The Biological and Immunological Effects of Irreversible Electroporation and Combination Therapy Options for Improving the Treatment of Pancreatic Cancer

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ABSTRACT

While cancer treatments have advanced for multiple cancers, pancreatic cancer remains a lethal cancer with few therapy options available. This is due to the inaccessibility of the tumor by surgical and thermal ablative means, high potential for chemoresistance and metastasis, and strongly immunosuppressive tumor microenvironment that makes new treatment measures ineffective in clinic. Irreversible electroporation (IRE) utilizes short, high voltage electrical pulses to form microlesions in cell membranes and induce cell death. While IRE has had significant impact in pancreatic cancer treatment in clinical trials, little is known on the biological and immunological effects of IRE on pancreatic cancer. By studying the effects of IRE on pancreatic tumor biology and the host immune system, I hypothesize I can identify potential combination therapy targets for IRE. I utilized *in vitro, ex vivo,* and *in vivo* animal models of both human and murine cancer to study the effects of IRE on pancreatic cancer progression and its potential for immunomodulation. My findings have shown that IRE can significantly delay cancer progression by inducing necroptosis in the tumor and altering the tumor microenvironment by increasing inflammatory signaling. IRE can also produce viable antigens for presentation to induce local and systemic immunosurveillance. However, these effects are limited by countering expression of programmed-cell death ligand 1 (PD-L1), a checkpoint protein that inhibits cytotoxic lymphocyte activity and allows the tumors to recur. The effects of IRE can therefore be expanded by multiple combination therapy approaches, such as chemotherapeutic application (potentially with nanoparticle packaging), PD-1/PD-L1 antibody immunotherapies, and small molecule inhibitors directed at tumor growth signaling that previously showed limited efficacy in clinic.

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GENERAL AUDIENCE ABSTRACT

While cancer treatments have advanced for multiple cancers, pancreatic cancer remains a lethal cancer with few therapy options available. This is due to limited surgical candidacy, resistance to chemotherapy, high potential for secondary tumor formation, and the cloaking of the tumor from the immune system that make new treatment measures ineffective in clinic. Irreversible electroporation (IRE) utilizes short, high voltage electrical pulses to form permanent pores in cell membranes and induce cell death. While IRE has had significant impact in pancreatic cancer treatment in clinical trials, little is known on how IRE affects pancreatic cancer biological or how it can alter the immune system. By studying the effects of IRE on pancreatic tumor biology and the host immune system, I hypothesize I can identify potential combination therapy targets for IRE. I utilized cell, tissue, and animal models of both human and mouse pancreatic cancer to study the effects of IRE on disease progression and its potential for inducing immune responses. My findings have shown that IRE can significantly delay cancer progression by inducing controlled inflammatory cell death in the tumor and altering the supportive cells populations in the tumor that allows for immune system recognition. IRE can also produce markers specific to the tumor for presentation to induce recognition of the primary tumor and secondary lesions in the body. However, these effects are limited by countering expression of programmed-cell death ligand 1 (PD-L1), a checkpoint protein that reduces immune cell activity and allows the tumors to recur. The effects of IRE can therefore be expanded by multiple combination therapy approaches, such as chemotherapeutic application (potentially with nanoparticle packaging), PD-1/PD-L1 antibody immunotherapies, and small molecule inhibitors directed at tumor growth signaling that previously showed limited efficacy in clinic.

DEDICATIONS

While I may live hundreds and thousands of miles apart from my family, they are always in my heart. I would like to dedicate my work to all of those in my family who supported me throughout my life and told me I could do anything. To my father who put me in a blazer in elementary school for career day and said, "You're a doctor. No, not that kind of doctor. The really smart kind that can teach at college or work for a company." To my brother who has called me "Dr. Becky" since I was a kid and can now do so for legitimate reasons, who always knew I had it in me even when I was stressed out and panicking. To my Aunt Marsha who helped me when I was in between schools to get back on my feet and follow my dreams. To my Oma, liebe Luise Oma, daß meine Arbeit und die amerikanisches PhD-System wirklich nicht verstehen hat, aber sie immer in ihre Beten mich gehelt. And to the friends that I hold as family that gave me love, support, life advice, companionship, and most of all adventure that helped color my life with such vivid experiences that made taking on a PhD program seem so much more possible.

