP34-2), CD4-AmCyan (clone L200), CD95-FITC (clone DX2) (BD Pharmingen), CD8-AF-405 (clone MHCD0826; Invitrogen), CD28-PerCP Cy5.5 (clone CD28.2; BioLegend, San Diego, CA, USA), CCR7-APC (clone 150503, R&D Systems Inc, Minneapolis, MN, USA), and CD45RA-AF700 (clone F8-11-13; ABD Serotec, Oxford, UK). The intracellular staining was performed using IFN-γ-PE Cy7 (clone B27; BD Pharmingen) and Granzyme B-PE (clone GB12; Invitrogen) antibodies. PBMC were cultured in medium without peptide pools as negative control or stimulated with phorbol myristate acetate (PMA) and calcium ionophore (Sigma, St. Louis, MO, USA) as positive control. Positive samples were defined as those having a frequency of IFN-γ T cells in the peptide-stimulated sample that was >2-fold higher than the frequency obtained in unstimulated (minus peptide) medium-only control sample. At least 10^5 T cells from each sample were acquired on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Results

SIV *env* DNA delivered via intradermal route followed by *in vivo* electroporation elicits humoral and cellular immunity in mice

We vaccinated BALB/c mice by ID DNA delivery followed by *in vivo* electroporation (ID/EP) using 2, 10, or 50 μg of a plasmid expressing SIVmac239 gp160. The mice (N = 5 per group) were vaccinated at week 0 and week 4, and 2 weeks after the last vaccination plasma and spleen were collected to monitor immune responses (Fig. 1A). Binding antibodies to SIVmac251 gp120 were measured by ELISA in pooled plasma from each group. Mice receiving the 10 or 50 μg of DNA developed similar levels of antibodies, which were higher than those measured in the group receiving the 2 μg vaccine dose (Fig. 1B). These results indicated that 10 μg DNA was sufficient to induce maximal antibody responses in mice when delivered using ID/EP. We next determined the cellular immune responses in these mice by measuring the frequency of peptide-induced IFN-γ T cells from pooled splenocytes. We noted that mice vaccinated with 10 or 50 μg DNA developed comparable levels of cellular immunity, mainly mediated by

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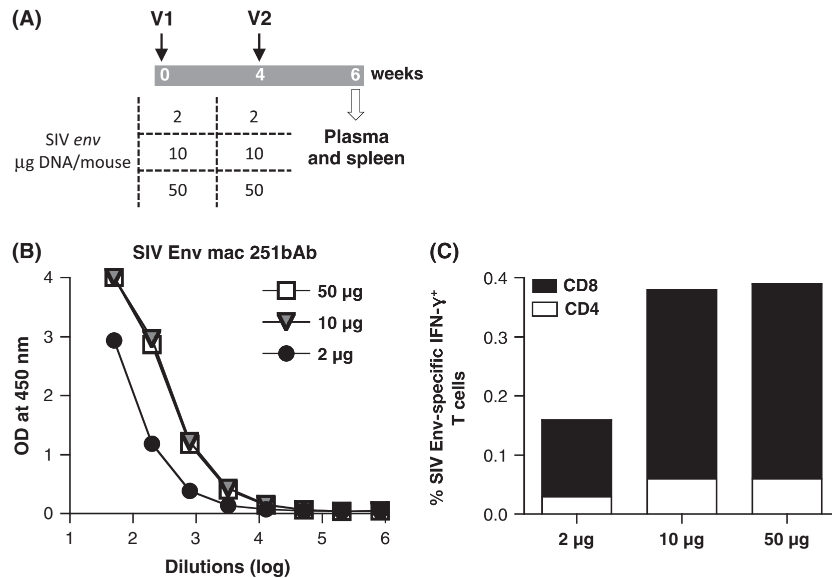

Fig. 1 Immunogenicity of ID/EP delivered Env DNAs in mice. (**A**) Outline of vaccination study of BALB/c mice. Mice (N = 5/group) were vaccinated via the ID route followed by *in vivo* electroporation at 0 and 4 weeks with three doses of SIV Env DNA. The mice were sacrificed 2 weeks after the 2nd vaccination. Spleen and blood were analyzed. (**B**) The Env-specific binding antibodies titers against SIVmac251 Env were measured by ELISA using serial dilutions of pooled plasma samples. (**C**) Splenocytes were stimulated with the SIVmac239 Env peptide pool (15-mer, 11 AA overlap), and the IFN-γ-producing T cells were measured by flow cytometry.

