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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*.

Keywords

Electroporation; triple negative breast cancer; thymic stromal lymphopoietin (TSLP); immunotherapy

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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 (Figure 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 (Jourabchi et al., 2014). 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 (Edd et al., 2006). In addition to the aforementioned variables, pulse polarity and width can be modified to avoid nerve stimulation and muscle contraction (Arena et al., 2011), as well as to target cells with higher nuclear to cytoplasmic ratio, a morphology which is a hallmark of many cancers (Ivey et al., 2015; Sano et al., 2014). 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 (Bastianpillai et al., 2015). 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 (Al-Sakere et al., 2007). 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 hours 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 (Li et al., 2012). 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 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 (Neal et al., 2013). 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 (Poo and Robinson, 1977; Zagjansky and Jard, 1979). Moreover, the redistribution and its permanence are functions of both the applied field strength and the duration of exposure (Groves and Boxer, 1995; Poo, 1981). 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 (500–800 V/cm) fields, such as those used in IRE (Figure 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 (Lv et al., 2013). 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% CO₂. 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 Figure 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 Figure S1. Short pulses of 100 µs were delivered at an interval of one second. 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 phosphatidylserine expression as an early death marker

Following recovery time (~45 minutes) after treatments, cells were centrifuged at 300 r.c.f. for 9 minutes 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; Biotium Inc.) 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 minutes 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% CO₂ overnight (~12–14 hours). 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) two hours 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% CO₂. After an overnight incubation, cells were homogenized using TRIzol detergent (LifeTechnologies). RNA was then isolated from the homogenized samples using a Phenol-Chloroform extraction technique and standard protocol provided by the manufacturer (Life Technologies manuscript number MAN0001271). After the isolation, equal amounts of nucleic acid were converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems catalog number: 4368814) in accordance with manufacturer's instructions. The cDNA obtained was used for rt-PCR using primer probes for TSLP (TaqMan Mouse: Mm01157588_m1; Human: Hs00263639_m1), IL-6 (TaqMan Mm00446190_m1), TNF (TaqMan Mm00443260_g1), CCL2 (TaqMan Mm00441242_m1) and CSF2 (TaqMan Mm01290062_m1). Expressions of these genes were normalized to the housekeeping gene 18s (TaqMan Hs99999901_s1). Relative gene fold expression change was calculated using the Ct method.

2.6 Assessment of supernatant protein concentration using ELISA

Cells were transferred to a 6 well tissue culture plate and incubated at 37 °C in 5% CO₂. After an overnight incubation (~12–14 hours), supernatants were collected and stored at –80 °C for downstream analysis of protein expressions using enzyme-linked immunosorbent assay (ELISA). Simultaneously, the number of live cells in each well was also measured (with Trypan Blue assay alone or combined with Alamar Blue) to account for the variable number of living cells across the treatment groups. The concentrations of protein in conditioned media were measured using an ELISA kit (Affymetrix eBioscience) in accordance with the manufacturer's instructions. Protein levels were then normalized per million live cells for each treatment group.

2.7 Digitonin as a chemical poration treatment

The protocol for Digitonin treatment was based on a published protocol (Tissera et al., 2010). Briefly, cell suspensions were washed with PBS and re-suspended in basal media. DMSO was used as the carrier vehicle for Digitonin that was added such that the final concentration was 50 µg/mL. The carrier vehicle alone was added to the untreated controls. After incubating the samples in an ice-bath for 1–2 minutes, cells were centrifuged at 300 r.c.f. for 5 minutes in 4 °C. The supernatant was discarded and the resulting cell pellet was washed with ice-cold PBS and re-centrifuged. The cell pellet was then re-suspended in basal media after which cells were plated in 6 well-tissue culture plates. The cells were incubated at 37 °C in 5% CO₂ overnight before supernatant was collected for ELISA.

