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Enhancement of protein vaccine potency by in vivo electroporation mediated intramuscular injection

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Abstract

Protein-based vaccines have emerged as a potentially promising approach for the generation of antigen-specific immune responses. However, due to their low immunogenicity, there is a need for innovative approaches to enhance protein-based vaccine potency. One approach to enhance protein-based vaccine potency is the employment of toll-like receptor ligands, such as CpG oligonucleotides, to activate the antigen-specific T cell immune responses. Another approach involves employing a method capable of improving the delivery of protein-based vaccine intramuscularly to lead to the slow release of the protein, resulting in improved vaccine potency. In the current study, we aimed to determine whether intramuscular injection of protein-based vaccines in conjunction with CpG followed by electroporation can lead to increased delivery of the protein-based vaccine into muscle cells, resulting in enhanced protein-based vaccine potency. We found that intramuscular injection followed by electroporation can effectively transduce the protein-based vaccine into the muscle cells. Furthermore, we found that intramuscular vaccination with OVA protein in combination with CpG followed by electroporation generates the best OVA-specific CD8⁺ T cell immune responses as well as the best protective and therapeutic antitumor effects in vaccinated mice. CD8⁺ T cells were found to play an important role in the observed protective antitumor effects generated by the vaccination. Similar results were observed using the HPV-16 E7 protein-based vaccination system. Thus, our data indicate that intramuscular administration of protein-based vaccines in conjunction with CpG followed by electroporation can significantly enhance the antigen-specific CD8⁺ T cell immune responses. The clinical implications of the study are discussed.

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Keywords

electroporation; protein-based vaccine; CpG; human papillomavirus (HPV)

Introduction

Antigen-specific immunotherapy using protein-based vaccines has emerged as a potentially promising approach for the generation of antigen-specific immune responses. In general, protein-based vaccines are safe and have the ability to bypass MHC restriction (for reviews see [1,2]). Various protein-based vaccines have been tested in clinical trials and found to be safe and well tolerated [3–5]. However, they are weakly immunogenic and usually induce low level of CTL responses [6,7]. Consequently, most of the research in this area has focused on the identification of innovative adjuvants to enhance protein-based vaccine potency in generating antigen-specific CD8⁺ T cells (for review, see [8]).

One approach to enhance protein-based vaccine potency is the employment of “danger” signals such as toll-like receptor (TLR) ligands that stimulate dendritic cells to mature and differentiate into potent activators of antigen-specific T cells (for review, see [9]). It is clear that toll-like receptors (TLRs) play a crucial role in enhancing innate and adaptive immune responses (for reviews, see [10–13]). TLR ligands have emerged as a promising new class of vaccine adjuvants, particularly the oligodeoxynucleotides containing one or more unmethylated CpG dinucleotides (CpG-ODNs), which target TLR9 [14]. It has been shown that CpG-ODNs can stimulate innate immunity and confer protection against a variety of bacterial and viral infections [15,16]. CpG-ODNs have previously been used in combination with protein-based vaccines to improve vaccine potency in several studies [17–19]. In fact, CpG ODNs have been employed in combination with HPV-16 E7 long peptide-based vaccines to generate significant antitumor effects against E7-expressing tumors [20].

Another strategy for increasing protein-based vaccine potency is to identify methods for retaining the protein for slow release into the immune system. We reasoned that a method capable of improving the delivery of protein-based vaccine intramuscularly may potentially lead to the slow release of the protein, resulting in improved vaccine potency. In the current study, we have employed electroporation to improve the delivery of protein-based vaccines into muscle cells. Electroporation has been used to improve transfection efficiency of DNA into muscle cells to improve DNA vaccine potency [21–23]. Electroporation involves the administration of the vaccine into the muscle by needle injection, followed by a brief electrical pulse to the surrounding tissue. A transient increase in the permeability of the plasma membrane induced by the electrical current allows increased uptake of the DNA plasmid [24,25]. Thus, the technology may potentially be used to improve the uptake of protein-based vaccines by muscle cells.