$CD8^+$ T cells, which was more than ~2-fold higher than that observed with 2 μg DNA dose (Fig. 1C). Collectively, these results showed that ID/EP is a potent DNA delivery procedure and that a 10 μg DNA dose was optimal to induce both cellular and humoral immune responses in mice.

Vaccination via intradermal/EP route induces robust humoral immune responses in macaques

Next, we evaluated the ID/EP DNA delivery method in rhesus macaques. We tested the immunogenicity of a panel of SIV Env DNA expressing SIVmac239 combined with recently transmitted SIVmac251 Env [29]. The different Env forms tested included gp160, which is processed to produce the extracellular gp120 anchored to the cell membrane via its gp41 unit, the trimeric gp140, found both in the cell-associated and extracellular fraction, and soluble gp120, which is primarily found in the extracellular fraction. We selected a mixture of DNAs expressing different forms of SIV Env delivered in sequential vaccinations (V): gp160 (V1–3), gp120 (V4) followed by gp140 (V5) using mixtures of different recently transmitted Env and SIVmac239 as detailed in Table 1. We previously reported the phylogenetic analysis of these sequences which share 4.7–6.8% amino acid (AA) difference with SIVmac239 with the majority of the AA differences found in variable regions 1 and 4 [29]. Macaques were vaccinated using the sequential immunization scheme outlined in Fig. 2A, and end-point binding antibody (bAb) titers against SIVmac251 Env were determined as indicated. Vaccination with gp160 DNA induced bAb titers of ~5 log, measured 2 weeks post-V3 (Fig. 2B). The humoral responses persisted during the 5-month follow-up (V3wk2 to V4) with an average decline of ~1.5 log. Vaccination with DNAs expressing the secreted gp120 forms of Envs (Fig. 2B) increased the bAb end-point titers (V4wk2) to levels similar to those obtained with the gp160 DNA vaccine. Subsequent vaccination with gp140 maintained the antibody titers induced by the previous immunizations.

The macaques enrolled in this study were previously (~3–5 years prior to start of study) infected with SHIV. To rule out any contribution of pre-existing immunity of HIV Env to the SIV-specific Env bAb responses found upon the SIV *env* DNA ID/EP vaccination (Fig. 2B), we also measured the humoral responses to HIV-1 IIIb Env (Fig. 2C). The plasma samples were subjected to HIV Env ELISA at the start of the study (V1), at the day of V3, and 2 weeks later (V3wk2). No changes in HIV-specific ELISA responses were found in the four animals (Fig. 2C). Therefore, these data demonstrate the ~5-log increase in SIV Env bAb titers (Fig. 2B) was truly specific to the ID/EP delivered SIV *env* DNA vaccine and reflected induction of *de novo* responses.