2.8 Staurosporine as a kinase inhibitor treatment

The protocol for Staurosporine treatment was based on studies reported in the literature (Bastian et al., 2015; Shanker et al., 2008). Briefly, approximately 1 million 4T1 cells were seeded in a 6-well tissue culture plate and incubated overnight at 37 °C in 5% CO₂. DMSO

was used as a carrier vehicle for Staurosporine and was added to treatment wells at a final concentration of 0.5 μ M. The untreated controls were only exposed to the carrier vehicle DMSO. The cells were again incubated overnight before supernatant was collected for ELISA.

2.9 Statistical analyses

The software JMP® (version 12, SAS Institute Inc., Cary, NC) was used for statistical analyses. The effect of IRE electric field strengths were compared to untreated controls using one-way analysis of variance (ANOVA) followed by post hoc Tukey HSD to find means that were significantly different from each other. Factorial ANOVA was used to evaluate the effects of both electric field strength and number of pulses with Tukey HSD post-test. The difference between means of two sample data were tested by student t-test.

3. RESULTS

The effect of IRE pulse parameters (field strength and number of pulses) on cell viability was studied using live cell Annexin-V/DAPI staining and NAD(P)H dependent resazurin metabolic activity. Then, the effect of IRE treatment on cell mRNA transcription was investigated using real-time PCR. The question of whether IRE treatment affected mRNA to protein translation was explored using ELISA on cell supernatants. Finally, comparison of thymic stromal lymphopoietin (TSLP) in cells treated with thermal shock, kinase inhibitor Staurosporine and chemical porator Digitonin was also performed. The relevance of TSLP in human breast cancer is also discussed.

3.1 Cell death is a function of electric field parameters

Cell suspensions were exposed to electric field strengths (= Applied voltage/distance between electrodes) of 500 V/cm, 600 V/cm and 700 V/cm. The pulse duration was kept constant at 100 μ s (Figure S1), which is consistent with clinically used values (Martin II et al., 2012; Thomson et al., 2011). The interval between pulses was set to 1 s and the number of pulses applied was varied from 80 to 99 pulses to change the exposure time, parameters that were consistent with clinically used ranges. For pulses of 100 μ s duration, the electric field across the outer cell membrane can be calculated using a simple analytical expression [for derivation refer to (Schoenbach et al., 2007)]:

$$E_M = E_a D / 2d \quad \text{Eqn. 1}$$

The variables of this equation include E_M (the electric field across the membrane), E_a (the applied electric field), D (the cell diameter) and d (cell membrane thickness). The cell diameter in suspension is a stochastic distribution (Figure S2), so a mean cell diameter of 13 μ m and mean membrane thickness of 5 nm can be assumed (Schoenbach et al., 2007). Thus, the field across the cell membrane (E_M) for 500 V/cm, 600 V/cm and 700 V/cm may be approximated to be 0.65 MV/cm, 0.78 MV/cm and 0.91 MV/cm respectively.

Viabilities of the 4T1 cells treated with IRE as well as untreated controls were measured immediately after ablation using live cell Annexin-V/DAPI staining visualized by a flow-

cytometer. A hallmark of early death through either apoptosis or necrosis is the translocation of phosphatidylserine. Annexin-V, which has high affinity to phosphatidylserine, can detect this translocation (Vermes et al., 1995). While the translocation of phosphatidylserine is an early marker of cellular death, a simultaneous loss of membrane integrity is the true determination of necrotic death. Live cell staining with DAPI enabled the measurement of the percentage of cells with loss of membrane integrity (Kapuscinski, 1995). A representative flow-cytometer image is shown in Figure 1A. The percentage of DAPI positive only (DAPI+) cells is completely lysed, while the Annexin positive only (Ann+) percent population has translocation of phosphatidylserine (Figure 1A). The population of double positive (Ann+DAPI+) represents dead cells, while the unstained population represents live cells (Figure 1A). Based on flow-cytometer measurements, there was a statistically significant ($p < 0.05$) fold increase in the Ann+DAPI+ population in IRE treated cells for all the electric field strengths (Figure 1B). The reduced viability in IRE treated cells when compared to untreated controls was confirmed using Alamar Blue (resazurin) metabolic assay, performed after overnight (~12–14 hours) incubation (Figure 1C).

