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Persistent antibody and T cell responses induced by HIV-1 DNA vaccine delivered by electroporation

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Abstract

Intramuscular needle injection of HIV-1 DNA vaccines typically elicits weak immune responses in immunized individuals. To improve such responses, the immunogenicity of a vaccine consisting of electroporated DNA followed by intramuscular protein boost was evaluated in rabbits and macaques. In macaques, electroporation of low dose DNA encoding HIV-1 *env* followed by gp120 protein elicited Th1 cytokines and functional CTL that persisted for over 1 year. In both macaques and rabbits, robust anti-envelope antibodies, elicited by electroporated DNA, were augmented by gp120 protein and such responses neutralized sensitive SHIV isolates. These findings highlight efficient priming of immune responses by electroporated DNA that in conjunction with protein boost may give rise to long-term immunity in immunized hosts.

Keywords: HIV-1; Vaccine; Electroporation; Cellular immunity; Humoral immunity; DNA vaccines

Given that both arms of the immune system have been shown to be important for containment of HIV-1 infection, their induction remains a core requisite of vaccine development efforts [1,2]. We previously reported the results of studies highlighting induction of antibodies and T-cell responses using a polyvalent HIV-1 DNA prime/protein boost vaccine strategy [3–5]. From these studies and those of other laboratories, it is clear that DNA immunization by intramuscular (IM) or intradermal (ID) route elicits measurable but weak antibody and T-cell responses that can be boosted by other vaccine regimens including subunit proteins [6–9].

To enhance immunological priming by DNA immunization, several parameters including DNA delivery by electroporation have been investigated [10–12]. To-date, several electroporation platforms have generated encouraging data, yet, many of these studies have used constant voltage electroporators to facilitate plasmid delivery. Such instruments do not measure tissue resistance prior to and during electric pulse delivery and thus, may give rise to non-optimal plasmid expression, inflammation, and possible tissue damage. We therefore investigated the efficacy of the constant current electroporator, Cellectra™, previously shown to facilitate delivery and expression of plasmid DNA for therapeutic applications [13–15]. Our results demonstrate that electroporation of low dose HIV-1 *env* plasmid followed by gp120 protein yields Th1 and Th2 cytokines that persist for a 52-week period in macaques. Cellular responses include increased intracellular expression of perforin and cell surface CD107a in $CD8^+$ T lymphocytes. DNA electroporation also elicits stronger anti-gp120 binding antibodies compared to ID or IM administration. These antibodies can be further enhanced by gp120 protein boost and are capable of neutralizing sensitive viral isolates.

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Materials and methods

Antigens and peptides. CMV promoter-driven plasmids encoding codon optimized HIV-1 *env* (Ba-L) and p55 *gag* (96ZM651) genes used for electroporation were prepared as described [3,16]. For ID immunization of macaques, a similar CMV-driven plasmid encoding HIV-1 *env* (Ba-L) was used (gift from Marv Reitz, IHV, Baltimore, MD). Recombinant gp120 (clade B) expressed in stably transfected CHO cells, under control of the CMV promoter, was purified as reported [3,4,16]. Recombinant HIV-1 HXB2 p41 Gag protein was expressed and purified from transformed BL21 (DE3) *Escherichia coli* [3]. Proteins were formulated with QS-21 adjuvant (Antigenics Inc., Woburn, MA). HIV-1 Env (Ba-L) and Gag (HXB2) 15 mer peptides with 11 amino acid overlapping residues were synthesized (Infinity Biotech Research and Resource Inc., Aston, PA) and used as a single peptide pool (1 μg/ml).

Immunization protocols. Four New Zealand White rabbits were immunized with DNA encoding *env* at weeks 0, 4, 8, and 12. Group 1 (C2286, C2287) received intramuscularly administered DNA (1 mg) and Group 2 (C2288, C2289) received electroporated DNA (200 μg; Cellectra™, The Woodlands, TX). Both groups were immunized once with gp120 (100 μg) by IM route at week 21. Two cynomolgous macaques (516M, 520M) received electroporated DNA encoding *env* (250 μg) and *gag* (250 μg) genes at weeks 0, 4, 8, and 12 followed by protein boost via IM route, at week 21 with gp120 (75 μg) and p41 Gag (75 μg) proteins. Alternatively, two rhesus macaques (397L, 436L) were immunized ID at weeks 0, 4, 8, and 27 with 4 mg of plasmid DNA encoding *env* and boosted IM at week 40 with gp120 (300 μg).

