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Irreversible electroporation inhibits pro-cancer inflammatory signaling in triple negative breast cancer cells

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ABSTRACT

Low-level electric fields have been demonstrated to induce spatial re-distribution of cell membrane receptors when applied for minutes or hours. However, there is limited literature on the influence on cell signaling with short transient high-amplitude pulses typically used in irreversible electroporation (IRE) for cancer treatment. Moreover, literature on signaling pertaining to immune cell trafficking after IRE is conflicting. We hypothesized that pulse parameters (field strength and exposure time) influence cell signaling and subsequently impact immune-cell trafficking. This hypothesis was tested in-vitro on triple negative breast cancer cells treated with IRE, where the effects of pulse parameters on key cell signaling factors were investigated. Importantly, real time PCR mRNA measurements and ELISA protein analyses revealed that thymic stromal lymphopoietin (TSLP) signaling was down regulated by electric field strengths above a critical threshold, irrespective of exposure times spanning those typically used clinically. Comparison with other treatments (thermal shock, chemical poration, kinase inhibitors) revealed that IRE has a unique effect on TSLP. Because TSLP signaling has been demonstrated to drive pro-cancerous immune cell phenotypes in breast and pancreatic cancers, our finding motivates further investigation into the potential use of IRE for induction of an *anti*-tumor immune response in vivo.

1. Introduction

Treating tumors with irreversible electroporation (IRE) involves placing a pair of electrodes in the targeted tissue volume to deliver high-amplitude electric pulses (Fig. S1) that disrupts homeostasis by increasing membrane permeability leading to cell death in a non-thermal manner. Similar to radiotherapy treatment planning, conventional medical imaging modalities such as computed tomography, magnetic resonance imaging, and ultrasound imaging can be used for pre-procedural and peri-procedural treatment planning to determine effective tissue volume ablation [1]. The delivered electric pulse train can be tailored to each patient by modifying the number of pulses (exposure time), the electric field strength (ratio of applied voltage to electrode distance), and the interval between pulses (to synchronize with cardiac rhythm), such that critical features (blood vessels and extracellular matrix) can be spared [2]. In addition to the aforementioned variables, pulse polarity and width can be modified to avoid nerve stimulation and muscle contraction [3], as well as to target cells with higher nuclear to cytoplasmic ratio, a morphology which is a hallmark of many cancers [4,5]. The aforementioned factors make IRE an attractive non-conventional tumor ablation modality.

It is well known that immune cells play an important role in cancer progression; however, there is a lack of mechanistic studies showing the effect of IRE treatment on cell signaling and, consequently, immunological responses [6]. The first investigation of immune response post IRE treatment used immune-competent mice inoculated subcutaneously with methylcholanthrene-induced sarcoma cells to study immune cell recruitment [7]. Briefly, immunohistochemistry (IHC) was used on extracted tumor tissue samples to assess populations of CD4+ T lymphocytes, CD8+ T lymphocytes, and macrophages. Moreover, CD86, CD80 and CD11c receptors were evaluated to identify activated dendritic cells. Based on the observation that there were no differences between the populations of cells under study within the first 6 h after treatment, the authors concluded that IRE did not induce any change in immune cell infiltrates.

Contrary to the aforementioned report, an investigation performed on immune-competent Sprague-Dawley rats with osteosarcoma provided evidence of an anti-tumor immune response with IRE treatment [8]. Briefly, the investigation measured the CD8+ and CD4+ T lymphocytes along with serum soluble interleukin-2 receptors (sIL-2R) in a cohort of 118 rats. When compared to untreated controls, IRE treated rats

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had increased CD4 +/CD8 + ratios in T lymphocytes, which is associated with a better prognosis. Moreover, IRE treated rats had reduced levels of serum sIL-2R comparable to the non-tumor bearing population.

In a third study conducted by one of our research team members, Davalos and colleagues treated immune-deficient and immune-competent mice bearing renal cancer with IRE [9]. Morbidity and tumor volume of the mice populations were monitored. In another aspect of their study, the investigators re-challenged the immune-competent mice with cancerous cells 18 days after treatment and monitored the tumor growth. There were two conclusions drawn from the study. First, there was a significant tumor recession in immune-competent mice compared to immune-deficient population. Second, re-challenged mice developed either no tumor or had a very small cancerous growth. An increased infiltration of CD3 + T cells into the treatment area was observed in some of the treated immune-competent re-challenged mice. Thus, the literature on cell communication pertaining to immune cell trafficking after IRE treatment is conflicting, yet there is strong evidence of an anti-tumor immune response resulting from IRE in some studies.

The motivation of this study was to develop an understanding of the influence of IRE on immune cell trafficking through its direct effects on tumor cell signaling. Experimental evidence in the literature suggests that a constant electric field applied in parallel to a cell's surface leads to the redistribution of charged receptors on the cell membrane [10,11]. Moreover, the redistribution and its permanence are functions of both the applied field strength and the duration of exposure [12,13]. The aforementioned literature used constant electric field strengths in the range of 1–25 V/cm applied over a long duration of time (order of minutes to hours). To our knowledge, there is no published work that has investigated the influence of short transient high-amplitude (500–1000V/cm) fields, such as those used in IRE (Fig. S1), on cell signaling related to immune-cell trafficking. Based on the contradictory findings in the literature relating to immune response with IRE treatment, it was hypothesized that pulse parameters (e.g. electric field strength and number of pulses) influence cell signaling and subsequently influence local and systemic immune response.