In the current study, we plan to determine whether intramuscular injection of protein-based vaccines in conjunction with CpG followed by electroporation can lead to increased delivery of the protein-based vaccine into muscle cells to enhance protein-based vaccine potency. We found that intramuscular injection followed by electroporation can most effectively transduce the protein-based vaccine into the muscle cells. Furthermore, we found that intramuscular vaccination with OVA protein in combination with CpG followed by electroporation generates the best OVA-specific CD8⁺ T cell immune responses as well as the best protective and therapeutic antitumor effects in vaccinated mice. CD8⁺ T cells were found to play an important role in the observed protective antitumor effects generated by the vaccination. Similar results were observed using the HPV-16 E7 protein-based vaccination system. Thus, our data indicate that intramuscular administration of protein-based vaccines

in conjunction with CpG followed by electroporation can significantly enhance the antigen-specific CD8⁺ T cell immune responses. The clinical implications of the study are discussed.

Materials and Methods

Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Cells

The OVA-expressing murine tumor model, B16-OVA, has been described previously [26]. This cell line was cultured *in vitro* in DMEM supplemented with 10% fetal bovine serum, 50units/ml of penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, and 2mM non-essential amino acids, and grown at 37°C with 5% CO₂.

In vivo protein delivery and vaccination

For in vivo immunization, mice were vaccinated three times 1 week interval using OVA protein (1 or 10ug), E7 long peptide (a.a. 43–62)(1ug), OVA protein (1 or 10ug) mixed CpG-1826 (1 or 10ug), or E7 long peptide (1ug) mixed CpG-1826 (1ug) by subcutaneous injection, intramuscular injection or electroporation-mediated intramuscular injection. For the subcutaneous injection-mediated protein vaccination, protein only or CpG mixed protein were delivered subcutaneously by syringe needle injection. For the intramuscular injection-mediated protein vaccination, protein only or CpG mixed protein were delivered intramuscularly by syringe needle injection. The electroporation-mediated intramuscular injection method was described previously [27]. Briefly, hair around the quadriceps femoris muscle of mice was removed. The muscle was then injected with protein only or CpG mixed protein using the TriGrid Electroporation Delivery System (Ichor Medical Systems). The TriGrid Electroporation system consists of an electric pulse generator and a TriGrid array comprising four electrodes 2 mm in length and 2.5 mm interelectrode spacing. A 0.3 ml syringe with a 0.5 inch 30 gauge needle (BD Ultra-Fine, 328431, Becton Dickinson, Franklin Lakes, NJ) is attached through the center of the electrode array. The EP protocol was completed within 0.5 seconds, comprising rectangular wave, direct current pulses applied at 220 volts/cm peak amplitude and 8% duty cycle.

Intracellular cytokine staining and flow cytometry analysis

From vaccinated mice, splenocytes were harvested 1 week after the last vaccination. Before intracellular cytokine staining, 6×10⁵ pooled splenocytes from each vaccination group were incubated for 16 hours with either 1 µg/ml of OVA peptide (SIINFEKL) [28] or E7 peptide (RAHYNIVTF) [29] containing an MHC class I epitope for detecting OVA or E7-specific CD8⁺ T cell. Cell surface marker staining of CD8 and intracellular cytokine staining for IFN-γ using Phycoerythrin-conjugated monoclonal rat anti-mouse CD8 antibody (BD Pharmingen, San Diego, CA) and FITC-conjugated anti-mouse IFN-γ antibodies (BD Pharmingen, San Diego, CA) as well as flow cytometry analysis, were performed using conditions described previously [30]. Analyses were done on a Becton-Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

Luciferase-expressing E7-specific CD8 T cells

Luciferase-expressing E7-specific CD8 T cells were generated as described previously [31]. This cell line was cultured in RPMI 1640 medium containing 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, nonessential amino acid, 100 IU penicillin/streptomycin and 10% fetal bovine serum in a 37°C incubator with 5% CO₂.