Immunological assays. Serum samples were tested for Env gp120- and Gag-specific antibodies using an enzyme-linked immunosorbent assay (ELISA) as described [17]. Sera from immunized animals were tested for neutralization of $SHIV_{Ba\text{-}L}$ and $SHIV_{162P4}$ isolates using TZM-bl cells (NIH AIDS Research and Reference Reagent Program) as described [18]. IFNγ ELISPOT assays were performed with rhesus peripheral blood mononuclear cells (PBMC) [3]. Th1 and Th2 cytokines were quantitated from supernatants of unstimulated and peptide-stimulated PBMC using the cytometric bead array assay (BD Biosciences, CA) [19]. Intracellular levels of perforin and cell surface expression of CD107a were assessed in $CD3^+CD8^+$ T cells following peptide-stimulation of macaque PBMC. Cells were resuspended in complete RPMI (1×10^6 cells/ml) and were either left unstimulated or were stimulated for 6 h using Env (1 μg/ml) or Gag (1 μg/ml) peptide pools in the presence of CD28 (1 μg/ml) and CD49d (1 μg/ml) at 37 °C, 5% CO_2. Cell surface markers were assessed using anti-human CD3-APC, CD8-PE, and CD107a-PE-Cy5 antibodies (BD Biosciences, CA). Intracellular detection of perforin was performed using an anti-Pf-344-FITC (Mabtech, MarieMont, OH) antibody. The percentage of vaccine-specific $CD3^+CD8^+perforin^+$ or $CD3^+CD8^+CD107a^+$ cells was determined by subtracting background levels obtained in unstimulated samples from peptide-stimulated samples.

Results and discussion

Induction of cell mediated immune responses in macaques following electroporation of DNA vaccines and intramuscular protein boost

In order to evaluate cellular immune responses elicited by electroporated DNA, a study was initiated with two cynomolgous macaques. Both macaques received DNA encoding *env* and *gag* genes via electroporation followed by IM delivery of gp120 and Gag proteins in QS-21 adjuvant (Materials and methods). Env-specific IFNγ expression, as measured by ELISPOT, was found to be induced following three DNA immunizations (Fig. 1A, left panel). While a fourth DNA administration and IM protein boost did not further augment IFNγ levels, cellular responses were persistent up to week 62. To assess electroporation versus ID needle injection, the results of this study were compared to those of a previous study where two macaques were immunized ID with 4 mg of plasmid DNA encoding *env* and boosted with gp120 protein (Materials and methods). A sixteenfold greater dose of DNA (4 mg), delivered intradermally, elicited comparable or lower levels of IFNγ compared to DNA electroporation (Fig. 1A, right panel). Moreover, ID or IM needle injection of macaques with a polyvalent DNA vaccine containing 500 μg of HIV-1 Ba-L *env* followed by protein boost yielded markedly lower levels of IFNγ compared to levels noted in this electroporation study following priming and boosting phases [3]. The persistent ELISPOT responses, following electroporation, were also not observed previously using ID or IM immunization [3]. Consistent with the ELISPOT results, secreted Th1 (IL-2, TNFα, and IFNγ) but not Th2 (IL-4, IL-5, and IL-6) cytokines were elevated following DNA electroporation (Fig. 1B, upper panels). Enhancement of Th2 but not Th1 responses was observed following gp120 protein boost and cellular responses were found to persist for 1 year (Fig. 1B, lower panels). An immunophenotypic analysis, performed at week 65 of the study, suggests that this Env-specific recall response is of central memory phenotype and involves both $CD8^+$ and $CD4^+$ T cells (data not shown).

In addition to vaccine-induced Th1 and Th2 cytokines, other phenotypic markers, including perforin and CD107a, indicative of functional CTL activity, were evaluated following immunization. Env-specific increases in the percentage of $CD3^+CD8^+perforin^+$ (Fig. 1C) and $CD3^+CD8^+CD107a^+$ (Fig. 1D) CTL were detected in macaque M516 following four DNA immunizations. While similar responses were detected in macaque M520, levels were found to be lower (data not shown). As expected, perforin and CD107a were not detected in $CD3^+CD4^+$ T cells demonstrating the specificity of such measurements for CD8-mediated CTL activity (data not shown). Cellular responses, such as cytokine production, increased perforin and CD107a and central memory T-cells were also noted in Gag peptide-stimulated PBMC; however, these responses were weaker than those induced by envelope peptides (data not shown).