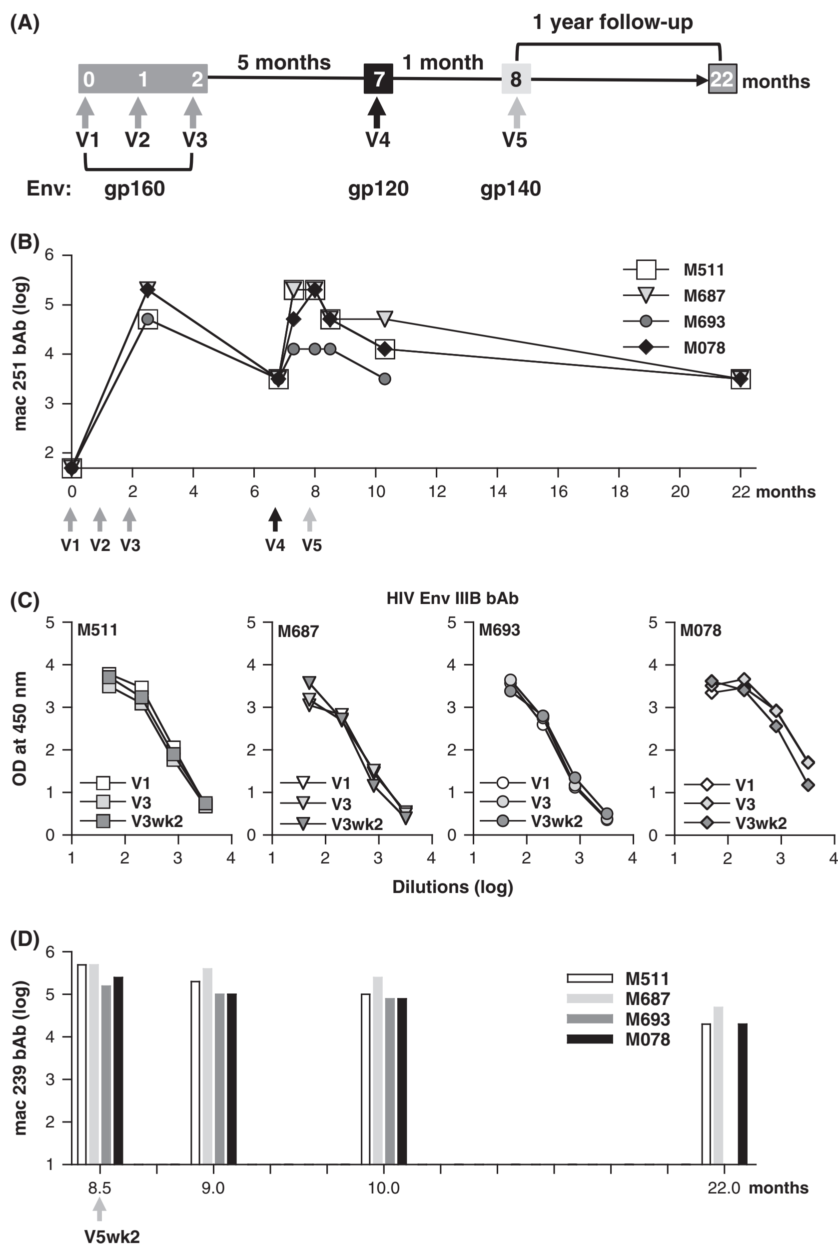

Fig. 2 Analysis of humoral immune responses upon ID/EP vaccination of macaques with *env* DNA. (**A**) Outline of vaccination study of macaques indicating the sequential immunization with DNA mixtures expressing a cocktail of multivalent SIV Env gp160 (V1–V3), gp120 (V4), and gp140 (V5). (**B**) Reciprocal end-point mac251 Env binding Ab titers (in log) are shown over the course of the study. (**C**) The HIV-1 Env-specific binding antibodies were measured against IIIB gp120 Env by ELISA using serial dilutions of plasma samples at the indicated time points: V1, V3, and 2 weeks post-V3. (**D**) Reciprocal end-point mac239 Env binding Ab titers (in log) are shown at V5 and over the 1-year follow-up. The data for animal M693 were only available until 2 months after V5.

We further investigated the SIV bAb responses for durability. We measured the bAb responses to SIVmac251 and SIVmac239 for ~1 year after V5, and we noted an impressive persistence of the humoral responses with only a ~1.2-log decline in SIVmac251 (Fig. 2B) and SIVmac239 (Fig. 2D) responses. The avidity for the SIVmac239 bAb was further tested 2 weeks after the last vaccination and ~1 year later (Table 2). The avidity index had a range of ~30–40% at 2 weeks post-V5 and was monitored over the follow-up time. We found that the avidity of the SIVmac239 responses persisted for ~1 year when the study was terminated (Table 2). Therefore, these data demonstrate that ID/EP DNA vaccination was able to induce robust

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Table 2 Avidity of SIVmac239 Env binding Ab

bAb avidity (in %) to SIVmac239 after V5				
	V5wk2	V5wk4	V5wk9	V5wk53
M511	36	40	46.6	37.2
M687	29.6	34.2	42.5	42.1
M693	39.4	37.1	39.7	ND
M078	34.5	39.7	31.4	31.9

ND, not determined.

and durable systemic bAb with persistent avidity, stable over ~1 year of follow-up.