Luciferase-based bioluminescence imaging

For the protein transduction experiment, 1 µg of recombinant luciferase protein (Luc) (Promega) was injected in vivo by subcutaneous injection, intramuscular injection, or electroporation-mediated intramuscular injection into the mice. The intensity of bioluminescence was checked on days 1 and 2 after injection as described previously [32]. The levels of light from the bioluminescent cells were detected by IVIS camera system, integrated, and digitized. Region of interest from displayed images was designated around the injection sites and quantified as total photon counts using Living Image 2.50 software (Xenogen). For the in vivo T cell attraction experiment, mice were injected using E7 long peptide (a.a. 43–62)(10 µg), CpG-1826 (10 µg) or E7 long peptide(10 µg) mixed CpG-1826 (10 µg) by intramuscular injection or electroporation-mediated intramuscular injection. One day after injection, 5×10⁶ of luciferase expressing E7-specific CD8⁺ T cells were adoptively transferred intravenously through the tail vein into the mice. The levels of light from the bioluminescent cells were detected as described above.

Long-term immunization

Mice were vaccinated three times 1 week interval using OVA protein (1 µg) mixed CpG-1826 (1 µg) by electroporation-mediated intramuscular injection. Seven weeks after the last vaccination, intracellular cytokine staining was followed by flow cytometry analysis as described previously to count E7-specific CD8⁺ T cells 1 week and 7 weeks.

ELISA

The presence of anti-ovalbumin Ab's in the sera was characterized by a direct ELISA as described previously[33]. Mice were immunized as described above. Sera were prepared from mice on day 7 after final immunization. The ELISA plate was read with a standard ELISA reader at 450 nm.

In vivo tumor treatment experiments

For in vivo tumor treatment experiments, mice (five per group) were challenged with 5×10⁴ B16-OVA tumor cells/mouse into the tail vein to simulate hematogenous spread of tumors. Three days after tumor challenge, mice were vaccinated and boosted twice more at 1-week intervals with OVA protein, CpG or OVA protein mixed CpG via subcutaneous injection, intramuscular injection, or electroporation-mediated intramuscular injection. Mice were monitored twice a week and sacrificed on day 30 after the last vaccination. The mean weight of lung in each mouse was determined by experimenters blinded to the sample identity.

In vivo Ab depletion of immune cells

For *in vivo* Ab depletion experiments, C57BL/6 mice (five per group) were vaccinated as described above. One week after the last vaccination, mice were subcutaneously challenged with 5×10⁴ B16-OVA cells/mouse in the right leg. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until day 60. *In vivo* antibody depletions were performed as described previously [34]. Briefly, C57BL/6 mice (five per group) were vaccinated three times with 1 µg of OVA protein mixed 1 µg of CpG, and challenged with 5×10⁴ cells/mouse B16-OVA tumor cells one week after the last vaccination. Antibody depletion was started 1 week before tumor challenge. The mAb

GK1.5 was used for CD4 depletion, mAb 2.43 was used for CD8 depletion, and mAb PK136 was used for NK1.1 depletion. Depletion was terminated on day 60 after tumor challenge.

Statistical analysis

All data are expressed as means $\pm$ standard deviation (S.D.) and are representative of at least two separate experiments. Results for intracellular cytokine staining with flow cytometry analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). In the tumor protection experiments, the principal outcome measure was time to tumor development. All analyses were performed in SAS version 9.2 (SAS institute, Inc., Cary, NC, USA), and the significance level was set at 0.05. The product limit estimate of tumor development was determined by the Kaplan-Meier method, **which indicates time-to-tumor development**. Log-Rank test was determined to test the statistical significance of differences among Kaplan-Meier curves. The Kruskal-Wallis tests were applied to test the difference among the weight of lung with tumor for the tumor treatment experiments.

Results

Vaccination with OVA protein in combination with CpG via electroporation generates the best OVA-specific CD8+ T cell immune responses

In order to determine which route of vaccination can generate the best OVA-specific CD8+ T cell immune responses, C57BL/6 mice (three per group) were vaccinated with OVA protein with or without CpG via subcutaneous injection or intramuscular injection with or without electroporation. As shown in Figure 1, mice vaccinated intramuscularly with 1 μ g of OVA protein mixed with 1 μ g of CpG followed by electroporation demonstrated significantly higher OVA-specific CD8+ T cell immune responses compared to mice vaccinated via subcutaneous or intramuscular injection without electroporation ($p < 0.0001$). Likewise, mice vaccinated intramuscularly with a higher dose of OVA protein (10 μ g) mixed with a higher dose of CpG (10 μ g) followed by electroporation also demonstrated significantly higher OVA-specific CD8+ T cell immune responses compared to mice vaccinated via subcutaneous or intramuscular injection without electroporation ($p < 0.0001$). We observed that vaccination with the higher dose of OVA and CpG generated a slightly higher increment in the OVA-specific CD8+ T cell immune responses compared to vaccination with the lower dose of OVA and CpG.