Anti-envelope binding and neutralizing antibodies induced by electroporated DNA

To evaluate whether electroporated DNA could enhance antibody responses as compared to IM delivery, rabbits were immunized as outlined (Materials and methods). Anti-gp120 antibodies were detected following a single administration of DNA by electroporation that was not observed when DNA was delivered by IM route (Fig. 2A, black arrow). Titers were found to increase following three additional DNA administrations (Fig. 2A, black arrow) by either route and were found to be significantly greater

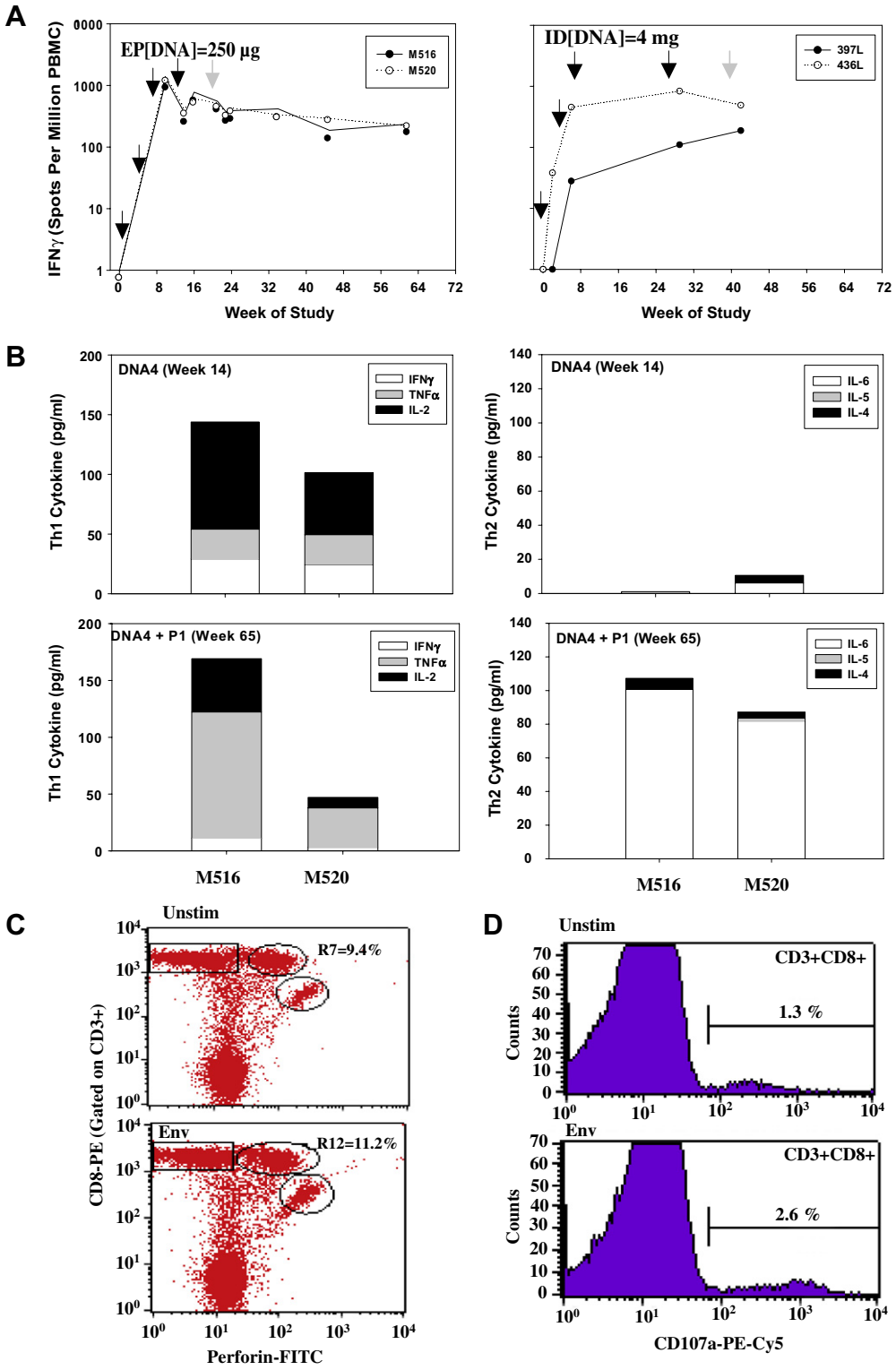
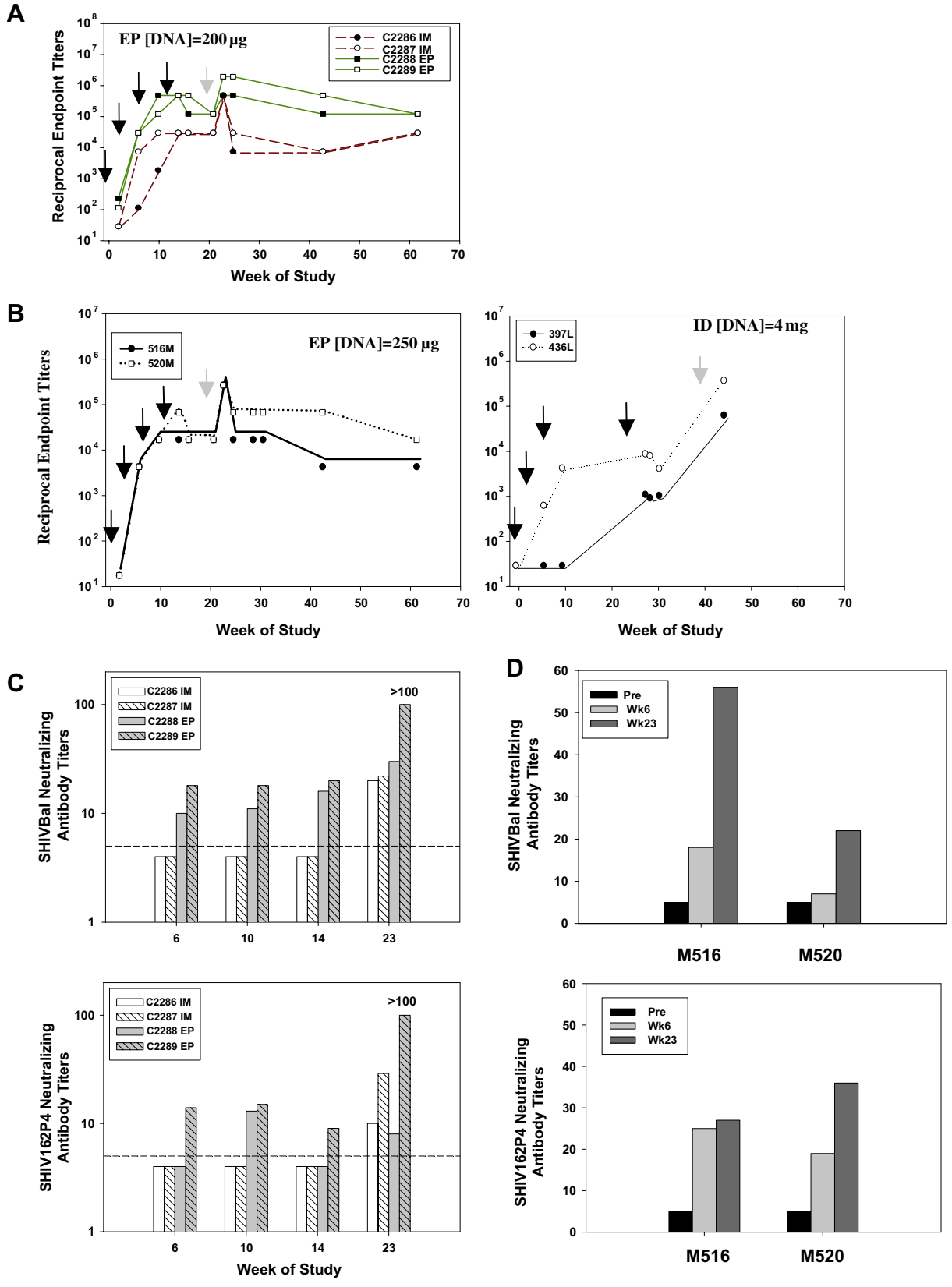


