Published in final edited form as:
Vaccine. 2009 September 4; 27(40): 5450–5459. doi:10.1016/j.vaccine.2009.07.005.

Administration of HPV DNA vaccine via electroporation elicits the strongest CD8+ T cell immune responses compared to intramuscular injection and intradermal gene gun delivery

Simon R. Best[1], **Shiwen Peng**[2,3], **Chi-Mou Juang**[6,7], **Chien-Fu Hung**[2,5], **Drew Hannaman**[8], **John R. Saunders**[1], **T.-C. Wu**[2,3,4,5], and **Sara I. Pai**[1]

[1] Department of Otolaryngology/Head and Neck Surgery, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[2] Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[3] Department of Obstetrics and Gynecology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[4] Department of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[5] Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[6] Department of Obstetrics and Gynecology, Taipei Veterans General Hospital, Taipei, Taiwan

[7] National Yang-Ming University, Taipei, Taiwan

[8] Ichor Medical Systems, Inc., San Diego, California, USA

Abstract

DNA vaccines are an attractive approach to eliciting antigen-specific immunity. Intracellular targeting of tumor antigens through its linkage to immunostimulatory molecules such as calreticulin (CRT) can improve antigen processing and presentation through the MHC Class I pathway and increase cytotoxic CD8+ T cell production. However, even with these enhancements, the efficacy of such immunotherapeutic strategies is dependent on the identification of an effective route and method of DNA administration. Electroporation and gene gun-mediated particle delivery are leading methods of DNA vaccine delivery that can generate protective and therapeutic levels of immune responses in experimental models. In this study, we perform a head-to-head comparison of three methods of vaccination – conventional intramuscular injection, electroporation mediated intramuscular delivery, and epidermal gene gun-mediated particle delivery - in the ability to generate antigen specific cytotoxic CD8+ T cell responses as well as anti-tumor immune responses against an HPV-16 E7 expressing tumor cell line using the pNGVL4a-CRT/E7(detox) DNA vaccine. Vaccination via electroporation generated the highest number of E7-specific cytotoxic CD8+ T cells, which correlated to improved outcomes in the treatment of growing tumors. In addition, we demonstrate that electroporation results in significantly higher levels of circulating protein compared to gene gun or intramuscular vaccination, which likely enhances calreticulin's role as a local tumor

Address correspondence to Sara I. Pai, MD, PhD, Department of Otolaryngology/Head and Neck Surgery, The Johns Hopkins University School of Medicine, CRB II Room 5M03, 1550 Orleans Street, Baltimore, MD 21231, USA. Phone: (410) 502-9825; Fax: (410) 502-2308; Email: spai@jhmi.edu.

anti-angiogenesis agent. We conclude that electroporation is a promising method for delivery of HPV DNA vaccines and should be considered for DNA vaccine delivery in human clinical trials.

Keywords

DNA vaccine; calreticulin (CRT); human papillomavirus (HPV); head and neck cancer; electroporation

INTRODUCTION

DNA vaccines are an attractive approach to eliciting antigen-specific immunity. DNA plasmids are relatively safe, cost efficient, and able to sustain reasonable levels of antigen expression within cells.[1,2] This endogenous antigen expression has been shown to induce broad cellular and humoral immune responses against the encoded antigens. In contrast to viral vectors, DNA vaccines do not elicit anti-vector immune responses in the vaccinated patient, and, therefore, are well suited for indications likely to require multiple administrations in order to achieve and maintain target immune responses. Furthermore, in contrast to recombinant proteins or peptides, DNA is relatively stable in cells and can provide long-term expression of the encoded immunogen which maybe favorable for the development of immunologic memory.[3–8]

Due to these advantages over conventional vaccines capable of inducing potent cellular responses, such as peptide or attenuated live pathogens, our laboratory has focused on the development of DNA vaccines to target cancers caused by the human papillomavirus type-16 (HPV-16), which include pre-malignant cervical intraepithelial neoplasia, squamous cell carcinoma of the cervix,[9] as well as a subset of head and neck squamous cell carcinomas (HNSCC).[10] The most promising vaccine candidate tested thus far uses an intracellular targeting strategy which conjugates the E7 antigen of HPV-16 to an immunostimulatory molecule, calreticulin (CRT).[11] The CRT/E7 DNA vaccine candidate has been shown to elicit higher levels of E7-specific cytotoxic CD8+ T cell precursors as compared to other strategies which link the HPV-16 E7 to the *Mycobacterium tuberculosis* heat shock protein 70 (HSP70),[12] or the translocation domain (domain II) of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)).[13]

Despite the application of various intracellular targeting strategies, the administration of DNA plasmids by conventional intramuscular needle injection still results in suboptimal immunogenicity, even when administered at high plasmid concentrations.[14] Therefore, various delivery strategies have been investigated, including co-administration with immunomodulatory molecules,[15] formulation with cationic lipids,[16] the use of polymers for controlled release of DNA plasmid,[17, 18] as well as evaluation of alternative routes and methods of DNA administration. The delivery method utilized during the development of the CRT/E7 DNA vaccine was the particle-mediated epidermal delivery system using a needleless gene gun system. [19] This method of delivery proved superior to other methods tested while using a relatively low dose of administered DNA plasmid (2 μg). The advantage to gene gun administration of a DNA plasmid is the targeting of intradermal Langerhans cells and other professional antigen-presenting cells[20, 21] which are able to migrate through the lymphatic system to draining lymph nodes, where they can prime antigen-specific T cells. Although it has not yet been tested using a vaccine targeting HPV antigens, another promising method of DNA vaccine delivery is electroporation. Electroporation involves the administration of a DNA plasmid into a target tissue, followed by the application of brief electrical pulses at the site of DNA distribution. A transient increase in the permeability of the plasma membrane induced by the electrical current allows increased uptake and, thus, expression of the DNA plasmid.[22, 23] Used most commonly for DNA vaccine delivery in skeletal muscle, the procedure not

only increases uptake of plasmid by myocytes and, thus, protein expression levels of the target antigen, but is also associated with upregulation of pro-inflammatory cytokines and recruitment of monocytes/macrophages to the site of vaccination, which can enhance antigen presentation to the immune system.[24]

The goal of this study is to perform a head-to-head comparison of three methods of vaccination – conventional intramuscular needle injection, electroporation mediated intramuscular delivery, and particle mediated epidermal delivery via gene gun - using a uniform dose of an HPV DNA vaccine across all vaccination methods in an *in vivo* E7-expressing tumor model. With a goal of translation into human clinical trials, we believe that the administered dose of the DNA vaccine in this preclinical model should reflect doses that can be feasibly escalated to human equivalency. Therefore, this study utilized a relatively low dose of DNA plasmid (2 μg), which we have previously demonstrated to be effective with gene gun delivery of DNA vaccines encoding HPV antigens. [19] Of note, due to limitations in the dose capacity inherent to the gene gun, this also represents the largest dose deliverable with a single injection in the clinical setting. This dose is also appropriate for evaluation of intramuscular delivery because 2 μg, when adjusted for body weight from mouse to human is within the dose range of current human Phase I clinical trials utilizing DNA vaccines delivered either by conventional intramuscular injection or electroporation.[25, 26] By using the same dose across all vaccination methods, we are able to perform direct comparisons of each technique rather than using different doses optimized for each method of administration which has been done in previous studies. After administration of the CRT/E7 DNA vaccine, we evaluate the humoral immune responses, antigen-specific cytotoxic T cell responses as well as anti-tumor immune responses elicited by each method of vaccination in *in vivo* tumor protection and tumor treatment studies. In addition, we compare the levels of antigen expression over time obtained by the various vaccination methods using a luciferase reporter system to evaluate its contribution to sustained immunologic responses.

RESULTS

Luminescence is higher when pCDNA3-luciferase plasmid is administered by electroporation compared to intramuscular injection

Administration of a luciferase DNA plasmid and subsequent luminescent imaging is a verified method of quantitatively evaluating the efficacy of gene transfer following electroporation. [27] The electroporation system that we utilized was the TriGrid™ Delivery System (TDS) developed by Ichor Medical Systems, Inc (San Diego, CA). The TDS electrode array consists of four electrodes arranged in two interlocking triangles around a central injection needle. With the configuration and dimensions of the electrode array selected to correspond to the fluid distribution pattern characteristic of the target tissue, co-localization of the DNA and electrical fields is facilitated. As verification of our administration techniques and validation of the TriGrid™ System, we investigated the effect of electroporation upon *in vivo* expression of a luciferase encoding plasmid compared to intramuscular injection. Since one of the goals of this study is to evaluate electroporation as a method of administration in comparison to the gene gun, we chose 2 μg of DNA plasmid as our reference dose, since this dose has been shown to have therapeutic anti-tumor responses in preclinical models.[19] We administered 2 μg of DNA plasmid by intramuscular injection and electroporation to compare levels of gene expression. Gene gun was excluded from this specific experiment as dermal expression of DNA plasmid would not be directly comparable to electroporation's intramuscular expression given the external measurement by a CCD camera.

