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In vivo electroporation enhances vaccine-mediated therapeutic control of human papilloma virus-associated tumors by the activation of multifunctional and effector memory CD8$^+$ T cells

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ABSTRACT

In vivo electroporation (EP) has reignited the clinical interest on DNA vaccines as immunotherapeutic approaches to control different types of cancer. EP has been associated with increased immune response potency, but its capacity in influencing immunomodulation remains unclear. Here we evaluated the impact of *in vivo* EP on the induction of cellular immune responses and therapeutic effects of a DNA vaccine targeting human papillomavirus-induced tumors. Our results demonstrate that association of EP with the conventional intramuscular administration route promoted a more efficient activation of multifunctional and effector memory CD8$^+$ T cells with enhanced cytotoxic activity. Furthermore, EP increased tumor infiltration of CD8$^+$ T cells and avoided tumor recurrences. Finally, our results demonstrated that EP promotes local migration of antigen presenting cells that enhances with vaccine co-delivery. Altogether the present evidences shed further light on the *in vivo* electroporation action and its impact on the immunogenicity of DNA vaccines.

1. Introduction

DNA vaccines represent a simple and safe alternative to activate cellular and humoral immune responses [1,2]. Although these vaccines demonstrated insufficient immunogenicity in the first clinical studies, different strategies were established to increase their potency in humans, including codon usage optimization, incorporation of adjuvants and more efficient gene delivery methods [3–6]. In this context, *in vivo* electroporation (EP) employs electrical pulses that generate transient pores in the cell membrane and promote DNA displacement [7–10]. The greater transfection efficiency related to this technique was shown to increase 10 to 100-fold the amount of antigen produced after plasmid administration by the intramuscular or intradermal routes associated with EP [11–15]. EP also promotes the recruitment of pro-inflammatory cells to the inoculation site and induces the local secretion of cytokines and chemokines, which can amplify the vaccine-induced immune responses [16–18]. *In vivo* EP has been evaluated in clinical studies to deliver DNA vaccines against infections, such as those caused by HIV [19] and hepatitis C [20]. The immunization with *in vivo* EP has been well tolerated and its capacity to activate robust cellular immune responses has directed this vaccine delivery technology toward the search of therapeutic vaccines against different types of cancer, including prostate [21], melanoma (ClinicalTrials NCT00471133) and HPV-induced cervical tumors [22,23].

Cervical cancer is the fourth cause of cancer death among women worldwide [24]. Persistent infections with high-risk HPVs are the causative agent of nearly all cervical cancer cases and significant numbers of other anogenital and oropharyngeal tumors [25–27]. HPV-16 and 18 are the virus types most commonly related to cancer induction and are responsible for 50 and 35% of the cervical cancer cases, respectively [28]. The viral oncoproteins E6 and E7, which are constitutively expressed by the tumor cells, promote cell transformation and maintenance of the malignant state, and therefore, represent potential targets for the development of therapeutic vaccine formulations against HPV-associated tumors [29].

Recently, intramuscular (i.m.) immunization with *in vivo* electroporation has been employed to deliver DNA vaccines to patients with Cervical Intraepithelial Neoplasia (CIN 2 or 3) associated to HPV-16 or 18. In the GX-188E phase I study, seven out of nine

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patients showed complete regression of lesions, which correlated with the induction of polyfunctional $CD8^+$ T cell responses [22]. In a phase 2 trial conducted with a distinct DNA vaccine, VGX-3100, histopathological regression of lesions was observed in 40% of the treated patients (n = 167) and the clinical outcome was related to an increased expression of molecules associated with the cytotoxicity of T cells [23]. Notably, EP of anti-cancer vaccines enhanced the number of polyfunctional $CD8^+$ T cells or their cytolytic activity, but the lack of control groups usually precludes the clear determination of the impact of this delivery method on the induced immune response with regard to conventional i.m. immunization. On the other hand, preclinical studies that directly compare parenteral vaccination alone or followed by *in vivo* EP mainly focus on the enhancement of the immune responses, but do not investigate the modulation of the induced cellular immune response [13,30–32].

Our group has developed a therapeutic vaccine formulation against HPV-associated tumors based on the expression of HPV-16 E7 genetically fused to the HSV-1 glycoprotein D (gD), that elicits the activation of E7-specific $CD8^+$ T cells and partial tumor protection in mice grafted with cells expressing the HPV-16 E6 and E7 proteins (TC-1 cells) after i.m. immunization [33]. In the present work, we evaluated the impact of *in vivo* EP on the immune responses and antitumor effects induced by the gD-based DNA vaccine, with particular emphasis on $CD8^+$ T lymphocytes activated during the immunization process and the cellular mechanisms involved with the observed adjuvant effects.