We further examined the long-term memory CD8+ T cell immune responses generated by vaccination with OVA protein mixed with CpG followed by electroporation. We found that mice vaccinated intramuscularly with OVA protein mixed with CpG followed by electroporation also demonstrated appreciable OVA-specific memory CD8+ T cell immune responses 7 weeks after the last immunization although the degree of immune responses was significantly lower than than at 1 week after the last vaccination ($p < 0.0002$) (See Supplementary Figure 1).

We also assessed the OVA-specific antibody responses generated by intramuscular administration of OVA protein mixed with CpG followed by electroporation. We found that mice vaccinated intramuscularly with OVA protein mixed with CpG followed by electroporation showed significantly higher OVA-specific antibody responses compared to the control (See Supplementary Figure 2). Thus, our data suggest that intramuscular vaccination with OVA protein mixed with CpG followed by electroporation generates the best OVA-specific CD8+ T cell immune responses among all the other vaccinated groups.

Intramuscular injection followed by electroporation can most effectively transduce the protein into the muscle cells

In order to determine if intramuscular injection followed by electroporation can effectively transduce a protein into muscle cells, C57BL/6 mice (2 per group) were injected with recombinant luciferase protein in vivo by subcutaneous injection, intramuscular injection with or without electroporation. The activity of luciferase was characterized by luminescence imaging on days 1 and 2 after injection. As shown in Figure 2, mice vaccinated intramuscularly with luciferase protein followed by electroporation demonstrated significantly the best luciferase activity compared to mice vaccinated via subcutaneous or intramuscular injection without electroporation ($p<0.05$). Thus, our data indicate that intramuscular injection followed by electroporation can most effectively transduce a protein into muscle cells.

Intramuscular vaccination with OVA protein in combination with CpG followed by electroporation generates the best protective antitumor effects in vaccinated mice

In order to determine if intramuscular vaccination with OVA protein in combination with CpG followed by electroporation can enhance the protective antitumor effects in vaccinated mice, C57BL/6 mice (5 per group) were immunized with OVA protein with or without CpG via subcutaneous injection, intramuscular injection with or without electroporation. Mice were then subcutaneously challenged with B16-OVA tumor one week after the last vaccination. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed at day 60. As shown in Figure 3, mice vaccinated intramuscularly with OVA protein in combination with CpG followed by electroporation demonstrated the best protective antitumor effects compared to mice vaccinated via subcutaneous or intramuscular injection without electroporation ($p<0.05$). Thus, our data indicate that intramuscular vaccination with OVA protein in combination with CpG followed by electroporation generates the best protective antitumor effects in vaccinated mice.

CD8+ T cells play an important role in the observed protective antitumor effects generated by intramuscular vaccination with OVA in combination with CpG followed by electroporation

In order to determine the subset of lymphocytes that are important for the observed protective antitumor effects generated by intramuscular vaccination with OVA in combination with CpG followed by electroporation, C57BL/6 mice (five per group) were immunized intramuscularly with OVA protein with CpG followed by electroporation and challenged with B16-OVA tumor cells one week after the last vaccination. The depletion of different subset of lymphocytes including CD4, CD8, and NK1.1 was initiated 1 week before tumor challenge and lasted 60 days after tumor challenge. As shown in Figure 4, the vaccinated mice depleted of CD8+ T cells demonstrated complete loss of antitumor effects ($p<0.05$). We also observed that vaccinated mice depleted of NK cells demonstrated slight loss of antitumor effects compared to vaccinated mice without depletion. Depletion of CD4+ T cells did not lead to any loss in antitumor effects. Thus, our data indicate that CD8+ T cells play the most important role in the observed protective antitumor effects generated by intramuscular vaccination with OVA in combination with CpG followed by electroporation.