The trans-cell membrane electric field (E_M) that leads to irreversible pore formation and lethal membrane damage is a function of cell diameter and membrane thickness at a given applied field (Eqn. 1). For a heterogeneous distribution of cell size in suspension (Figure S2), the fraction of cells that experience a lethal electric field increases with increasing applied field strengths (E_a). Consistent with this prediction, the fraction of non-viable cells increased with higher applied field strength as confirmed by both flow-cytometry and Alamar Blue (resazurin) assay (Figures 1B & C). A higher exposure time to IRE led to increased cell death in populations treated with 99 pulses as compared to 80 pulses (Figures 1C). One plausible reason for this may be due to loss of homeostasis from pore formation that increases with time (Saulis, 1997; Tieleman et al., 2003). Increased exposure time could also lead to additional time for electrical pathways into the cell leading to intracellular fields (Esser et al., 2010a). However, the factors and mechanisms of cellular death due to IRE are complex and beyond the scope of this study. The interested reader is directed to relevant literature (Deipolyi et al., 2014; Mašek-Lebar and Miklavcic, 2001).

3.2 IRE influences TSLP in 4T1 murine triple negative breast cancer cells

Comparison of pro-inflammatory cytokines in conditioned media of metastatic 4T1 cells to cytokines present in non-metastatic clones provided specific cytokines that were up-regulated in metastatic counterparts of 4T1 cells (Olkhanud et al., 2011). These included the cytokines CCL17, CXCL1 and TSLP. Epithelial cells are one of the primary producers of TSLP, which has been implicated in polarizing a gamut of immune cells to the Th-2 phenotype. Polarization of immune cells to the Th-2 phenotype has been implicated in progression of human breast and pancreatic cancers (De Monte et al., 2011; Ziegler et al., 2013). The list of hematopoietic cells influenced by TSLP includes myeloid-derived dendritic cells (DCs), CD4+ and CD8+ T cells, basophils, natural killer T cells, eosinophils, and B cells (Kitajima et al., 2011; Omori and Ziegler, 2007; Watanabe et al., 2004; Ziegler et al., 2013). Previous murine studies have not only implicated TSLP in cancer escape and progression but have also proven that its inactivation leads to a complete blockade of lung metastasis (Olkhanud et al., 2011). Interestingly, a recent murine study reported that

increasing the amount of systemic TSLP prevented progression in early stage breast cancer (Demehri et al., 2016). Moreover, the study demonstrated that breast cancer cells that express more TSLP actually block tumor progression in this model. This recent result suggests that the role of TSLP in cancer progression is likely tumor and stage-dependent; further motivating focused studies such as ours. The assay by Olkhanud *et. al.* also provided other cytokine candidates that were expressed more in non-metastatic clones compared to the metastatic versions; these included TNF, IL-6 and CCL-2. The cytokine CSF-2 was highly expressed in the conditioned media of only one metastatic clone of 4T1. Thus, based on the literature implicating these factors as key regulators of tumor progression, the effect of IRE on TSLP, IL-6, TNF, CSF-2, and CCL-2 was further explored in our investigation.

Analysis using rt-PCR was performed on cells incubated overnight after treatment, indicating that IL-6 and TNF mRNA were up-regulated in IRE treated cells (Figure 2 A&B). This became significant ($p < 0.05$) at an applied electric field of 700 V/cm, corresponding to $E_M > 0.78$ MV/cm. Results also showed that TSLP mRNA was down-regulated at applied field strengths above 600 V/cm ($E_M > 0.78$ MV/cm; Figure 2C, $p < 0.05$). No trend was observed in CCL2 (Figure 2D) or CSF-2 (data not shown).