DNA plasmid was delivered to the tibialis muscle of mice via electroporation and intramuscular injection, and the anesthetized mice were imaged one hour after plasmid delivery. These mice were then reimaged at one, seven, fourteen, and twenty-eight days after plasmid administration.

Figure 1(a) demonstrates the fusion images obtained from the optical and CCD camera. Figure 1(b) quantitates the maximal luminescence, and shows the sustained increase in luminescent intensity achieved by electroporation as compared to conventional intramuscular injection. At all time points, this difference in intensity was one to two-log higher in the electroporation group as compared to the intramuscular group. These results indicate that delivery of the luciferase plasmid by electroporation results in a higher efficacy in gene transfer as compared to conventional intramuscular injection. In addition, gene expression can be detected in as little as one hour after DNA administration and can be sustained as long as 28 days with electroporation mediated gene delivery as compared to intramuscular administration.

Electroporation results in higher, sustained levels of circulating protein than other methods of DNA administration

Circulating levels of calreticulin from CRT/E7 DNA vaccination have been shown to exert a tumor anti-angiogenesis effect independent of immune cell response. [28, 29] Therefore, we measured the levels of circulating protein after DNA administration using different methods. Direct comparisons by using the same plasmid dose (2 μg) among the various groups (intramuscular, electroporation, and gene gun) was performed and circulating secreted alkaline phosphatase (SEAP) levels was evaluated after pSEAP2 plasmid administration. [30]

Several studies demonstrated that 2 μg of reporter gene delivered by gene gun resulted in detectable expression levels as early as 24 hours and persisted up to 7–10 days.[31, 32] A previous study in mice had utilized 10 μg of SEAP plasmid to study electroporation-mediated intramuscular plasmid delivery;[30] therefore, both a 2 μg and 10 μg dose were used to verify experiment technique and results. Three mice were assigned to each group, including a control group which received no plasmid. Serum was extracted from mice seven days after DNA administration. The pooled results from each group, including naïve mice, are shown in Figure 2.

Mice receiving conventional intramuscular (48 ± 2 ng/mL) administration of 2 μg of pSEAP2-Control plasmid achieved serum levels of SEAP that were not significantly different from naïve mice (32 ± 17, $p = 0.27$). Gene gun (118 ± 8 ng/mL, $p = <0.001$) delivery resulted in detectable serum levels that were higher than control mice. However, mice administered plasmid by electroporation (1224 ± 469 ng/mL, $p = 0.001$) achieved the highest levels of serum protein expression on day 7 after vaccination. When the dose of administered plasmid was increased to 10 μg, the intramuscular group achieved serum levels statistically higher than naïve mice (64 ± 8 ng/mL, $p = 0.018$). Gene gun (121 ± 21 ng/mL) delivery at 10 μg of DNA did not show any significant difference as compared to the group which received 2 μg of DNA ($p = 0.82$). This is not unexpected since most of the DNA-transfected cells would have likely sloughed off by day 7. Thus, dose dependent gene expression would not likely be observed in the cohort of mice vaccinated via gene gun. However, electroporation mediated delivery demonstrated a dose response between the group which received 2 μg of DNA as compared to the group which received 10 μg of DNA (5048 ± 1452 ng/mL, $p = <0.001$), with a detectable five-fold increase in serum levels. These results establish that electroporation is superior to either conventional intramuscular injection or gene gun in generating sustained, circulating levels of protein when equivalent doses of DNA plasmid are administered.

Gene gun vaccination results in higher levels of circulating E7-specific antibody responses as compared to other methods of vaccination

We also compared the circulating levels of E7-specific antibodies generated by the various vaccination methods. A cohort of mice (5 per group) were inoculated subcutaneously with an E7-expressing tumor cell line, TC-1, and vaccinated on Day 3 after tumor inoculation with 2 μg of pNGVL4a-CRT/E7(detox) DNA plasmid via conventional intramuscular injection,

electroporation, or gene gun. Subsequently, mice received a booster vaccination with pNGVL4a-CRT/E7(detox) DNA plasmid in alternating hind legs on Days 6 and 9 for a total of three vaccinations. Serum from these mice was isolated fourteen days after the last vaccination and analyzed for circulating levels of antibody which recognized the E7 protein by ELISA.

Figure 3 depicts serial dilutions of pooled sera from the vaccinated mice, and demonstrates that both gene gun vaccination (1:100 dilution, mean value 0.566, $p<0.05$) and electroporation (0.499, $p<0.05$) generate significantly higher levels of E7-specific antibodies than control mice (0.109, $p<0.05$) or those vaccinated by intramuscular injection (0.135, $p<0.05$). However, gene gun vaccination generates the highest levels of E7-specific antibodies as compared to electroporation or intramuscular injection (all p values <0.05). These results suggest that epidermal delivery with the gene gun is the most effective method of vaccination for generating circulating E7-specific antibodies following delivery of the pNGVL4a-CRT/E7(detox) DNA vaccine at the dose and immunization schedule tested.

pNGVL4a-CRT/E7(detox) DNA vaccine administration via electroporation generates the greatest levels of E7-specific CD8+ T cells

We then compared the ability of the pNGVL4a-CRT/E7(detox) DNA vaccine to elicit E7-specific CD8+ T cells by performing intracellular cytokine staining on splenocytes obtained from vaccinated mice. 2 μg of DNA plasmid was administered to all groups through various methods, and we performed a direct dose comparison of immune stimulation after DNA administration. Splenocytes from naïve or vaccinated groups of mice were incubated with or without the MHC class I (H-2 D^b)-restricted E7 peptide (amino acid 49–57) to assay for E7-specific CD8+ T cells. Five mice were used in each group, and experiments were performed in triplicate.

As shown in Figure 4, mice vaccinated with 2 μg of DNA plasmid via electroporation ($208 \pm 50/3\times10^5$ splenocytes, $p = 0.002$) and gene gun ($88 \pm 15/3\times10^5$ splenocytes, 0.0005) exhibited higher numbers of E7-specific CD8+ T cells as compared to naïve mice ($9 \pm 2/3\times10^5$ splenocytes) and mice vaccinated by conventional intramuscular injection ($35 \pm 14/3\times10^5$ splenocytes, $p = 0.09$). In addition, electroporation was better than gene gun ($p = 0.045$) in generating the greatest number of E7-specific CD8+ T cells. Our data suggests that electroporation may be an effective method of vaccination in generating E7-specific CD8+ T cells when administered at the DNA dose and immunization schedule tested.

Mice vaccinated via electroporation or gene gun display superior protection against E7-expressing tumors

We then challenged mice with E7-expressing tumor cells after vaccination with the pNGVL4a-CRT/E7(detox) DNA plasmid which was administered by various methods. Briefly, C57BL/6 mice (five in each group) were vaccinated with 2 μg of pNGVL4a-CRT/E7(detox) DNA plasmid via conventional intramuscular injection, electroporation or gene gun. Mice received a booster vaccination on Day 7 and were inoculated with 5×10^4 TC-1 tumor cells subcutaneously on Day 14, and then were followed for palpable tumor development.

Figure 5 shows the tumor free survival of mice expressed as a Kaplan-Meier plot. Vaccination by electroporation and gene gun resulted in almost complete tumor protection, whereas the majority of mice in the intramuscular and naïve groups developed palpable tumors. Electroporation and gene gun were both superior to intramuscular injection ($p = 0.01$, $p = 0.01$). This data indicates that pNGVL4a-CRT/E7(detox) DNA vaccination via electroporation and gene gun are both superior to intramuscular injection in providing protection against the growth of E7-expressing tumors.