Tumor-bearing mice treated intramuscularly with OVA protein in combination with CpG followed by electroporation generates the best therapeutic antitumor effects

We also characterized the therapeutic antitumor effects generated by intramuscular vaccination with OVA protein in combination with CpG followed by electroporation. C57BL/6 mice (five per group) were first challenged with B16-OVA tumor cells via tail

vein injection. Three days after tumor challenge, mice were vaccinated with OVA protein with or without CpG via subcutaneous injection, intramuscular injection with or without electroporation. The data was analyzed by Kruskal-Wallis test and depicted using box plot. As shown in Figure 5, tumor-bearing mice treated intramuscularly with OVA protein in combination with CpG followed by electroporation demonstrated significantly lower lung weight compared to tumor-bearing mice vaccinated via subcutaneous or intramuscular injection without electroporation ($p<0.01$). Furthermore, tumor-bearing mice treated intramuscularly with OVA protein in combination with CpG followed by electroporation demonstrated significantly lower lung weight compared to tumor-bearing mice treated intramuscularly with OVA protein or CpG alone followed by electroporation ($p<0.01$). In addition, the lung weight of tumor-bearing mice treated intramuscularly with OVA protein in combination with CpG followed by electroporation was comparable to that of naïve mice. Thus, our data indicate that intramuscular vaccination with OVA protein in combination with CpG followed by electroporation generates the best therapeutic antitumor effects in tumor-bearing mice.

Intramuscular vaccination with E7 long peptide in combination with CpG followed by electroporation generates the best E7-specific CD8⁺ T cell immune responses

In order to determine if the observed immune responses and antitumor effects observed with electroporation were applicable to other antigenic systems, C57BL/6 mice (three per group) were vaccinated with E7 long peptide (a.a 43–62) with or without CpG via subcutaneous injection, intramuscular injection with or without electroporation. One week after the last vaccination, the splenocytes were isolated and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 6, mice vaccinated intramuscularly with E7 peptide in combination with CpG demonstrated significantly higher E7-specific CD8⁺ T cell immune responses compared to mice vaccinated with E7 peptide without CpG regardless of the route of administration ($p<0.001$). In addition, intramuscular vaccination of E7 peptide mixed with CpG followed by electroporation generated significantly higher E7-specific CD8⁺ T cell immune responses compared to subcutaneous or intramuscular vaccination of E7 peptide mixed with CpG without electroporation ($p<0.001$). Taken together, our data suggest that intramuscular administration of protein-based vaccines in conjunction with CpG followed by electroporation can significantly enhance the antigen-specific CD8⁺ T cell immune responses.

Intramuscular vaccination with E7 long peptide in combination with CpG followed by electroporation leads to accumulation of E7-specific CD8⁺ T cells at the vaccination site

In order to determine if intramuscular injection of E7 long peptide (a.a 43–62) with CpG followed by electroporation can lead to accumulation of E7-specific CD8⁺ T cells at the vaccination site, C57BL/6 mice (three per group) were vaccinated with E7 long peptide (a.a 43–62) with or without CpG via intramuscular injection with or without electroporation. One week after the last vaccination, luciferase-expressing E7-specific T cells were adoptively transferred intravenously via the tail vein into the vaccinated mice. As shown in Figure 7, mice vaccinated intramuscularly with E7 peptide in combination with CpG followed by electroporation demonstrated the greatest number of luciferase-expressing E7-specific CD8⁺ T cells at the vaccination site among all the vaccination groups ($p<0.001$). Thus, our data suggest that intramuscular administration of protein-based vaccines in conjunction with CpG followed by electroporation can lead to accumulation and/or expansion of antigen-specific CD8⁺ T cell immune responses at the vaccination site.

Discussion

In the current study, we found that intramuscular vaccination with OVA protein in combination with CpG via electroporation generated the best OVA-specific CD8⁺ T cell immune responses and protective and therapeutic antitumor effects in vaccinated mice. Intramuscular vaccination followed by electroporation was found to effectively transduce the protein-based vaccine into muscle cells. We showed that CD8⁺ T cells play an important role in the observed protective antitumor effects generated by intramuscular vaccination with OVA in combination with CpG followed by electroporation. The same strategy has also shown to be effective in HPV-16 E7 protein-based vaccine system. Thus, our strategy may potentially be applicable to other antigenic systems for the control of infectious disease and/or cancer.