We next investigated whether the changes in mRNA transcription led to differences in the translation and secretion of proteins as measured by probing protein concentrations in conditioned media after overnight incubation post-treatment. The concentrations of supernatant proteins evaluated by ELISA were normalized to number of live cells (per million) assessed using both Alamar Blue metabolic assay and Trypan Blue exclusion tests. Because of the important roles of TSLP in tumor progression reported in the literature, we chose to further focus on secreted TSLP protein level, necessary for their subsequent impact on immune cell phenotypes. The Tukey HSD analysis indicated that TSLP was down-regulated at electric field strengths of 600 V/cm and above ($E_M > 0.78$ MV/cm; Figure 3A) when compared to controls ($p < 0.05$). Moreover, there was no statistical difference between the populations exposed to 80 and 99 pulses within the same field strength as determined by factorial ANOVA analysis. We also determined the concentration of IL-6 in the cell supernatant. We observed a slight increase in IL-6 expression in treated cells compared to controls, although this was not statistically significant (Figure S3). Based on our analysis, an applied electric field above 600 V/cm contributed to the down-regulation of TSLP for IRE-treated 4T1 cells when compared to untreated controls, at both the mRNA and secreted protein levels. Notably, at these field strengths the duration of exposure (or number of pulses) did not have any significant influence.

3.3 Effects of thermal shock and chemical poration on TSLP

Exposure of cell suspensions to electric field pulses causes cell membranes to be exposed to a thermal load that is directly proportional to the pulse width and the magnitude of the field (Schoenbach et al., 2007). Because of concerns that such temperature changes may impact our observed signaling results, we analyzed the increase of bulk temperature in the suspension media at the tested electric field strengths (500 V/cm, 600 V/cm, 700 V/cm) and durations (80 and 99 pulses). Both mean and maximum temperature increases were measured in suspension media without cells (Figure S4A). The mean and maximum

temperature rises were highest at 700 V/cm, which were approximately 2 °C and 3.5 °C, respectively (Figures S4B & S4C). An experimental setup was developed in which the bulk temperature increase, as seen in a typical electric ablation treatment (Figure S4A), was replicated in a water bath (Figure 4A). Cells were exposed to mean and maximum temperature rises of approximately 3.5 and 5 °C, respectively. After exposing the cell suspension to this temperature increase an overnight incubation was performed, following which the cell supernatant was collected for ELISA. The concentrations of TSLP obtained from the assay were normalized to number of live cells (per million) as measured by Trypan Blue exclusion studies. A t-test statistical analysis of the ELISA data indicated that TSLP was not down-regulated due to the temperature change (Figure 4B) when compared to untreated control ($p > 0.05$).

We next hypothesized that the TSLP down-regulation observed may have resulted from the loss of homeostasis due to cell membrane permeabilization, as opposed to being a direct result of electric field exposure. While there are different methods of permeabilizing cells including chemical (Jamur and Oliver, 2010) and mechanical (Tomizawa et al., 2013) agents, we chose to perform a non-electrical control experiment using Digitonin as a poration agent. Digitonin is known to preferentially permeabilize the plasma membrane without affecting the nuclear envelope (Griffis et al., 2003; Tissera et al., 2010). The 4T1 cells were treated with 50 µg/mL of digitonin, and after overnight incubation, results indicated approximately 26% viability ($\pm 6\%$; determined using Trypan Blue exclusion test). Cell supernatants were collected after overnight incubation followed by analysis using ELISA. The concentration of TSLP in the supernatant was normalized to live cells (per million) and t-test analysis indicated that there was no statistical ($p > 0.05$) difference between digitonin treated and untreated cells (Figure 4C). Thus, the ELISA data indicated that TSLP was not down-regulated due to poration using Digitonin (Figure 4C).