Mice vaccinated via electroporation display superior anti-tumor responses against growing E7-expressing tumors

The most stringent model of vaccine efficacy is *in vivo* activity against a growing tumor. Therefore, we compared the activity of the pNGVL4a-CRT/E7(detox) DNA plasmid against an E7-expressing tumor cell line, TC-1, using the different methods of vaccination. Briefly, mice were inoculated with 5×10^4 TC-1 tumor cells on Day 0, and subsequently vaccinated on Day 3 with 2 μg of pNGVL4a-CRT/E7(detox) DNA plasmid via intramuscular injection, electroporation or gene gun. Subsequently, mice received a booster vaccination with pNGVL4a-CRT/E7(detox) DNA on Days 6 and 9 in alternating hind legs for a total of three vaccinations. This form of cluster vaccination has been found more effective in controlling tumors than vaccinations separated at one week intervals.[33] Tumor growth was then measured twice a week and tumor volumes calculated. Mice were euthanized according to the animal care protocols at Johns Hopkins University School of Medicine when tumors exceeded the size criteria outlined by the ethic protocols.

The results in Figure 6 show that mice in the naïve group experienced rapid and exponential tumor growth, which was delayed by only three days in the intramuscular group. All mice in these groups had to be euthanized between days 27–30 due to the bulky size of the tumor. Both the electroporation and gene gun groups demonstrated potent anti-tumor responses. However, the electroporation group seemed to elicit a sustained anti-tumor response as compared to the gene gun group since differences in tumor volume between the two groups was observed starting on Day 30 and reached statistical significance on Day 38 ($p = 0.045$). Mice in the gene gun group had stabilized controlled growth of their tumors within the first 30 days of vaccination; however, acceleration in their tumor growth was observed between days 38–42 which required their subsequent euthanasia by Day 42. In the electroporation group, three of five mice achieved complete tumor regression by Day 27 with no evidence of palpable tumor. Two of these mice eventually recurred, but one mouse achieved complete tumor response until the termination of the experiment at Day 60. This finding differs from the other vaccinated groups since this complete response was not observed in any other group. Interestingly, the remaining two mice in the electroporation group exhibited progressive tumor growth requiring euthanasia at Day 42 following initial disease stabilization, possibly reflecting an underlying variability in immune susceptibility of a heterogenic tumor population or a need for a booster vaccination at a later time point.

DISCUSSION

We performed a head-to-head comparison of three different methods of administering an HPV DNA vaccine in order to determine the most effective method of delivery for this vaccine candidate before initiating human clinical trials. Instead of utilizing traditional “known effective doses” for each vaccination method, a uniform dose of 2 μg of pNGVL4a-CRT/E7 (detox) DNA plasmid was administered at a single site, thereby facilitating direct comparisons among the various administration delivery methods. Although a uniform dose of 2 μg was administered to tissue, we acknowledge that there may be slight variations in the quantity of DNA transfected into cells which is inherent to the various administration methods.

The 2 μg dose provides a relevant basis for comparison of delivery methods since it is known to be an effective therapeutic dose in mice when delivered using the gene gun and is the largest dose that can be delivered with a single gene gun administration. In addition, induction of cellular and humoral immune responses has been demonstrated in humans with multiple DNA vaccines, including an influenza HA encoding DNA vaccine[34, 35] and hepatitis B surface antigen vaccine,[36] following gene gun administration at DNA doses ranging from 1–4 μg.[34, 36,37]

Induction of antigen specific cellular and humoral responses has also been demonstrated in multiple human clinical trials of DNA immunization by conventional intramuscular injection using a variety of antigens.[38–41] However, in contrast to gene gun delivery, induction of immunological responses in humans has required intramuscular administration of DNA doses in the range of 0.5 – 5 mg and protection against disease in humans has not yet been demonstrated. While electroporation has clearly enhanced DNA vaccine potency in a broad range of animal models and multiple clinical trials of electroporation mediated DNA vaccine delivery are now ongoing (reviewed in [42]), there is limited data regarding immune responses that has been published to date.

Therefore, intramuscular delivery methods (conventional injection and electroporation based delivery) were also evaluated at the 2 μg dose in mice since this dose corresponds, on a body mass basis, to the dose range of completed and ongoing human Phase I clinical trials utilizing intramuscular delivery of DNA vaccines[25, 26,38–41]. Although there remains uncertainty regarding the ability of rodent species to predict vaccine responses in the clinical setting, effective induction of anti-E7 specific immune responses at this DNA dose would provide encouragement regarding the potential for extrapolation into the clinical setting.

Based on multiple immunologic parameters, our data indicates that electroporation is an effective method of administering the pNGVL4a-CRT/E7(detox) DNA plasmid. Electroporation generates the greatest levels of E7 specific cytotoxic CD8+ T cell precursors when compared to other methods of vaccination, including gene gun. For conventional intramuscular injection, previous studies often required 50 μg of DNA in order to generate robust numbers of CD8+ T cells;[19] therefore, our finding that 2 μg of DNA plasmid administered by intramuscular injection did not generate significant numbers of antigen specific CD8+ T cells was not unexpected. Both electroporation and gene gun demonstrated superior tumor protection compared to conventional intramuscular injection. Furthermore, we found that administration of DNA vaccine via electroporation seemed to maintain a sustained anti-tumor response as compared to the gene gun group since differences in tumor volume between the two groups was observed starting on Day 30. Mice in the gene gun group had stabilized controlled growth of their tumors within the first 30 days of vaccination; however, acceleration in their tumor growth was observed between days 38–42 which required their subsequent euthanasia by Day 42. In contrast, in the electroporation group, three of five mice achieved complete tumor regression.

While the improved outcomes observed with electroporation mediated delivery are most likely due to the increased CD8+ T cell response induced following immunization, the mechanisms underlying the observed differences in immunogenicity between electroporation and the gene gun have not been definitively established. Nonetheless, it is likely that differences in antigen expression, presentation, and processing between the different cell types transfected in skin and muscle tissue are important factors.

With regards to antigen expression, we and others have demonstrated that electroporation based DNA intramuscular delivery results in higher and more persistent levels of protein expression using SEAP and luciferase.[27] In our study, we detected antigen expression as early as one hour after vaccination and high levels of expression were maintained up to 28 days. The increased levels of expression are a function not only of the efficient delivery associated with electroporation, but also the inherent mitotic stability of skeletal myofibers compared to the relatively rapid turnover of cells of the epidermis. It is possible that the quick onset and sustained high levels of antigen expression associated with electroporation may be favorable for a higher magnitude of CD8+ T-cell responses and the development of immunologic memory in the setting of processing of the E7 antigen through the cross priming pathway where high levels of antigen production are likely to be favorable.[43]

In contrast to the CD8+ T-cell response, we found that the gene gun was superior to electroporation or conventional intramuscular injection for induction of an E7-specific antibody response. These results are consistent with some previous comparisons of antibody responses induced by electroporation or gene gun based DNA vaccine delivery,[44] but not others,[45] indicating that the optimal method of administration may be antigen specific. The observed differences could arise due to differences in immunological presentation intrinsic to muscle and skin. It is also possible that antigen expression in different cell types could lead to differences in the glycosylation and/or folding of the antigen, thereby affecting the induction of antibody responses cross reactive to the native antigen. Finally, it should also be noted that the immunization regimen used for assessment of antibody responses (2 μg dose at Days 0, 3, and 6), was optimized for gene gun delivery. Given the differences in kinetics of antigen expression with electroporation and gene gun, it is possible that the antibody responses achieved with electroporation could be improved through the use of alternative immunization schedules.

With regards to CD4+ T cell responses, we have previously published results indicating an inability to successfully detect a measurable increase in the number of E7-specific IFN-γ secreting or IL-4 secreting CD4+ T cells in mice vaccinated with CRT/E7 DNA via gene gun. [43] However, vaccination with CRT/E7 DNA via gene gun did result in significantly high titers of anti-E7 antibody in the sera of mice which corroborates our findings in this study. Although the mechanism for enhancement of antibody response is not clear, we cannot exclude the possibility that there is T helper cells generated against CRT, although we are not able to measure it through our current assays.

In addition to differences in immunogenicity between the different delivery methods, the higher levels of circulating protein achievable with electroporation mediated intramuscular delivery of the DNA may also make this administration method uniquely well suited for use with the pNGVL4a-CRT/E7(detox) DNA vaccine due to the incorporation of the calreticulin molecule. Calreticulin has been shown to have an anti-tumor effect independent of immune cell function in CD4+/CD8+ depleted mice.[43] Immune deficient mice vaccinated with the pNGVL4a-CRT/E7(detox) DNA demonstrated anti-tumor responses which were attributed to calreticulin's anti-angiogenesis effect.[32] Therefore, it is possible that elevated circulating calreticulin levels in our electroporation group may have also contributed to improved anti-tumor responses independent of immune cell function.