Our study showed that the coadministration of the TLR9 agonist CpG is essential for the generation of antigen-specific CD8⁺ T cell immune responses (Figures 1 and 6) and antitumor effects by the protein/peptide-based vaccine (Figures 3, 4 and 5). CpG with increase in the local and systemic T-dependent antitumor responses [35]. CpG delivered intramuscularly followed by electroporation into the muscle cells may lead to increased uptake of CpG into the muscle cells since electroporation has been shown to improve transfection efficiency of DNA [21–23]. Furthermore, the increased uptake of CpG by the muscle cells may lead to a continuous local inflammatory response, which may result in the recruitment of lymphocytes to the local environment. This may be important in the enhancement in the antigen-specific CD8⁺ T cell immune responses since muscle cells have been shown to be capable of serving as antigen-presenting cells to activate antigen-specific CD8⁺ T cells [36].

DNA transfection into muscle cells has been shown to stimulate muscle cells and upregulate the expression of MHC class I and co-stimulatory molecules [36]. In addition, DNA transfection in muscle cells has been shown to produce cytokines and chemokines, leading to local inflammation [36]. In fact, our data demonstrates that intramuscular vaccination with E7 long peptide in combination with CpG followed by electroporation leads to accumulation and expansion of E7-specific CD8⁺ T cells at the vaccination site (Figure 7). This indicates that chemokines may cause local inflammation, which in turn leads to migration and accumulation of antigen-specific CD8⁺ T cells to the muscle cells. Furthermore, it has been shown that these transfected muscle cells are capable of directly presenting antigen to CD8⁺ T cells through an IFN-regulatory factor 3-dependent process [36]. Thus, intramuscular administration of CpG followed by electroporation may enable muscle cells to present antigen to CD8⁺ T cells, leading to antigen-specific immune responses in the local inflammatory environment.

The improved delivery of protein-based vaccine into muscle cells by electroporation (Figure 2) may potentially lead to increased uptake of the protein, resulting in improved antigen presentation by muscle cells through the MHC class I pathway to activate antigen-specific CD8⁺ T cells. Thus, the combination of CpG with the improved delivery of protein-based vaccine by electroporation may contribute to the observed enhancement in antigen-specific CD8⁺ T cell immune responses generated by intramuscular injection of protein-based vaccines.

In summary, our study demonstrates that intramuscular administration of protein-based vaccines in conjunction with CpG followed by electroporation can significantly enhance the antigen-specific CD8⁺ T cell immune responses and antitumor effects in vaccinated mice. Since CpG, protein-based vaccines and electroporation have been employed in clinics, the combination of these technologies may be potentially suitable for future clinical translation.

However, it is important to optimize the dose and regimen of treatment before this strategy can be translated to clinics. Furthermore, our approach can be extended to other antigenic systems to control infectious diseases and/or cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of OVA-specific CD8⁺ T cell immune responses generated by different routes of administration with OVA protein

C57BL/6 mice (three per group) were vaccinated with 1 μ g/mouse (**A & B**) or 10 μ g/mouse (**C & D**) of OVA protein with or without 1 μ g/mouse (**A & B**) or 10 μ g/mouse (**C & D**) of CpG via subcutaneous injection or intramuscular injection with or without electroporation. Splenocytes from vaccinated mice were collected 1 week after last immunization and the OVA-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. (**A & C**) Representative flow cytometry data depicting the number of OVA-specific CD8⁺ T cells. (**B & D**) Bar graph representing the

number of OVA-specific CD8⁺ T cells/ 3×10^5 splenocytes (mean $\pm$ SD). Data shown are representative of two experiments performed.

Figure 2. Characterization of the luciferase activity in mice injected with recombinant luciferase protein via different routes of administration

C57BL/6 mice (two per group) were injected with 1 μ g/mouse of recombinant luciferase protein in vivo by subcutaneous injection, intramuscular injection with or without electroporation. The intensity of bioluminescence was checked on days 1 and 2 after injection. (A) Representative images representing the luciferase activity in mice at different time points. (B) Bar graph depicting quantification of luminescent activity (mean $\pm$ S.D.). The data presented in this figure are from one representative experiment of three performed.