ID/EP delivery induced cross-neutralizing antibodies

The neutralization capability of the antibody responses induced in the vaccinated macaques was measured against a panel of pseudotyped viruses containing different SIV Env variants, including the homologous T-cell-adapted (TCLA) SIVmac251 (Fig. 3A), two transmitted SIVmac251 Env M766 (Fig. 3B), and 35014_7 (Fig. 3C), as well as SIVmac251_15 3R1 (not shown) and CR2.RU.3R1 (not shown). Plasma samples collected 2 weeks after the gp160 (V3wk2), gp120 (V4wk2), and gp140 (V5wk2) DNA vaccinations were analyzed. Overall, similar NAb titers were detected using ID/EP vaccination regimen (Fig. 3A–C) upon vaccination with different forms of Env (gp160, gp120, gp140), which is in agreement with the similar bAb levels (see above Fig. 2). We noted a distinct pattern in neutralization of the transmitted Env mac_M766 (Fig. 3B) and mac_35014_7 (Fig. 3C), with M766 being easier to neutralize and showing higher NAb titers. We also tested neutralization using another 3 SIVmac251 Env (mac251_15, CR2.RU.3R1 and the difficult to neutralize SIVmac239), which were also included in the DNA vaccine mixture. None of these Env were neutralized by any of the plasma samples tested. Therefore, mac251_15 and CR2.RU.3R1, like mac239, constitute a group of Env difficult to neutralize (not shown).

The plasma samples were also tested for their ability to neutralize the heterologous SIVsmE660 Env (Fig. 3D, E) CG7G and CG7V, which differ by ~20% from the SIVmac251 Envs present in the vaccine mixture. The plasma samples were able to neutralize the tier-1a-like smE660_CG7G (Fig. 3D) and to a lesser extent the tier-1b-like CG7V (Fig. 3E). We noted a slightly more robust neutralization ability to smE660_CG7G (Fig. 3D) after additional vaccinations including gp120 and gp140 forms of Env (V4, V5). In addition, like the bAb, NAb titers, although low titers, were detectable ~1 year after the last vaccination, supporting persistency of systemic humoral responses.

Cellular immune responses induced upon intradermal and intramuscular delivery of Env DNA plasmids

Next, we examined the induction of Env-specific cellular immune responses. PBMC were stimulated with a SIVmac239 Env peptide pool (15-mer overlapping by 11 AA), and the production of IFN-γ was measured by intracellular cytokine staining followed by flow cytometry. The four ID/EP immunized animals showed positive responses, albeit with low frequencies, reaching 0.03–0.08% of SIV Env-specific IFN-γ^+ T cells 2 weeks after the 3rd vaccination with gp160 DNA (Fig. 4A). The Env-specific responses were mediated by both $CD4^+$ and $CD8^+$ T cells with transitional ($CD28^+$ $CD95^+$ $CCR7^-$) and effector ($CD28^-$ $CD95^+$ $CCR7^-$) memory T-cell phenotype (Fig. 4B). The Env-specific T lymphocytes harbored the cytotoxic marker granzyme B (GzmB) (Fig. 4C), a desired vaccine-induced T-cell response. Importantly, although low in magnitude in PBMC during the vaccination period, the T-cell responses were also long-lasting and could be detected in three of the four animals for ~1 year after the last vaccination (Fig. 4).