3.4 Positive control - TSLP expression is regulated by a known modulator

The effect of the PKC inhibitor Staurosporine on TSLP was tested as a positive control to validate the relevance of TSLP variations in our experiments. A recent study reported increased induction of TSLP in primary human fibroblasts with IL-1B and TNF stimulation (Nomura et al., 2012). In the same study, the up-regulation of TSLP with IL-1B was inhibited when pre-treated with pan-protein kinase c (pan-PKC) inhibitor. Staurosporine is an alkaloid component that is known to be a potent inhibitor of PKC (Tamaoki et al., 1986) and is known to cause apoptosis (Zhang et al., 2004). The 4T1 cells were treated with 0.5 µM of Staurosporine with approximately 53% viability ($\pm 12\%$; determined using Trypan Blue exclusion test) after treatment. Cell supernatants were collected after overnight incubation followed by performing ELISA. When the concentration of TSLP in the supernatant was normalized to live cells (per million), t-test statistical analysis indicated that TSLP concentration in cell supernatants was lower in the treated group as compared to untreated controls (Figure 4D, $p < 0.05$), consistent with existing literature.

3.5 IRE influences TSLP in human triple negative breast cancer cells

We evaluated TSLP gene expression in the MCF10 human breast epithelial cell line progression series, and found that the pre-malignant 10AT1 cells had lower TSLP expression

compared to fibrotic epithelium 10A and ductal carcinoma in situ DCIS cells (Figure 5A). This demonstrated the correlation of TSLP with grade of breast cancer, increasing in expression from pre-malignant (10AT1) to DCIS. Moreover, we measured lower TSLP gene expression in human triple negative breast cancer MDA-MB-231 cells incubated overnight after exposure to 600 V/cm (99 pulses) when compared to untreated controls (Figure 5B). Thus the molecular effect on TSLP was independent of breast cancer cell species.

4. DISCUSSION

4.1 IRE has a direct molecular effect on TSLP

The previously mentioned results indicate that chemical poration and temperature change do not influence TSLP signaling in 4T1 cells. However, an applied electric field threshold demonstrated that TSLP signaling is affected. Based on our results, we conclude that IRE has a direct molecular effect on TSLP for fields over 600 V/cm. Although the mechanism behind this phenomenon is outside the scope of this work, we will discuss two possible explanations to be tested in future experiments.

Spatial reorganization of cell membrane protein receptors is known to occur due to electrophoretic forces exerted by an applied electric field. When exposed to an electric field, cells experience both a trans-membrane field (E_M) and a tangential-field on the cell surface (E_θ) that is a function of the azimuthal angle θ and the applied field E_a (McLaughlin and Poo, 1981):

$$E_\theta = f E_a \sin \theta \quad \text{Eqn. 2}$$

The shape of the cell is accounted for in this relation by using a factor 'f' (f = 1.5 for spheres). Tangential fields (E_θ) are known to influence distribution of protein receptors in the cell membrane. For example, a tangential field of 25 V/cm applied for over 30 minutes in a planar supported bilayer membrane showed irreversible clustering of charged components on the lipid layer (Groves and Boxer, 1995). Cultures of embryonic frog muscle cells exposed to 10 V/cm tangential fields for 30 and 180 minutes showed irreversible clustering of concanavalin A receptors on the membrane surface (Poo, 1981). Such reorganization and clustering of phospholipids are known to induce changes in cell signaling (Zhou et al., 2015), and may therefore be relevant to the signaling changes we report here.