Delivery of DNA vaccines using electroporation has already been tested successfully in a wide range of disease models. Electroporation has been used to enhance immune responses using DNA vaccines directed against infectious diseases (such as Influenza,[44] HIV,[46, 47] Hepatitis C,[48] malaria,[49] anthrax,[50] and others) or to treat or prevent the development of tumors. Breast cancer, [51, 52] prostate cancer, [53] and melanoma[54] are examples of tumor models where the development of cytotoxic CD8+ T cells is essential for cancer control. In our study, we show that electroporation is also an effective vaccination technique for the treatment of HPV induced cancers using the pNGVL4a-CRT/E7(detox) DNA vaccine. Both the generation of cytotoxic CD8+ T cells and circulating protein contribute to tumor control in this model, and we and others have concluded that due to these properties, electroporation is a mode of vaccination of sufficient interest to translate into human clinical trials.[55] The electroporation device that we used in this study is the TriGrid™ delivery system (TDS) (Ichor Medical Systems, Inc, San Diego, CA), an available commercial device which is now being used in multiple ongoing Phase I clinical trials. These include delivery of a DNA vaccine encoding a melansomal antigen in patients with Stage IIB – IV melanoma (www.clinicaltrials.gov ID#NCT00471133) as well as a study comparing TDS-IM and conventional intramuscular administration of a preventative HIV vaccine candidate in healthy volunteers (www.clinicaltrials.gov ID# NCT00545987). This is the first study that directly compares electroporation mediated intramuscular delivery

to gene gun using the same dose of DNA vaccine delivered at a single site. Three previous studies in which electroporation and gene gun were compared varied the number of administration sites and used 16 to 100 times the amount of DNA vaccine dose for the electroporation groups.[44, 45, 51] While this approach is useful for evaluation of the *maximal* efficacy of a given vaccine method, conclusions regarding relative performance are limited by the inability to determine if observed differences in treatment outcomes are attributable to the delivery method or dosage. Therefore, we have conducted this study using a consistent 2 μg of pNGVL4a-CRT/E7(detox) DNA plasmid dose across all techniques. By demonstrating the activity of the vaccine candidate at a relatively low dose level that can be extrapolated into larger species, we are also well positioned to translate these findings into the clinical setting. Based on its ability to induce high levels of antigen expression and potent, antigen specific cellular immune responses, we conclude that electroporation is an effective method of delivery of the pNGVL4a-CRT/E7(detox) DNA vaccine and holds great promise for the administration of other DNA vaccines targeting oncogenic HPV-16 antigens.

MATERIALS AND METHODS

Mice

Six to eight week old C57BL/6 female mice were purchased from the National Cancer Institute-Frederick Animal Production Area (Frederick, Maryland) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals.

DNA Vaccines

The construction of the CRT/E7 plasmid DNA vaccine (pNGVL4a-CRT/E7(detox)) used in this study has been described previously.[19] Briefly, the plasmid backbone, pNGVL-4a, obtained from the NIH National Gene Vector Laboratory is a human approved vector which encodes a kanamycin resistance gene, a transcription unit consisting of a CMV promoter, multi-cloning site, and a poly-A tail. In addition, the plasmid includes two immunostimulatory sequences consisting of tandem repeats of a CpG dinucleotide.[56] Human calreticulin (CRT) and HPV-16 E7 oncogenic protein DNA sequences were cloned into this plasmid, with amino acid substitutions at positions 24 (cysteine to glycine) and 26 (glutamic acid to glycine) of E7 which ablates the Retinoblastoma protein binding site and, thus, prevents malignant transformation of transfected cells. pCDNA3-Luciferase DNA plasmid was a generous gift from Dr. Hyam I. Levitsky (Johns Hopkins Medical Institutions, Baltimore, MD). Luciferin substrate was obtained commercially (potassium salt; Xenogen Corp, Alameda, CA).

The DNA plasmid encoding secreted alkaline phosphatase (SEAP) was obtained from Clontech (Mountain View, CA) along with the Great EscAPe Reporter System 3 for serum detection of expressed SEAP. The pSEAP2-Control vector was used as a control vector.

Electroporation mediated DNA vaccination

The Ichor TriGrid™ Electroporation Delivery System (TDS) (Ichor Medical Systems, Inc., San Diego CA) consists of an electrode array, a pulse generator, and a foot pedal for pulse activation. Disposable 30G needles (Becton-Dickinson, Franklin Lakes, NJ) are used in the center of the electrode grid to administer DNA plasmid equidistant from each of the four electrode probes. Mice were injected in the tibialis muscle of the shaved hind leg with activation of the pulse generator as instructed by the manufacturer and as previously described.[57] The appropriate concentration of DNA plasmid was diluted in a total volume of 20 uL of PBS. When vaccination schedules required a booster vaccination, the contralateral leg was used for vaccination, and subsequent vaccinations used alternating hind legs.

Needle intramuscular mediated DNA vaccination

For intramuscular (IM) injections, the tibialis muscle was shaved and exposed in a similar manner to electroporation vaccination. Using a disconnected electroporation grid with a 30G needle (Ichor Medical Systems, Inc. San Diego, CA) 50 μL of diluted plasmid was delivered into the tibialis muscle without activation of the pulse generator. Full insertion of the electrode grid was performed identical to the method of delivery for electroporation to achieve consistent intramuscular injections. Similar to mice vaccinated via electroporation, the contralateral tibialis muscle was used for booster vaccinations as indicated in the vaccination protocol.

Gene gun mediated DNA vaccination

DNA-coated gold particles were delivered to the shaved abdominal region of mice using a helium-driven gene gun (BioRad, Hercules, CA) with a discharge pressure of 400 psi.[19] DNA bullets were created so that the total vaccination dose of DNA was divided between two bullets for each set of vaccinations. Subsequent boosts were administered to areas of fresh epidermis to avoid the scar created from previous sites of vaccinations.

Luciferase expression and kinetics

Mice were vaccinated with 2 μg of pCDNA3-Luciferase plasmid in the left leg, either by intramuscular injection or electroporation, using the techniques described above. As a control, the contralateral leg received an injection of PBS containing no plasmid.

Fifty minutes after vaccination, 200 uL of Luciferin diluted in PBS (3.9 mg/mL) was injected intraperitoneally. After ten minutes, mice were anesthesized with Isoflurane and optical imaging performed with a CCD camera and black box at the Small Animal Imaging Resource Program (SAIRP) at Johns Hopkins Hospital (Baltimore, MD). The duration of luminescence acquisition was 120 seconds. The maximal level of luminescence was acquired from a region of interest drawn over the luminescent zone on the optical image. Luciferin injection and optical imaging were repeated at one, seven, fourteen, and twenty-eight days after vaccination.

Secreted alkaline phosphatase (SEAP) expression

Mice were vaccinated by intramuscular injection, electroporation, or gene gun with either 2 μg or 10 μg of secreted alkaline phosphatase (SEAP) or pSEAP2-Control plasmid using the protocols described above. Seven days after vaccination, serum was extracted from the tail vein. Using the Chemiluminescent SEAP Assay (Clontech, Mountainview, CA), 3 μL of serum was used to measure SEAP concentration with a 96 well plate luminometer (Molecular Devices, Sunnyvale, CA). The serum chemiluminescent reaction was measured in triplicate for each mouse.

Detection of E7-specific antibodies in the serum of vaccinated mice

The presence of anti-HPV-16 E7 antibodies in the sera was characterized by a direct Enzyme Linked Immunosorbent Assay (ELISA) as described previously.[58] A cohort of mice were immunized with 2 μg of pNGVL4a-CRT/E7(detox) vaccine by intramuscular injection, electroporation, or gene gun three days after tumor challenge and received two booster vaccinations at days six and nine. Sera were prepared from mice on day fourteen after the last vaccination. The ELISA plate was read with a standard ELISA reader at 450 nm using serial dilutions of prepared sera.

Intracellular cytokine staining with flow cytometric analysis to detect IFN-γ secretion by E7-specific CD8+ T cells

Cell surface marker staining for CD8 and intracellular cytokine staining for IFN-γ as well as FACScan analysis were performed using conditions described previously.[8] Eleven mice in

each group (intramuscular injection, electroporation, gene gun) were vaccinated with 2 μg of pNGVL4a-CRT/E7(detox) and received a booster vaccination one week later. One week after the last vaccination, splenocytes were collected and incubated for 20 hours either with or without 1 μg/mL of MHC class I restricted E7 peptide (amino acids 49–57, RAHYNIVTF). The number of IFN-γ-secreting $CD8^+$ T cells was analyzed using flow cytometry. Analysis was performed on a Becton-Dickinson FACScan with CELLQuest software (Becton-Dickinson Immunocytometry System, Moutain View, CA).