Fig. 1. Env-specific Th1 and Th2 cytokine production and functional CTL activity in rhesus macaques following immunization with electroporated DNA and intramuscular protein. PBMC of macaques primed with DNA (black arrows) delivered by electroporation (EP) or intradermal (ID) route and boosted with QS-21-formulated protein (gray arrows) were assayed for IFNγ production by ELISPOT (A). Th1 (TNFα, IFNγ, and IL2) and Th2 (IL4, IL5, and IL6) cytokines were quantitated from the supernatants of unstimulated and stimulated PBMC on weeks 14 and 65 (B). PBMC of macaques immunized with electroporated DNA and IM protein boost were assayed for induction of $CD3^+CD8^+perforin^+$ (C) and $CD3^+CD8^+CD107a^+$ (D) T cells. Representative dot blots are shown for cells (macaque M516) stimulated *ex vivo* with no peptide (Unstim) or Env peptide pool (Env). The percentage of intracellular $perforin^+$ cells is shown after gating on $CD3^+CD8^{bright}$ T cells for unstimulated (9.4%) and Env-stimulated (11.2%) samples (C). The percentage of $CD107a^+$ cells is shown after gating on $CD3^+CD8^+$ T cells for unstimulated (1.3%) and Env-stimulated (2.6%) samples (D).

($p < 0.0001$) in rabbits immunized by electroporation as compared to those immunized by IM route at 2 weeks post-final DNA immunization (week 14). This finding is of particular interest given that fivefold less DNA was used for immunization by electroporation (200 μg) as compared to IM (1 mg) delivery. A single administration of QS-21-



formulated gp120 protein by IM route (Fig. 2A, gray arrow) yielded antibody titers that were comparable in both DNA-primed groups. When rabbits were rested, antibody levels in the group primed with IM-delivered DNA declined with more rapid kinetics compared to levels observed in the electroporation group as evident from the titers observed on week 62 ($p < 0.0001$).

Since macaques were electroporated with plasmids encoding both *env* and *gag* genes, induction of antibodies to both antigens was assessed following immunization. Measurable anti-gp120 antibodies were detected following two DNA immunizations that were increased following a third immunization (Fig. 2B, left panel). Consistent with our findings in rabbits (Fig. 2A), a fourth DNA administration did not further augment antibody titers markedly. Anti-Env antibody titers were further enhanced following gp120 protein boost and persisted over the course of the next 41 weeks as demonstrated from titers measured on week 62. Similar induction of anti-Gag antibodies was also noted following DNA electroporation and recombinant p41 Gag protein boost although titers were lower and decayed at a faster rate than those observed following gp120 boost (data not shown). To assess antibody responses using different delivery methods, the results of this study were compared to a separate study described above where two macaques were immunized intradermally with 4 mg of DNA. Lower levels of anti-Env antibodies were observed in ID-injected animals (Fig. 2B, right panel) as compared to levels observed in macaques electroporated with DNA (Fig. 2B, left panel). However, following recombinant protein boost, antibody titers were augmented to similar levels in both groups. The level of antibody titers observed here following DNA electroporation was also found to be substantially higher than levels observed following IM or ID needle injection of a polyvalent DNA vaccine [3,4,16]. Although protein immunization boosted DNA-primed antibodies to comparable levels using either delivery method, a more rapid decay of antibody titers was observed in the ID/IM DNA-primed animals [4,16]. Functional properties of the immune sera were evaluated by assaying neutralization of homologous ($SHIV_{Ba\text{-}L}$) and heterologous ($SHIV_{162P4}$) isolates. Neutralizing antibodies to $SHIV_{Ba\text{-}L}$ (Fig. 2C, upper panel) and $SHIV_{162P4}$ (Fig. 2C, lower panel) were not detected in rabbit serum by week 14 following four IM DNA immunizations. However, such antibodies were clearly detected to both neutralization-sensitive isolates in the sera of rabbits immunized by electroporation following two DNA administrations. When DNA-primed rabbits were boosted with a single gp120 IM administration, neutralizing antibody titers increased in both DNA-primed groups, yet the levels were higher in rabbits where DNA was delivered by electroporation as compared to IM route. Similar neutralizing antibodies to $SHIV_{Ba\text{-}L}$ (Fig. 2D, upper panel) and $SHIV_{162P4}$ (Fig. 2D, lower panel) were detected in the serum of macaques following two DNA electroporations (week 6). In addition, these titers were further boosted at 2 weeks following an IM protein immunization (week 23). Although neutralizing antibodies were detected against homologous and heterologous isolates using this vaccine strategy, we do not believe that this response, elicited by a single Env immunogen, will broadly neutralize primary HIV-1 isolates. Future studies will evaluate whether this vaccine delivery approach, using modified envelope constructs or multivalent DNA and protein combinations, will elicit robust broadly reactive neutralizing antibodies.