Additionally, electro-permeabilization of the outer cell membrane may be a relevant mechanism inducing signaling changes in our studies. It has been widely argued that at electric pulse width above the charging time of the outer membrane (~ 75 ns – 1 μ s) the intracellular compartments are shielded from the electric field (L. Retelj et al., 2013; PAULY and SCHWAN, 1959; Sundararajan, 2008). Moreover, it was also hypothesized that pulse frequencies above 100 kHz allow the applied electric field to reach the intracellular structures (Batista Napotnik et al., 2016; Gowrishankar and Weaver, 2003; Schoenbach et al., 2001). Thus, it was believed that electric pulse trains that have nano-second pulse widths and frequencies above 100 kHz allow electric perturbation of voltage sensitive intracellular processes of the endoplasmic reticulum, mitochondria, and the nucleus (Chopinet et al.,

2013; Schoenbach et al., 2004). However, the Weaver group has recently shown through modeling that longer pulses also induce intracellular effects (Esser et al., 2010b). Their mathematical model predicted that intracellular electric fields are generated after electro-permeabilization using an exponential pulse of amplitude 1000 V/cm and pulse width of 40 μ s. Although their prediction needs experimental validation, an internal electric field could exist for the electrical pulse used in our investigation (pulse width: 100 μ s; frequency: 1 Hz). Experimental investigation has reported that cells subjected to millisecond pulses (10 pulses, 5 ms pulse width, 1 Hz frequency, 700 V/cm square pulse) demonstrate phospholipid internalization and scrambling, contingent upon electro-permeabilization (Escoffre et al., 2014). However, such interactions are also contingent upon the temporal dynamics of the pulse (e.g. microsecond vs. nanosecond pulse width). For example, a translocation similar to reported by Escoffre et. al. for millisecond pulses was demonstrated using field strengths of 20 kV/cm delivered by 50 pulses of 7 ns each (Vernier et al., 2006). Moreover, Vernier et. al. also demonstrated differences in translocation between unipolar and bi-polar pulses when the translocations at the anodic and cathodic sides were investigated. Further work beyond the scope of this study will test our mechanistic hypotheses regarding the theories described here.

4.2 Clinical implications and future work

The treatment of triple negative breast cancer is challenging due to the lack of target receptors. Although samples from triple negative breast cancer tumors provide evidence of a large number of tumor-associated antigens (Shah et al., 2012; Stagg and Allard, 2013), tumor cell signaling skews the microenvironment to promote cancer progression and growth. For example, programmed cell death protein (PD-1) and its ligands PD-L1 and PD-L2 are highly expressed in tumor cells and infiltrating immune cells that prevent T cell immune-surveillance (Barber et al., 2006; Wang and Chen, 2011). The tumor cell signaling is known to polarize dendritic cells to express high levels of OX-40 proteins that aid pulmonary metastasis through macrophage re-programming, and promote neoplastic epithelia survival and chemotherapy resistance (Coussens et al., 2013). These findings have led to clinical trials of immunotherapy drugs aimed at blocking PD-1 (Herbst et al., 2014; Tumei et al., 2014) and OX40 (Curti et al., 2013) pathways. Our results with respect to TSLP signal down-regulation has significance in this context. For instance, it has been demonstrated that TSLP up-regulates production of OX40 ligands in dendritic cells through NF- κ B genes (Arima et al., 2010). In this study, it was also shown that TSLP preferentially phosphorylates STAT6 signaling, inducing positive regulation of PD-L2 (Rozali et al., 2012). Our results provide avenues of exploring IRE as another option in inducing an anti-tumor response in immune cells that are “exhausted” (Jiang et al., 2015) by tumor cell signaling.

This investigation shows TSLP down-regulation with IRE treatment. Efforts are currently underway to examine the systemic functional analysis of TSLP signaling (by investigating STAT phosphorylation and NF- κ B pathway analysis), which are anticipated to provide evidence on the mechanistic influence of IRE. Assessment of influence on phenotype and migration of immune cells (such as macrophages and dendritic cells) responding to IRE treated cancer cells are also ongoing. As the field pulse width and polarity may also influence cell signaling, these factors will also be further considered.