In vivo tumor protection

For all tumor experiments, an HPV-16 E6 and E7 expressing cell line, TC-1, was used. The generation of this cell line has been previously described.[59] Five mice per group were vaccinated with 2 μg of pNGVL4a-CRT/E7(detox) DNA vaccine via intramuscular injection, electroporation, or gene gun followed by a booster vaccination one week later using the same method of vaccination. One week after the last vaccination, mice were challenged subcutaneously with 5×10^4 TC-1 cells/mice including five naïve mice who did not receive any vaccination. All mice were followed for 60 days to monitor tumor progression. Tumor growth was determined by direct palpation twice a week and the percentages of tumor free mice were recorded.

In vivo tumor treatment

Five mice per group were challenged subcutaneously with 5×10^4 TC-1 cells/mice. Three days after tumor challenge, mice were vaccinated with 2 μg of pNGVL4a-CRT/E7(detox) DNA via intramuscular injection, electroporation, or gene gun. Mice received a booster vaccination at three day intervals for a total of three vaccinations using alternating hind legs. Tumor size was measured with electronic calipers and tumor volumes calculated starting at day seven and then twice per week.

Statistical analysis

All data are expressed as means ± S.E. where indicated. Data for intracellular cytokine staining with flow cytometric analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). Comparisons between individual data points were made using a Student's *t*-test. In the tumor protection experiments, the principal outcome of interest was time to development of tumor. The event time distributions for different mice were compared by the Kaplan-Meier method and by use of the log-rank statistic. All p-values <0.05 were considered significant.

Acknowledgments

This work was funded by the National Institute of Dental and Craniofacial Research (NIDCR) SPORE program in Head and Neck Cancer Research P50 CA96784-01 and The Milton J. Dance, Jr. Head and Neck Center at Greater Baltimore Medical Center.

References

1. Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 2002;20:621–667. [PubMed: 11861614]
2. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization*. Annu Rev Immunol 2000;18:927–974. [PubMed: 10837079]
3. Pardoll DM. Cancer vaccines. Nat Med 1998 May;4(5 Suppl):525–531. [PubMed: 9585204]
4. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol 1997;15:617–648. [PubMed: 9143702]

5. Robinson HL. Nucleic acid vaccines: an overview. Vaccine 1997 Jun;15(8):785–787. [PubMed: 9234513]
6. Chaudhuri D, Suriano R, Mittelman A, Tiwari RK. Targeting the immune system in cancer. Curr Pharm Biotechnol 2009 Feb;10(2):166–84. [PubMed: 19199949]
7. Abdulhaqq SA, Weiner DB. DNA vaccines: developing new strategies to enhance immune responses. Immunol Res 2008;42(1–3):156–72.
8. Braun S. Muscular gene transfer using nonviral vectors. Curr Gene Ther 2008 Oct;8(5):391–405. [PubMed: 18855634]
9. Paavonen J. Human papillomavirus infection and the development of cervical cancer and related genital neoplasias. Int J Infect Dis 2007 Nov;11 Suppl 2:S3–9. [PubMed: 18162244]
10. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med 2007 May 10;356(19):1944–1956. [PubMed: 17494927]
11. Kim JW, Hung CF, Juang J, He L, Kim TW, Armstrong DK, et al. Comparison of HPV DNA vaccines employing intracellular targeting strategies. Gene Ther 2004 Jun;11(12):1011–1018. [PubMed: 14985791]
12. Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM, et al. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. Cancer Res 2000 Feb 15;60(4):1035–1042. [PubMed: 10706121]
13. Hung CF, Cheng WF, Hsu KF, Chai CY, He L, Ling M, et al. Cancer immunotherapy using a DNA vaccine encoding the translocation domain of a bacterial toxin linked to a tumor antigen. Cancer Res 2001 May 1;61(9):3698–3703. [PubMed: 11325841]
14. Bansal A, Jackson B, West K, Wang S, Lu S, Kennedy JS, et al. Multifunctional T-cell characteristics induced by a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 vaccine regimen given to healthy adults are dependent on the route and dose of administration. J Virol 2008 Jul;82(13):6458–6469. [PubMed: 18448544]
15. Sasaki S, Takeshita F, Xin KQ, Ishii N, Okuda K. Adjuvant formulations and delivery systems for DNA vaccines. Methods 2003 Nov;31(3):243–254. [PubMed: 14511957]
16. Hermanson G, Whitlow V, Parker S, Tonsky K, Rusalov D, Ferrari M, et al. A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. Proc Natl Acad Sci USA 2004 Sep 14;101(37):13601–13606. [PubMed: 15342913]
17. O'Hagan DT, Singh M, Ulmer JB. Microparticles for the delivery of DNA vaccines. Immunol Rev 2004 Jun;199:191–200. [PubMed: 15233735]
18. Mollenkopf HJ, Dietrich G, Fensterle J, Grode L, Diehl KD, Knapp B, et al. Enhanced protective efficacy of a tuberculosis DNA vaccine by adsorption onto cationic PLG microparticles. Vaccine 2004 Jul 29;22(21–22):2690–2695. [PubMed: 15309815]
19. Trimble C, Lin CT, Hung CF, Pai S, Juang J, He L, et al. Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. Vaccine 2003 Sep 8;21(25–26):4036–4042. [PubMed: 12922140]
20. Stoecklinger A, Grieshuber I, Scheiblhofer S, Weiss R, Ritter U, Kissenpfennig A, et al. Epidermal langerhans cells are dispensable for humoral and cell-mediated immunity elicited by gene gun immunization. J Immunol 2007 Jul 15;179(2):886–893. [PubMed: 17617579]
21. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. J Exp Med 1998 Sep 21;188(6):1075–1082. [PubMed: 9743526]
22. Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci USA 1999 Apr 13;96(8):4262–4267. [PubMed: 10200250]
23. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. Nat Biotechnol 1998 Sep;16(9):867–870. [PubMed: 9743122]
24. Peng B, Zhao Y, Xu L, Xu Y. Electric pulses applied prior to intramuscular DNA vaccination greatly improve the vaccine immunogenicity. Vaccine 2007 Mar 1;25(11):2064–2073. [PubMed: 17239494]

25. Study of a Potential Preventive Vaccine Against HIV in Healthy Volunteers (ADVAX-EP). NCT00545987. 2007. Available at: http://www.clinicaltrials.gov
26. Phase I of Human Papillomavirus (HPV) DNA Plasmid (VGX-3100) + Electroporation for CIN 2 or 3. NCT00685412. 2008. Available at: http://www.clinicaltrials.gov
27. Bloquel C, Trollet C, Pradines E, Seguin J, Scherman D, Bureau MF. Optical imaging of luminescence for in vivo quantification of gene electrotransfer in mouse muscle and knee. BMC Biotechnol 2006 Mar 8;6:16. [PubMed: 16524461]
28. Pike SE, Yao L, Jones KD, Cherney B, Appella E, Sakaguchi K, et al. Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. J Exp Med 1998 Dec 21;188(12): 2349–2356. [PubMed: 9858521]
29. Pike SE, Yao L, Setsuda J, Jones KD, Cherney B, Appella E, et al. Calreticulin and calreticulin fragments are endothelial cell inhibitors that suppress tumor growth. Blood 1999 Oct 1;94(7):2461–2468. [PubMed: 10498619]
30. Gronevik E, von Steyern FV, Kalhovde JM, Tjelle TE, Mathiesen I. Gene expression and immune response kinetics using electroporation-mediated DNA delivery to muscle. J Gene Med 2005 Feb;7 (2):218–227. [PubMed: 15515140]
31. Williams RS, Johnston SA, Riedy M, DeVit MJ, McElligott SG, Sanford JC. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc Natl Acad Sci USA 1991;88:2726–2730. [PubMed: 2011582]
32. http://www.biocompare.com/Articles/TechnicalArticle/1198/Detection-Of-Reporter-Gene-Activity-In-Cell-Cultures-And-Murine-Epidermis-After-Helios-Gene-Gun-Mediated-Particle-Bombardment.html
33. Peng S, Trimble C, Alvarez RD, Huh WK, Lin Z, Monie A, et al. Cluster intradermal DNA vaccination rapidly induces E7-specific CD8(+) T-cell immune responses leading to therapeutic antitumor effects. Gene Ther. 2008 Apr 10;
34. Drape RJ, Macklin MD, Barr LJ, Jones S, Haynes JR, Dean HJ. Epidermal DNA vaccine for influenza is immunogenic in humans. Vaccine 2006 May 22;24(21):4475–4481. [PubMed: 16150518]
35. Jones S, Evans K, McElwaine-Johnn H, Sharpe M, Oxford J, Lambkin-Williams R, Mant T, Nolan A, Zambon M, Ellis J, Beadle J, Loudon PT. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial. Vaccine 2009 Apr 21;27(18): 2506–12. [PubMed: 19368793]
36. Roy MJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 2000;19:764–778. [PubMed: 11115698]
37. McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G, et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. Nat Med 2003;9(6):729–735. [PubMed: 12766765]
38. Wloch MK, Smith LR, Boutsaboualoy S, Reyes L, Han C, Kehler J, Smith HD, Selk L, Nakamura R, Brown JM, Marbury T, Wald A, Rolland A, Kaslow D, Evans T, Boeckh M. Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy adult subjects. J Infect Dis 2008 Jun 15;197(12):1634–42. [PubMed: 18444883]
39. Martin JE, Louder MK, Holman LA, Gordon IJ, Enama ME, Larkin BD, Andrews CA, Vogel L, Koup RA, Roederer M, Bailer RT, Gomez PL, Nason M, Mascola JR, Nabel GJ, Graham BS. VRC 301 Study Team. A SARS DNA vaccine induces neutralizing antibody and cellular immune responses in healthy adults in a Phase I clinical trial. Vaccine 2008 Nov 25;26(50):6338–43. [PubMed: 18824060]
40. Keitel WA, Treanor JJ, El Sahly HM, Evans TG, Kopper S, Whitlow V, Selinsky C, Kaslow DC, Rolland A, Smith LR, Lalor PA. Evaluation of a plasmid DNA-based anthrax vaccine in rabbits, nonhuman primates and healthy adults. Hum Vaccin 2009 Aug 14;5(8)
41. Martin JE, Pierson TC, Hubka S, Rucker S, Gordon IJ, Enama ME, Andrews CA, Xu Q, Davis BS, Nason M, Fay M, Koup RA, Roederer M, Bailer RT, Gomez PL, Mascola JR, Chang GJ, Nabel GJ, Graham BS. A West Nile virus DNA vaccine induces neutralizing antibody in healthy adults during a phase 1 clinical trial. J Infect Dis 2007 Dec 15;196(12):1732–40. [PubMed: 18190252]