To-date, plasmid DNA delivered by electroporation technology has yielded encouraging immunological data in HIV-1 vaccine research [10,11]. While other studies have shown either strong CMI or antibody responses, our study clearly demonstrates robust induction of both types of responses in macaques. Additionally, our study demonstrates that induction of immunological responses persists for a prolonged period. With respect to cellular immunity, this leads to the generation of central memory T cells. For these reasons, the current study supports and extends the findings of others where electroporation was used for efficient priming of immune responses by DNA vaccines. Electroporation devices used in several of these studies were primarily constant voltage electroporators. One of the advantages of the constant current electroporation technique, as used in this study, is that an impedance check to assess the individual tissue resistance is automatically performed by the electrodes before plasmid injection and electroporation occurs. This avoids loss of efficacy by injection into a sub-optimal location (blood vessel, adipose tissue, and fascia) and reduces subject-to-subject variability due to inadequate delivery.

Plasmids delivered by IM or ID routes can remain at the site of injection for prolonged periods [20–22]. Hence, lower doses of DNA would potentially facilitate clearance of plasmids from injection site more rapidly thereby resulting in lower risks of reactogenicity and toxicity. Although delivery of DNA by gene gun is highly effective in eliciting

Fig. 2. Binding and neutralizing antibodies induced in rabbits and macaques by HIV-1 vaccine comprised of electroporated DNA and intramuscular protein boost. ELISA (A,B) and neutralization (C,D) assays were performed using rabbit and macaque sera. Rabbits (A,C) and macaques (B, left panel; D) were immunized with electroporated DNA. Alternatively, macaques were immunized intradermally with DNA (B, right panel) at the time points indicated (black arrows). Protein was administered by IM route (gray arrow). Antibody titers are based on the dilution of immune serum producing two times the optical density at 450 nm compared with the corresponding dilution of preimmune serum (A,B). Neutralization assays were conducted against $SHIV_{Ba\text{-}L}$ (C,D; upper panels) and $SHIV_{162P4}$ (C,D; lower panels) isolates using TZMb1 cells. Titers are shown as dilution of immune serum showing 50% inhibition of infection compared to untreated controls. The lowest serum dilution tested in the assay was 1:5 as indicated (dotted line). For macaques (D) sera were assayed prior to immunization (pre) and on weeks 6 and 23 of the study.

immune responses with low dose of immunogen, such responses are often skewed towards Th2 responses [23,24]. In our study, Th1-polarized cytokine responses and CTL activity were elicited by low doses of electroporated DNA (Fig. 1). Further, optimal priming of immune responses could be achieved with as few as two administrations of DNA, thereby, making clinical development of an HIV-1 vaccine highly practical.

A successful prophylactic HIV-1 vaccine would elicit persistent humoral and cellular immune responses in immunized individuals. In the current study, antibody levels in non-human primates (Fig. 2) were found to persist for up to week 62 of the study. Similarly, the kinetics of antibody decline in rabbits (Fig. 2) was observed to be more rapid in animals immunized by IM route as compared to those immunized by electroporation. Long lasting cellular immune responses were similarly noted following electroporated DNA/protein boost vaccination, as measured by IFNγ (Fig. 1) production that persisted to week 65 of the study. Further, this persistence of CMI responses was indicative of central memory T cells capable of producing Th1 cytokines (data not shown). Taken together, these findings highlight that constant current electroporation can induce humoral and cellular immune responses using lower doses of DNA as compared to injection by IM or ID routes. Such responses are robust and persistent when combined with IM protein boost vaccination. Future studies with larger numbers of animals will evaluate whether such robust immune responses elicited by electroporation of DNA vaccines are capable of protecting macaques against pathogenic SHIV challenge.

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