5. CONCLUSION

Irreversible electroporation (IRE) is a highly tunable, safe, and minimally-invasive cancer treatment based on irreversible electric field-induced cellular damage, which provides the potential to preferentially target malignant cells while preserving critical supporting structures in the tumor microenvironment such as extracellular matrix, nerves and blood vessels. While the immunological impact of IRE cancer treatment has to date not received a significant amount of attention, we provide evidence that pulse parameters not only influence lethality to cells, but can also influence cell signaling with the potential to impact immune-cell trafficking. This study demonstrates that for a short transient applied voltage used in IRE, there is a threshold at which applied field can influence thymic stromal lymphopoietin (TSLP) signaling, a molecule with a known pro-tumor role via immune cell phenotype modulation. This study provides the foundation to further explore the influence of the broad spectrum of IRE pulse parameters such as polarity and pulse width on tumor cell signaling responses, specifically to develop safe and effective means of triggering an anti-tumor immune response in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biographies

Goswami received his bachelors and masters degrees in mechanical engineering from Vellore Institute of Technology (India) and University of Cincinnati, respectively. He is currently a PhD student in the Department of Mechanical Engineering at Virginia Tech. He has been a recipient of Hord Fellowship at Virginia Tech, University Graduate Scholarship at University of Cincinnati, and Vellore Institute of Technology merit scholarship. His broad research interests are in thermodynamics and medical devices.

I received my DVM from VA-MD College of Veterinary Medicine in May 2011. Upon graduation, I spent 3 years at the University of Georgia where I trained as a veterinary anatomic pathologist. I passed my board certification exams in September 2014. I then returned to VA-MD College of Veterinary Medicine in July 2014 to begin working on my PhD as an NIH T-32 Fellow. I am currently a postdoctoral candidate in Dr. Irving Allen's lab where I am using animal models to investigate mechanisms of inflammation and tumorigenesis.

As I enter my fifth year at Virginia Polytechnic and State University as an electrical engineering major with a minor in biomedical engineering, I have begun to search for medical school programs that will allow me to apply my engineering skills to develop technologies that dramatically improve patient treatment and care. I hope to bring a multidisciplinary scientific approach to the development of processes to help physicians bridge the gap between ailments diagnosed and the range of treatment options available.

As an immunologist and cancer researcher, I am interested in understanding the complex tumor microenvironment and gaining a greater understanding of the interplay that occurs between the immune system and tumor. The overarching goal of my research program is to elucidate mechanisms associated with the regulation of immune system homeostasis and methods to modulate host immunity during cancer. To this end, we invest heavily in transdisciplinary approaches that complement our basic, translational, and pre-clinical model expertise. It is our belief that incorporation of diverse research expertise will drive

innovation and move beyond discipline-specific approaches to improve therapeutic strategies targeting cancer.

Dr. Davalos is a Professor in the Virginia Tech – Wake Forest University School of Biomedical Engineering and Sciences. Davalos received his BS from Cornell University and PhD from the University of California, Berkeley. Prior to joining Virginia Tech, Davalos served as Principal Technical Staff at Sandia National Laboratories. Davalos received the 2006 HENAAC Nation's Most Promising Engineer Award, the NSF CAREER, the TechNite 2015 Innovator award, and the Virginia Tech Most Outstanding Dissertation Advisor award. Dr. Davalos is a Coulter Fellow and an ASME Fellow. His research interests are in microfluidic tissue mimics, biotransport, electroporation, and dielectrophoresis.

Dr. Verbridge is an Assistant Professor in the Virginia Tech – Wake Forest University School of Biomedical Engineering and Sciences, and Department of Biomedical Engineering and Mechanics. Dr. Verbridge has B.S. degrees in physics and applied mathematics from the University of Rochester, and M.S. and Ph.D. degrees in physics from Cornell University. Prior to joining the Virginia Tech faculty, Dr. Verbridge did his postdoctoral work in the departments of Chemical and Biomedical Engineering at Cornell. Dr. Verbridge's broad interests lie at the intersection of cancer biology, tissue engineering, and the development of novel physics-based therapeutics.