Discussion

In this exploratory study, we used a cocktail of plasmid DNAs expressing different forms (gp160, gp140, and gp120) of recently transmitted SIV Env and SIVmac239. We found that the ID/EP, as DNA vaccine delivery method, induces robust and durable humoral Env-specific immune responses. The antibodies induced by gp160 DNA vaccine reached levels that were not further increased by the subsequent vaccinations with different forms of Env, such as the primarily secreted gp120 and the trimeric gp140. These results are comparable to the data that we obtained from another study where the same DNA cocktail was delivered by IM/EP following a similar vaccination schedule, and the gp120 DNA vaccine led only to a small increase over the peak bAb induced by immunization with gp160 [29]. Thus, we conclude that the ID/EP method used herein induced maximal responses with the gp160 DNA vaccination in these animals. Comparing the magnitude of the bAb responses using different DNA vaccine delivery methods, such as the one described in this work, a different ID/EP delivery method [29], IM/EP [24, 29], or Vaxfectin® [30] showed overall similar magnitude of responses of ~4 log, using the same ELISA assay to determine the bAb titers.

To understand whether the inclusion of plasmids encoding several Env proteins in the DNA vaccine cocktail induced Ab with broader cross-reactivity, we

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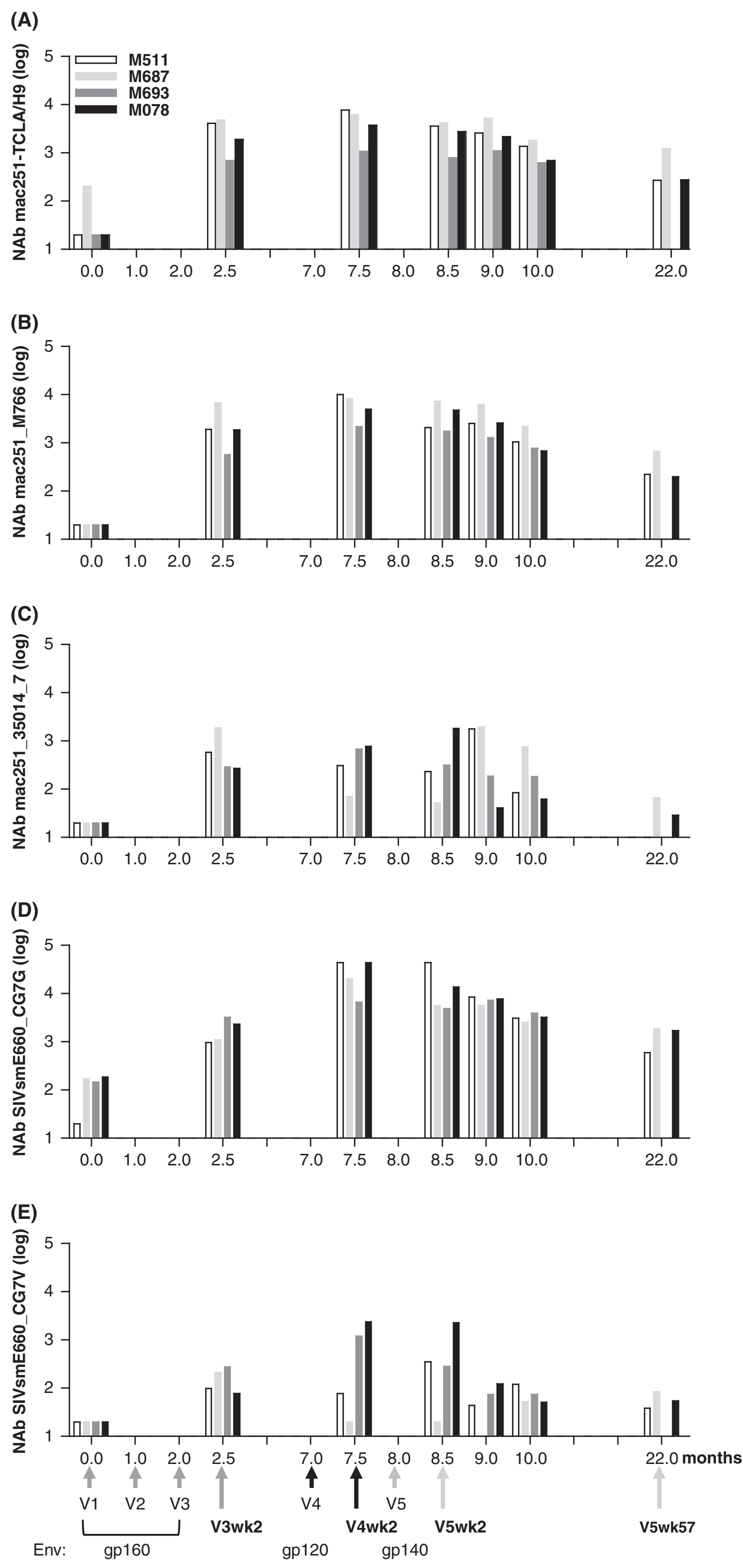