This hypothesis was tested on triple negative breast cancer cells which lack three cell membrane receptors commonly leveraged for effective drug targeting (e.g. aromatase inhibitors as blockade of estrogen production and selective estrogen receptor modulators as *anti*-estrogen activity agents). As IRE leverages physical mechanisms as opposed to relying on the expression of specific membrane receptors, IRE could be an ideal alternative or complementary treatment for triple negative breast cancer. In this study, murine 4T1 triple negative breast cancer cells were used, as this model closely replicates human stage IV triple negative breast cancer [14]. Specifically, we investigated the influence of IRE on signaling factors tumor necrosis factor (TNF), interleukin 6 (IL6), thymic stromal lymphopoietin (TSLP) and chemokine (C-C motif) ligand 2 (CCL2). To develop an understanding of the influence of IRE parameters on cell signaling, lethality of pulse parameters and their effect on the aforementioned signaling factors were studied.

2. Materials & methods

2.1. Cell line and culture conditions

The triple negative breast cancer murine cell line 4T1 was purchased from American Type Culture Collection (ATCC, Catalog number: CRL-2539). The human progressive breast cancer cell lines MCF-10A, MCF-10AT1 and MCF-DCIS.com were obtained from Dr. Eva Schmelz at Virginia Tech (Blacksburg, USA). The human triple negative breast cancer cell line MDA-MB-231 was purchased from ATCC (Catalog number: HTB-26). The 4T1 and MDA-MB-231 cells were maintained in RPMI-1640 and DMEM-F12 culture medium respectively, supplemented with 10% (by volume) fetal bovine serum (FBS) and 1% (by volume) penicillin streptomycin (PS). The human progressive breast cancer cell lines (MCF-10A, MCF-10AT1, MCF-DCIS.com) were grown in DMEM-F12 culture media supplemented with 5% (by volume) horse serum, 20 ng/mL endothelial growth factor (EGF), 0.5 mg/mL hydrocortisone, 10 mg/mL insulin and 1% (by volume) PS. Cells were sustained in humidified incubators at 37 °C and 5% CO_2. Cells were sub-cultured at approximately 80% confluence and 0.25% Trypsin-EDTA solution was used for detachment. All experiments were performed within the first ten sub-cultures.

2.2. Irreversible electroporation procedure

After reaching near confluence (~80–90%), the obtained cell pellet was washed with phosphate buffered saline without calcium and magnesium ($PBS^{-/-}$; Santa Cruz Biotechnology) and re-suspended in basal growth media with neither serum nor PS. Approximately 1 million cells/cuvette were transferred to 4 mm electroporation cuvettes (Mirus Bio LLC) in a volume of 600 μL. As shown in the schematic of the experimental setup in Fig. S1 (Supplement figure), the cuvette was placed in a holder through which the cells were exposed to electric field. The electric voltage required for the ablation was generated using an electroporation unit (Harvard Apparatus). A representative electric pulse train used to ablate the cells is shown in Fig. S1. Short pulses of 100 μs were delivered at an interval of 1 s. Changing the number of these short pulses from 80 to 99 pulses varied the duration of exposure. The electric field strength reported in this article was calculated by taking the ratio of electric voltage applied to the distance between the electrodes in the cuvette (4 mm). For the untreated controls, cells were transferred to cuvettes and kept in the cuvette for the same duration as treatment groups without application of electric field.

2.3. Assessment of phospatidylserine expression as an early death marker

Following recovery time (~45 min) after treatments, cells were centrifuged at 300 r.c.f. for 9 min at 4 °C. Cell pellets were washed with $PBS^{-/-}$ followed by centrifugation and re-suspension in 300 μL of Annexin V binding buffer (Biotium Inc.). A 100 μL volume from each sample was used for staining with 10 μL of Annexin V conjugated with fluorescent allophycocyanin (APC; Ex: 633 nm Em: 660 nm; Biolegend) and approximately 3 μM of nucleic acid stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Ex: 364 nm Em: 454 nm; Sigma-Aldrich). After 15 min of incubation at room temperature, the percentage of sample population expressing Annexin V and DAPI was measured using fluorescence-activated cell sorting (Amnis imaging flow cytometer, EMD Millipore).

2.4. Assessment of cell viability using NAD(P)H dependent mitochondrial metabolic activity

Following recovery time after treatments, cell concentration was determined in samples from each condition and cells were transferred to 96 well tissue culture plates such that the total cell number was approximately 10,000 cells/well for all treatment groups. The samples were incubated at 37 °C in 5% CO_2 overnight (~12–14 h). Alamar Blue dye (Bio-Rad Laboratories Inc.) was then added (5:1 media and dye ratio) and fluorescence (Ex: 530 nm; Em: 600 nm) was read using a spectrophotometer (Molecular Devices) 2 h after the addition of dye. Viability of cells for a given treatment condition was defined as the ratio of the raw fluorescence signal from the treated wells to the fluorescence signal from the control wells.

2.5. Assessment of gene expression using real time PCR (rt-PCR)

Cells were transferred to a 6 well tissue culture plate (approximately 1 million cells/well) and incubated at 37 °C in 5% CO_2. After an overnight incubation, cells were homogenized using TRIzol detergent (LifeTechnologies). RNA was then isolated from the homogenized