Figure 3. *In vivo* tumor protection experiments in mice vaccinated with OVA protein by different routes of administration

Kaplan-Meier analysis indicating time-to-tumor development of vaccinated mice challenged with B16-OVA tumors. C57BL/6 mice (five per group) were immunized with 1 μ g/mouse of OVA protein with or without 1 μ g/mouse of CpG via subcutaneous injection, intramuscular injection with or without electroporation. Mice were then subcutaneously challenged with 5×10^4 /mouse of B16-OVA tumor in the right leg one week after the last vaccination. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed at day 60.

Figure 4. *In vivo* antibody depletion experiments in vaccinated mice

C57BL/6 mice (five per group) were immunized with 1 μ g/mouse of OVA protein with 1 μ g/mouse of CpG via intramuscular injection followed by electroporation and challenged with 5×10^4 /mouse of B16-OVA tumor cells one week after the last vaccination. CD4, CD8, and NK1.1 depletions were initiated 1 week before tumor challenge and lasted 60 days after tumor challenge. Kaplan & Meier analysis indicating time-to-tumor development of vaccinated mice challenged with B16-OVA tumors and depleted of CD4, CD8 or NK cells. The data shown here are from one representative experiment of two performed.

Figure 5. *In vivo* tumor treatment experiments in mice vaccinated with OVA protein by different routes of administration

C57BL/6 mice (five per group) were first challenged with 5×10^4 /mouse of B16-OVA tumor cells via tail vein injection. Three days after tumor challenge, mice were treated with OVA protein mixed with or without CpG via subcutaneous injection, intramuscular injection with or without electroporation. Mice vaccinated with CpG alone followed by electroporation were included as controls. Vaccinated mice were boosted twice at 1-week intervals with the same dose and regimen. Mice were sacrificed on day 30 after the last vaccination. The mean weight of lung in each mouse was determined by experimenters blinded to the sample identity. Box plot with median depicting the difference in the lung weight of tumor-bearing mice vaccinated with the different regimens. The data was analyzed using the Kruskal-Wallis test. Each box plot describes the 25th, 50th and 75th percentile of the lung weight distribution among 5 mice with 1.5 times Inter Quartile Range for the lower and upper whiskers. The bottom and top of the box are the 25th and 75th percentile and the band in the middle of the box is 50th percentile. The upper and lower adjacent values were defined as described previously by Tukey et al [37]. Upper adjacent value is 75th percentile value $+(3/2) \times$ inter quartile range, lower adjacent value is 25th percentile $-(3/2) \times$ inter quartile range, and inter quartile range: 75th percentile value $- 25^{\text{th}}$ percentile value. The data presented are from one representative experiment of the two performed.

Figure 6. Characterization of E7-specific CD8⁺ T cell immune responses generated by different routes of administration of E7 long peptide

C57BL/6 mice (three per group) were vaccinated with 1 μg/mouse of E7 long peptide (a.a 43–62) with or without 1 μg/mouse of CpG via subcutaneous injection or intramuscular injection with or without electroporation. Splenocytes from vaccinated mice were collected 1 week after last immunization and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. **(A)** Representative flow cytometry data depicting the number of OVA-specific CD8⁺ T cells. **(B)** Bar graph representing the number of E7-specific CD8⁺ T cells/3 × 10⁵ splenocytes (mean ± SD). Data shown are representative of two experiments performed.

Figure 7. Characterization of the aggregation of luciferase-expressing E7-specific CD8⁺ T cell immune responses generated by intramuscular injection of E7 long peptide with or without electroporation

C57BL/6 mice (three per group) were vaccinated with 1 μ g/mouse of E7 long peptide (a.a 43–62) with or without 1 μ g/mouse of CpG via intramuscular injection with or without electroporation. One day later, mice were adoptively transferred with luciferase-expressing E7-specific T cells intravenously through the tail vein into the mice. **(A)** Representative luminescence images depicting the luciferase activity of E7-specific CD8⁺ T cells at the vaccination site. **(B)** Bar graph representing the quantification of luciferase activity of the E7-specific CD8⁺ T cells (mean $\pm$ SD). Data shown are representative of three experiments performed.