Lissett Ramirez Bickford, PhD, is an Assistant Professor in the Department of Biomedical Engineering and Mechanics at Virginia Tech. She received her B.S. in Mechanical Engineering from Texas A&M University, her M.S. in Biomedical Engineering from The University of Texas at Austin, and her Ph.D. in Biomedical Engineering from Rice University. Her postdoctoral research at the Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill was focused on developing medical devices for local cancer treatment. Dr. Bickford's research has focused on improving therapeutic outcomes for cancer patients by closely examining immune response to cancer treatment.

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Highlights

- Pulsed electric field can be used to inhibit pro-cancer inflammatory TSLP signal.
- TSLP was uniquely reduced after electric exposure compared to chemical poration.
- The inhibition was not dependent on mammalian species (mouse vs. human).

Figure 1.

Influence of electric pulse parameters on cell viability of 4T1 triple negative breast cancer cells. (A) A representative flow-cytometry image obtained from staining cells with Annexin-V and DAPI, 45 minutes after treatment. Single cell images during cytometry were captured using AMNIS® flow cytometry. (B) Fold increase of Annexin-DAPI double positive population (number of sets/condition $n = 4$). (C) Viability of cells after overnight incubation measured using resazurin dependent metabolism ($n=3$). Significance was determined using Tukey HSD test. The “*” denotes significance ($p < 0.05$) in IRE treated samples when compared to control, while “#” denotes significance between 80 and 99 pulses within the group.

Figure 2.

Influence of electric pulse parameters on mRNA transcription in 4T1 triple negative breast cancer cells measured using real time PCR. Select group of genes involved in tumor cell signaling were studied: (A) Interleukin-6, (B) Tumor Necrosis Factor, (C) Thymic Stromal Lymphopoietin, (D) Chemokine C-C motif Ligand 2. Error bars show standard error and number of sets per condition $n = 3$. Significance of difference (*, $p < 0.05$) between IRE treated groups and control was determined by one-way ANOVA followed by Tukey HSD post-test.

Figure 3.

Influence of electric pulse parameters on TSLP cell signaling evaluated by measuring proteins secreted by 4T1 triple negative breast cancer cells in conditioned media using ELISA. Measured protein concentrations were normalized to million live cells. Error bars show standard deviation and number of sets per condition $n = 3$. Significance of difference (*, $p < 0.05$) between IRE treated groups and untreated controls were determined using one-way ANOVA followed by Tukey HSD post-test. The number of pulses did not influence supernatant TSLP, as determined by factorial ANOVA analysis.

Figure 4.

(A) A representative temperature profile (Temperature legends: —: Transient; — —: Initial; — ·: Mean; — · ·: Maximum) used for thermal shock treatments that replicated temperature rise seen in electric ablation treatments. Influence of different treatments on TSLP signaling evaluated by measuring proteins secreted by 4T1 triple negative breast cancer cells in conditioned media using ELISA: (B) Thermal shock, (C) Digitonin chemical poration, and (D) Staurosporine kinase inhibition. Measured protein concentrations were normalized to million live cells. Error bars show standard deviation and number of sets per condition $n = 3$. Significance (* $p < 0.05$) was determined using student's t-test.

Figure 5.

(A) TSLP gene fold change in human progressive cell lines: MCF-10AT1 lines: MCF-10AT1 (pre-malignant), MCF-10A (fibrotic epithelium), and MCF-DICS.com (ductal carcinoma in situ) shows the role of TSLP in progression of breast cancer (10AT1 vs. DCIS). (B) The IRE treatment on triple negative human MDA-MB-231 cells with 600 V/cm and 99 pulses reduced the TSLP gene expression compared to untreated controls. Error bars show standard error and number of sets per sample as follows: 10AT1= 4, 10A= 6. DCIS = 4, MDA-MB-231 = 3 per condition (control and 600 V/cm). The significance of difference to the control (10AT1 in A; Control in B) is denoted by ‘*’ for $p < 0.05$.