Fig. 3 Analysis of neutralizing antibody in macaques immunized via the ID/EP SIV DNA. Neutralizing antibody titers were determined against SIVmac251-TCLA were measured in M7-luc cells (**A**) and against pseudotyped viruses containing the following Env: mac251_35014 (M766) (**B**) and 35014_7 (**C**), the heterologous smE660 Env_CG7G (**D**), and CG7V (**E**) were measured using the TZM-bl assay. The animals were monitored for ~1 year after the last vaccination. 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.

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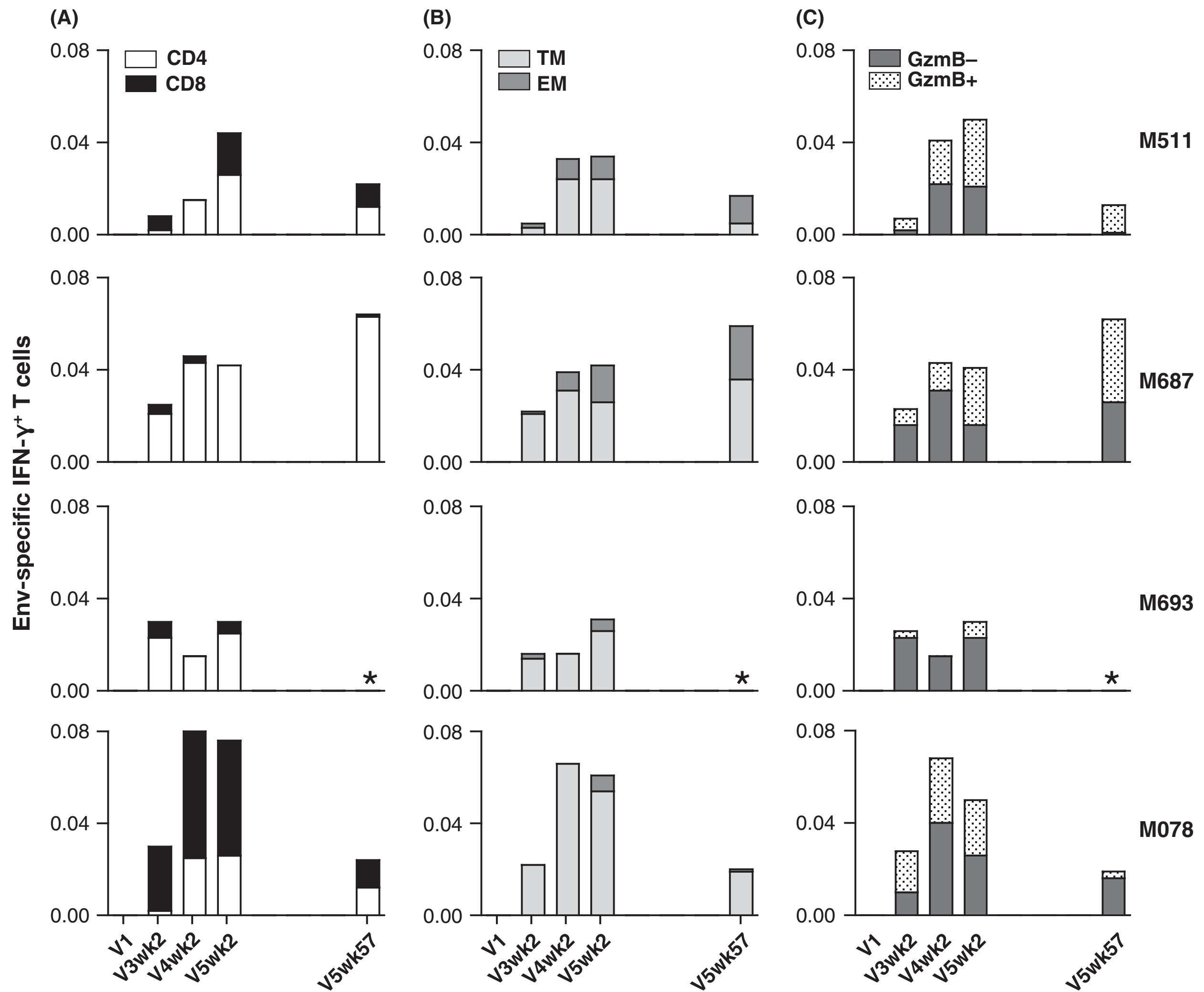