2. Materials and methods

2.1. Mice

C57BL/6 mice at 6–8 weeks of age were supplied by the Animal Breeding Center of the Parasitology Department from the Institute of Biomedical Sciences of the University of São Paulo. All the procedures involving animal handling followed the recommendations for the proper use and care of laboratory animals from the University of São Paulo Ethics Committee.

2.2. Plasmids

The DNA vaccine pgDE7h encodes the HPV-16 E7 oncoprotein genetically fused to the HSV-1 gD protein as previously described [33]. The gene sequence was cloned in the pUMVC3 vector (Aldevron, ND, USA). A plasmid encoding the luciferase protein (pLuc) was constructed after cloning the synthetic luciferase gene (*luc2*)-extracted from the vector pGL4 (Promega, Madison, WI, EUA)-using the sites *Bam*HI e *Xba*I of the pcDNA3.0 plasmid (Invitrogen, Waltham, MA EUA).

2.3. TC-1 cells

The TC-1 tumor cell line was kindly provided by Dr. T.C. Wu (John Hopkins University, Baltimore, MD, USA). This cell line is transfected with retrovirus vectors encoding v-Ha-ras, HPV-16 E6 and E7 [34]. The cells were propagated in RPMI medium supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 2 mM non-essential amino acids, 10 mM HEPES buffer, 50 units/ml penicillin/streptomycin, 0.4 mg/ml G418, 10% fetal bovine serum (FBS) and were kept at 37 °C at 5% CO_2. Prior to the inoculation, cells were harvested by trypsinization, washed twice, and suspended in serum-free media at the proper cell concentration.

2.4. In vivo EP

The CUY560-5-0.5 electrode (NepaGene Co., Ltd.; Japan) consisting of a pair of parallel 0.5 mm needles displayed 5 mm apart was used for the i.m. immunization associated with EP. Mice treated with EP were anesthetized i.p. with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). The electrodes were inserted immediately after vaccine injection in the tibialis anterior muscle using a conventional syringe. Two poring electric pulses were applied (130 V, 450 ms) followed by 4 transfer pulses (70 V, 450 ms) using the NEPA21 electroporator equipment (NepaGene Co., Ltd.; Japan).

2.5. Detection of luciferase expression in vivo

Animals were inoculated with pLuc plasmid by i.m. administration combined or not with EP. The bioluminescence results were obtained after inoculation of 200 μl of luciferin (Promega, USA) i.p. at a concentration of 150 mg/kg of body weight, and bioluminescence was observed in the IVIS® Spectrum (Caliper, England) 48 h or 7 days after transfection. The bioluminescence images were analyzed to obtain the total flux values, which refer to the number of photons per second (p/s).

2.6. Cell influx to the inoculation site

Mice were divided in five experimental groups: non-inoculated (Naive), inoculated with PBS, treated only with the electrical pulses (EP), or immunized with 50 μg of the pgDE7h plasmid via the conventional intramuscular route (IM) or followed by *in vivo* electroporation (IMEP). Muscle samples were collected 24 or 48 h after immunization and treated with collagenase D (Roche) (1 mg/ml) for 1 h. Samples were then filtered and stained with two sets of markers: (i) PerCP Cy5.5-anti-CD45, APC Cy 7-anti-CD3, APC-anti-CD8, BV605-anti-CD4, PE-anti-CD19 and FITC-anti-NK1.1 or (ii) PerCP Cy5.5-anti-CD45, Alexa700-anti-CD11b, BV421-anti-CD11c, PE-anti-Ly6G, FITC-anti-Ly6c and APC-anti-F4/80 (BD Bioscience). Cells were examined by flow cytometry using a FACS LSRFortessa (BD Biosciences) and data were analyzed using the FlowJo software (TreeStar, OR, USA).

2.7. Immunization and tumor cell challenge

Groups of five mice were challenged subcutaneously (s.c.) with 7.5×10^4 TC-1 tumor cells/mice suspended in 100 μl of serum-free media and injected into the right rear flank. Tumor growth was measured with a caliper three times a week for at least 60 days. Mice were scored as tumor-bearing when tumors reached a size of approximately 2 mm in diameter. Mice were euthanized once the tumors exceeded a diameter of 15 mm or became necrotic. To study the vaccine efficacy against tumor recurrence, mice that remained protected from tumor growth received a second tumor challenge with 7.5×10^5 TC-1 tumor cells/mice, 60 days after the first tumor graft. Mice were vaccinated with one dose of 5, 10, 25 or 50 μg of the i.m. DNA vaccine associated with *in vivo* electroporation (IMEP) or not (IM). The vaccine dose was administered 3, 7, 10 or 14 days after the tumor cells graft. For some experiments a second dose was administered 7 days after the first one. In some experiments, the EP and vaccine administration was carried out in different days. Some groups received the vaccine on day 1 and the EP on day 2 (IM-EP) while other groups received EP on day 1 and the vaccine on day 2 (EP-IM).

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