42. Luxembourg A, Evans CF, Hannaman D. Electroporation-based DNA immunisation: translation to the clinic. Expert Opin Biol Ther 2007 Nov;7(11):1647–64. [PubMed: 17961089]
43. Cheng WF, Hung CF, Chai CY, Hsu KF, He L, Ling M, et al. Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. J Clin Invest 2001 Sep;108(5):669–678. [PubMed: 11544272]
44. Wang S, Zhang C, Zhang L, Li J, Huang Z, Lu S. The relative immunogenicity of DNA vaccines delivered by the intramuscular needle injection, electroporation and gene gun methods. Vaccine 2008 Apr 16;26(17):2100–2110. [PubMed: 18378365]
45. Saha S, Takeshita F, Sasaki S, Matsuda T, Tanaka T, Tozuka M, Takase K, Matsumoto T, Okuda K, Ishii N, Yamaguchi K, Klinman DM, Xin KQ, Okuda K. Multivalent DNA vaccine protects mice against pulmonary infection caused by Pseudomonas aeruginosa. Vaccine 2006 Sep 11;24(37–39): 6240–9. [PubMed: 16806598]
46. Rosati M, Valentin A, Jalah R, Patel V, von Gegerfelt A, Bergamaschi C, et al. Increased immune responses in rhesus macaques by DNA vaccination combined with electroporation. Vaccine. 2008 Apr 21;
47. Widera G, Austin M, Rabussay D, Goldbeck C, Barnett SW, Chen M, et al. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. J Immunol 2000 May 1;164(9):4635–4640. [PubMed: 10779767]
48. Ahlen G, Soderholm J, Tjelle T, Kjeken R, Frelin L, Hoglund U, et al. In vivo electroporation enhances the immunogenicity of hepatitis C virus nonstructural 3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3+ T cells. J Immunol 2007 Oct 1;179(7): 4741–4753. [PubMed: 17878373]
49. LeBlanc R, Vasquez Y, Hannaman D, Kumar N. Markedly enhanced immunogenicity of a Pfs25 DNA-based malaria transmission-blocking vaccine by in vivo electroporation. Vaccine 2008 Jan 10;26(2):185–192. [PubMed: 18054817]
50. Luxembourg A, Hannaman D, Nolan E, Ellefsen B, Nakamura G, Chau L, et al. Potentiation of an anthrax DNA vaccine with electroporation. Vaccine. 2008 Apr 16;
51. Smorlesi A, Papalini F, Amici A, Orlando F, Pierpaoli S, Mancini C, et al. Evaluation of different plasmid DNA delivery systems for immunization against HER2/neu in a transgenic murine model of mammary carcinoma. Vaccine 2006 Mar 10;24(11):1766–1775. [PubMed: 16288939]
52. Cipriani B, Fridman A, Bendtsen C, Dharmapuri S, Mennuni C, Pak I, et al. Therapeutic Vaccination Halts Disease Progression in BALB-neuT Mice: The Amplitude of Elicited Immune Response Is Predictive of Vaccine Efficacy. Hum Gene Ther. 2008 Jun 6;
53. Roos AK, Moreno S, Leder C, Pavlenko M, King A, Pisa P. Enhancement of cellular immune response to a prostate cancer DNA vaccine by intradermal electroporation. Mol Ther 2006 Feb;13(2):320–327. [PubMed: 16185933]
54. Lucas ML, Heller L, Coppola D, Heller R. IL-12 plasmid delivery by in vivo electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. Mol Ther 2002 Jun;5(6):668–675. [PubMed: 12027550]
55. Liu MA, Ulmer JB. Human clinical trials of plasmid DNA vaccines. Adv Genet 2005;55:25–40. [PubMed: 16291211]
56. Jurk M, Vollmer J. Therapeutic applications of synthetic CpG oligodeoxynucleotides as TLR9 agonists for immune modulation. BioDrugs 2007;21(6):387–401. [PubMed: 18020622]
57. Luxembourg A, Hannaman D, Ellefsen B, Nakamura G, Bernard R. Enhancement of immune responses to an HBV DNA vaccine by electroporation. Vaccine 2006 May 22;24(21):4490–4493. [PubMed: 16140436]
58. Wu TC, Guarnieri FG, Staveley-O'Carroll KF, Viscidi RP, Levitsky HI, Hedrick L, et al. Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. Proc Natl Acad Sci USA 1995 Dec 5;92(25):11671–11675. [PubMed: 8524826]
59. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996 Jan 1;56(1):21–26. [PubMed: 8548765]

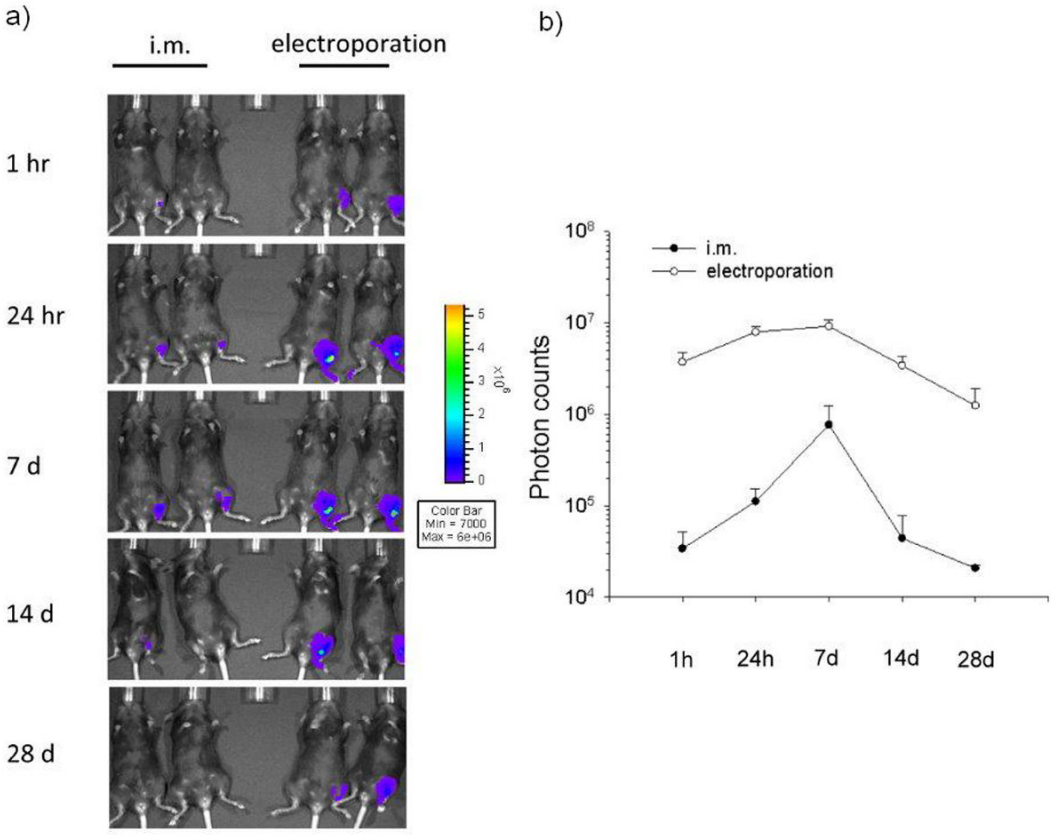

Figure 1. Comparison of electroporation (EP) and intramuscular (IM) delivery of a luciferase gene