Fig. 4 Magnitude and longevity of cellular responses in macaques vaccinated via the ID/EP route. Cellular immune responses were measured in PBMC upon stimulation with a mac239-specific peptide pool (15-mer overlapping by 11 AA) at start of the study (V1), 2 weeks after vaccination with gp160 (V3 wk2), gp120 (V4 wk2), and gp140 (V5 wk2). The responses are shown as % SIV Env-specific IFN-γ^+ T cells among $CD4^+$ and $CD8^+$ (**A**); transitional (TM; $CD28^+$ $CD95^+$ $CCR7^-$) and effector memory (EM; $CD28^-$ $CD95^+$ $CCR7^-$) (**B**) T cells. The frequency of the Env-specific IFN-γ^+ granzyme B^+ T cells is shown (**C**). The animals were monitored for ~1 year after the last vaccination, and the cellular responses were measured at V5 wk57. Asterisk marks sample not available.

compared the neutralization results shown in Fig. 3 to our previously reported data of macaques vaccinated with SIVmac239 gp160 DNA delivered by IM/EP in the absence of IL-12 DNA as adjuvant [24]. Using the same panel of pseudotyped viruses, we found similar humoral responses and cross-reactivity using a mixture of different SIVmac251 Env delivered by ID/EP (this report) compared with only SIVmac239 Env delivered by IM/EP [24]. Interestingly, we noted a trend that the ID/EP delivery is more potent in inducing more consistent and higher NAb to the difficult to neutralize 'tier-1b-like' E660_CG7V, although these NAb titers were still very low. Thus, the delivery method to the skin may induce antibodies with better neutralization breadth. This observation needs to be further investigated using a bigger group of animals.

Another aim of this work was the evaluation of the longevity of the vaccine-induced immune responses. Here, we report that the humoral responses (bAb, NAb) elicited by ID/EP have persistent high titers and preserved avidity during the ~1 year of follow-up after the last vaccination. This is comparable to the reported long-lasting immunity obtained with other vaccine delivery methods, including IM/EP and Vaxfectin® [23, 24,

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29, 30, 40]. In contrast to the IM/EP delivery using the same DNA mixture [29], the method of ID/EP DNA delivery used in this report induced low levels of cellular immunity. However, despite the lower magnitude, this vaccine delivery method induced long-lasting Env-specific T-cell memory responses with cytotoxic (granzyme B^+) potential, a desired characteristic of a HIV/SIV vaccine. Similar to the antibodies, the cellular responses were still detectable at ~1 year after the last vaccination, demonstrating extended durability. Thus, the EP delivery of *env* plasmid DNA via ID route as well as via the IM route shares the unique features of inducing both humoral and cellular immune responses that have impressive durability in the vaccinated macaques.

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