C57BL/6 mice were vaccinated with 2 μg of pCDNA3-Luciferase plasmid to compare the efficacy and duration of various gene transfer techniques. Both electroporation (EP) and intramuscular (IM) injections were performed in the left hind leg, with control PBS injections in the right hind leg. One hour after plasmid delivery, 200 μL of luciferin (3.9 mg/mL) was injected intraperitoneally. After allowing 10 minutes for reaction conjugation, luminescence measurements were taken within a region of interest drawn over each site of vaccination. Luciferin injections and imaging were repeated at 24 hours, 7, 14, and 28 days after vaccination. N = 2 per group.

(a) Optical photographs of anesthetized C57BL/6 mice with luminescence overlay shows the increased expression of pCDNA3-Luciferase plasmid by EP compared to IM injection.

(b) Quantification of maximum luminescence on a log scale. Data are expressed as means ± SE of maximum luminescence within a region of interest drawn over each site of vaccination.

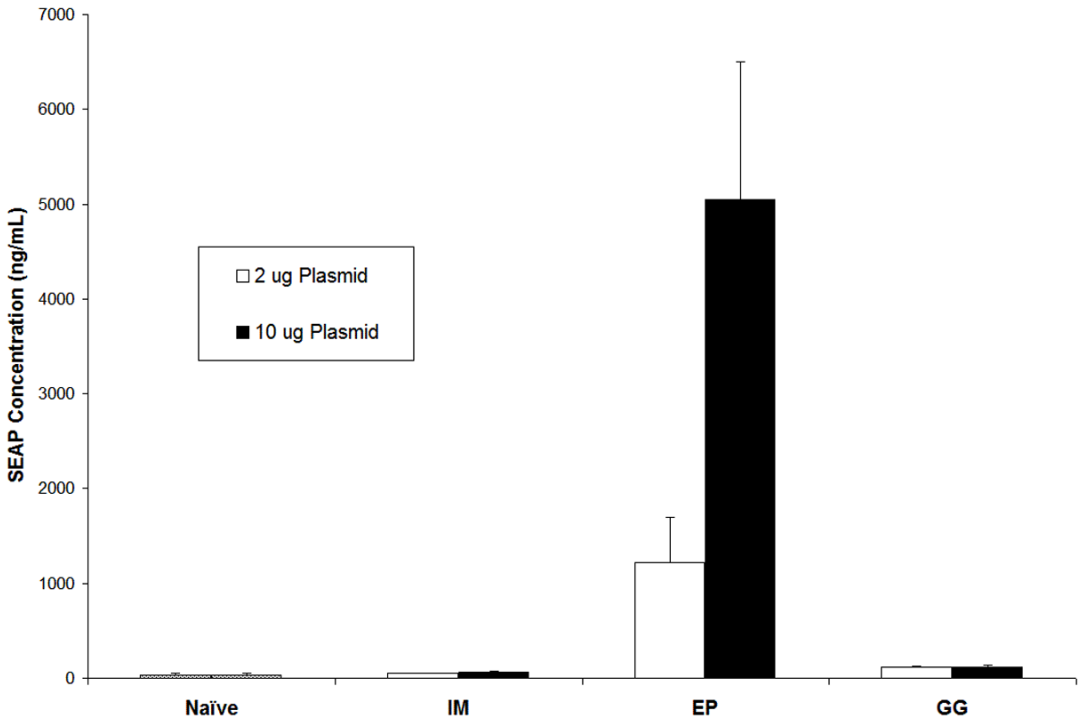

Figure 2. Serum expression levels of secreted alkaline phosphatase (SEAP) were measured in mice vaccinated via intramuscular injection (IM), electroporation (EP), or gene gun (GG)

C57BL/6 mice were vaccinated with the pSEAP2-control plasmid and serum levels were measured seven days after vaccination. Mice received either 2 μg or 10 μg of pSEAP2-control plasmid, and measurements were performed with a commercially available reaction kit (Clontech). Values are pooled means ± SE of triplicate chemiluminescent reactions. N = 3 per group.

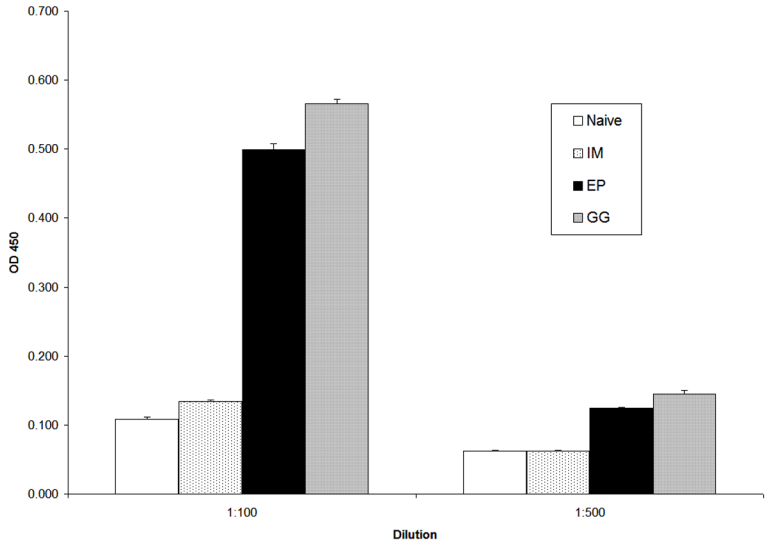

Figure 3. Serum levels of E7-specific antibodies elicited in mice vaccinated by intramuscular injection (IM), electroporation (EP), or gene gun (GG)

C57BL/6 mice were vaccinated with 2 μg of pNGVL4a-CRT/E7(detox) DNA plasmid in alternating hind legs on Days 0, 3, and 6. Serum was collected from the tail vein 14 days after the last vaccination and analyzed for E7-specific antibodies using direct ELISA at a mean absorbance of 450 nm. Values are pooled means ± SE of serial dilutions of triplicate ELISA reactions. N = 4 per group.

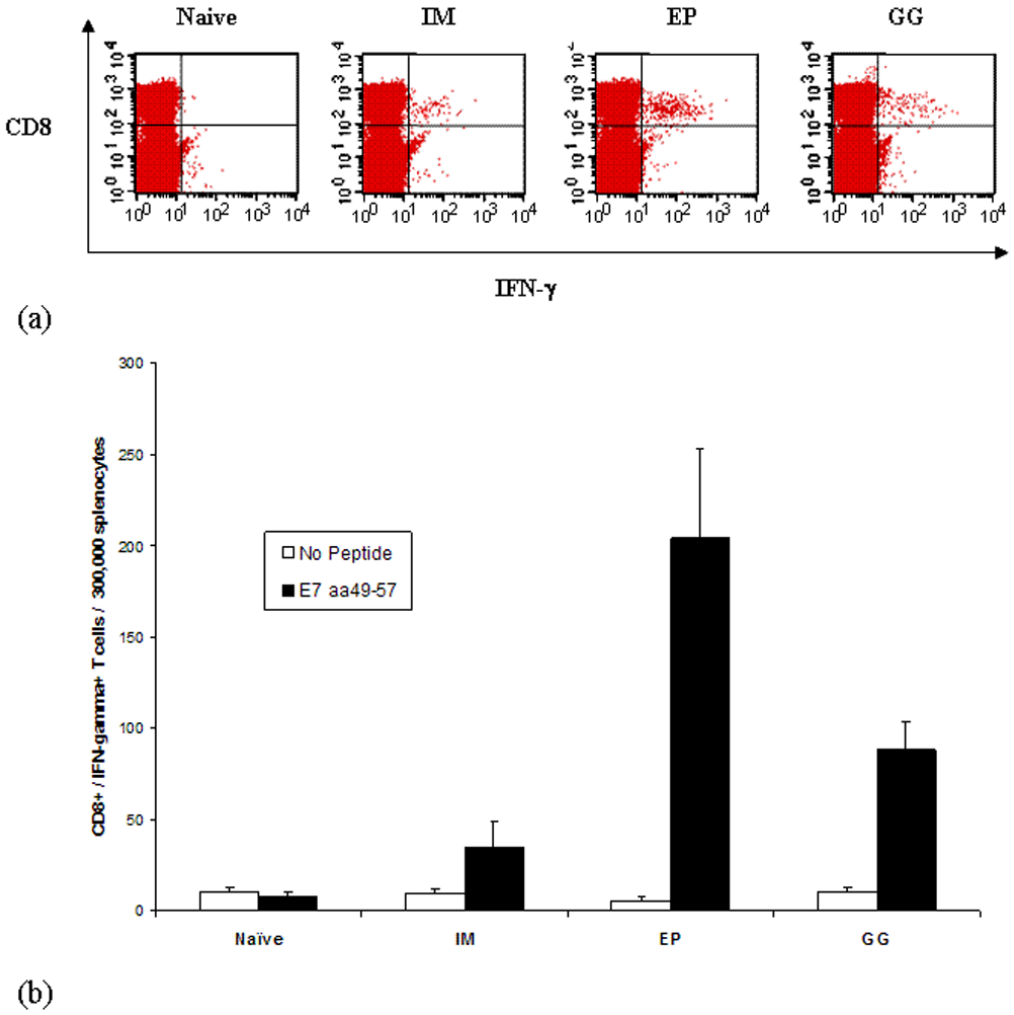

Figure 4. Intracellular cytokine staining and flow cytometry analysis to characterize IFN-γ-secreting E7-specific CD8+ T cell precursors in mice vaccinated with 2 μg of pNGVL4a-CRT/E7 (detox) DNA vaccine via intramuscular injection (IM), electroporation (EP), or gene gun (GG)

C57BL/6 mice were vaccinated with pNGVL4a-CRT/E7 (detox) DNA vaccine through various routes of administration and received a booster vaccination one week later. One week after the last vaccination, the splenocytes were harvested and cultured in vitro with or without the E7 peptide (amino acid 49–57) overnight and were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ-secreting $CD8^+$ T cell precursors in naïve and immunized mice were analyzed by flow cytometry.

(a) A representative figure of intracellular cytokine staining experiment. $CD8^+$ IFN- γ^+ T cells appear in the right upper quadrant of each flow cytometry plot.

(b) Composite results of flow cytometry analysis of $CD8^+$ IFN- γ^+ T cell precursors in naïve mice and mice vaccinated with pNGVL4a-CRT/E7 (detox) DNA vaccine via IM, EP, and GG. Data are expressed as the mean number of E7-specific $CD8^+$IFN-γ^+ T cells per 3×10^5 splenocytes ± SE. N = 11 mice for all groups.

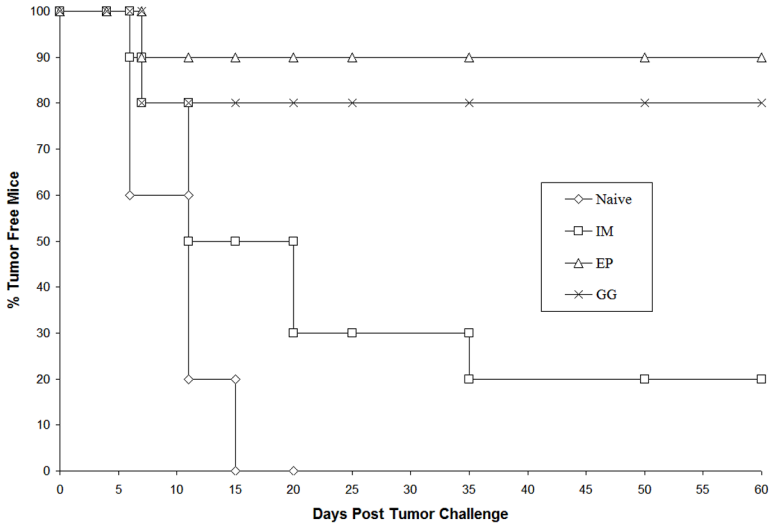

Figure 5. ***In vivo*** **tumor protection experiments to compare the antitumor effect mediated by different methods of vaccination using 2 μg of pNGVL4a-CRT/E7(detox) DNA plasmid**

C57BL/6 mice were vaccinated with pNGVL4a-CRT/E7 (detox) DNA vaccine via intramuscular injection (IM), electroporation (EP), or gene gun (GG) and received a booster vaccination one week later. Fourteen days after the first vaccination, mice were inoculated with 5×10^4 TC-1 tumor cells and followed bi-weekly for palpable tumor development. Data are expressed as percentage of tumor free mice. N = 5 for each group. Experiments were repeated in duplicate. The difference between the EP and IM groups reached statistical significance with a p-value of 0.01. The difference between the EP and GG groups were not statistically significant (NS).

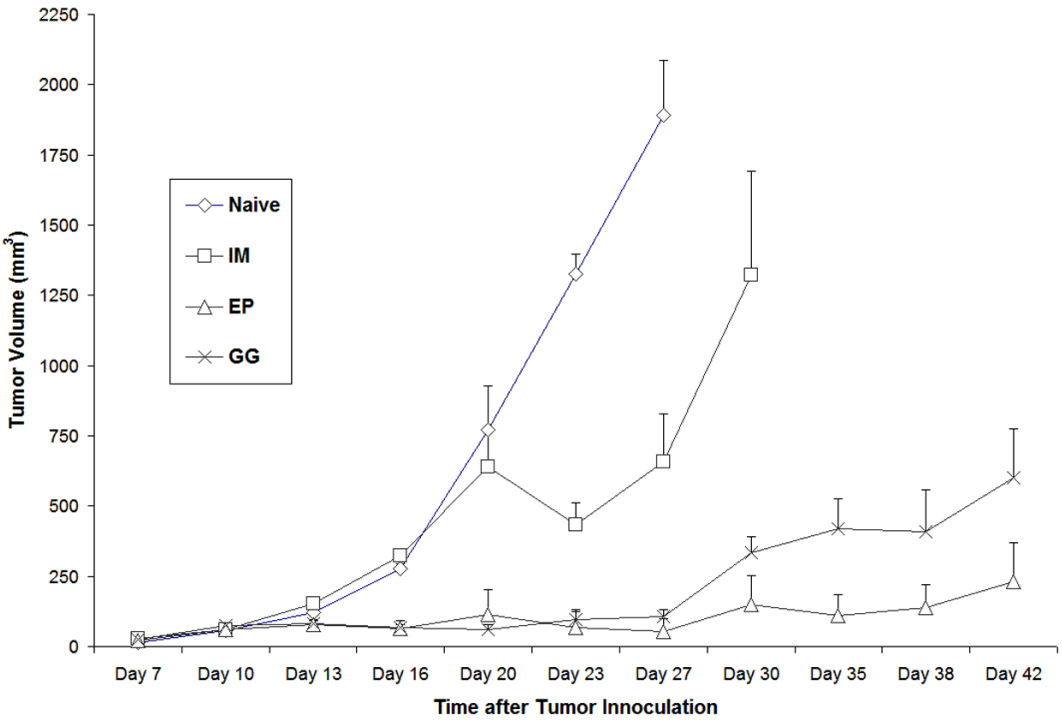

Figure 6. ***In vivo*** **tumor treatment experiments to compare the antitumor effect mediated by intramuscular (IM), electroporation (EP), or gene gun (GG) vaccination using 2 μg of pNGVL4a-CRT/E7(detox) DNA plasmid**

C57BL/6 mice were inoculated with 5×10^4 TC-1 tumor cells subcutaneously on Day 0 and were vaccinated with 2 μg of pNGVL4a-CRT/E7(detox) DNA in alternating hind legs on Days 3, 6, and 9. Mice were followed for tumor development by manual palpation as well as tumor growth by recording tumor measurements using digital calipers and calculating tumor volumes. Data are expressed as volume means ± SE. N= 5 for each group. The difference in tumor volumes between the EP and GG groups became statistically significant (p=0.045) on Day 38.