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ATTRIBUTIONS ON CO-AUTHORED PAPERS

Chapter Two: RB wrote original draft of this article, designed Figure 1, and incorporated suggested edits and comments from IA, NW, and RD. NW provided Figure 2 based on consultations with **RB**, RD, and IA.
Chapter Three: **RB** wrote original draft of this article, designed all figures, and incorporated suggested edits and comments from IA. AZ contributed to the writing of IRE section and editing.
Chapter Four: IA, RD, and NM contributed to the concept and design of the study. **RB** and VR monitored animals and collected animal tissues. NB performed *ex vivo* IRE and tissue property assessments. SC and DG assessed histology and provided photomicrographs. ML provided COMSOL modelling. **RB** performed tissue processing and RNA evaluation. IA analysed IPA results. NB, ML, SC, and IA wrote sections of the manuscript. **RB** wrote the original draft of the manuscript and all proposed edits.
Chapter Five: IA, RD, and NM contributed to the concept and design of the study. **RB** ran *in vitro* and *in vivo* models, monitored animals, and collected animal tissues. NB performed *in vitro and in vivo* IRE and temperature measurements. SC assessed histology. **RB** performed HA plasmid isolation, transfection of cells, tissue processing, RNA evaluations, flow staining, and analysis assessment. AH assisted with tissue processing and performed protein evaluation. IA analysed IPA results. NB and IA wrote sections of the manuscript. **RB** wrote the original draft of the manuscript and final formatting.

CHAPTER ONE
Introduction

While most cancers in the last twenty years have had improvement in treatment options and overall survival, pancreatic cancer remains a lethal diagnosis for tens of thousands of patients every year in the US. Due to the location of the primary tumor in often inoperable areas, high potential for metastatic disease, and resistance of pancreatic cancer to chemotherapeutics, the need for new pancreatic cancer treatments is vital(Ansari et al., 2015; Kamisawa et al., 2016). Unfortunately, many newer treatments such as thermal ablation and checkpoint inhibitors have had limited impact on patient survival(Wu et al., 2006; Aroldi and Zaniboni, 2017). This can be because of the danger of thermal ablation treatment near sensitive critical structures such as the mesenteric artery that primary malignancies can form around and the highly immunosuppressive tumor microenvironment that hides pancreatic cancer from anti-tumor immune effects.

My work presented here has focused on irreversible electroporation (IRE) as a novel treatment for pancreatic malignancies. IRE utilized short, high voltage electrical pulses to permanently open pores or lesions on cell membranes and induce cell death by loss in cell homeostasis(Davalos et al., 2005). IRE's success in clinical studies on pancreatic cancer has been astounding but often inconsistent on much of a impact IRE has on increasing patient survival. While some studies show a near doubling in patient survival, another may show an increase in only months(Belfiore et al., 2015; Martin et al., 2015; Scheffer et al., 2016). Still, in a cancer so lethal, any increase in patient survival, especially with treatments that are generally considered safe with limited potential for adverse events, is beneficial.

Interestingly, at the beginning of these studies little had been discovered about the impact of IRE on cancer biology and its potential for immunomodulation. As both of these play a key role in the efficacy of cancer treatments, this laid a rich foundation for research potential. In the last couple years, myself and others have contributed to this knowledge and identified potential combination therapy options based on our findings that may prove to enhance IRE's clinical outcomes and improve patient survival(Beitel-White et al., 2018; Zhao et al., 2019; Mercadal et al., 2020) (*subsequent chapters 2-5 currently in preparation or under review for publication*).

In this collection of work, I first review what is known about cell death and inflammation in electroporation-based modalities. There has been much debate on what type of cell death IRE induces, whether IRE-induced cell death is non-inflammatory apoptotic or inflammatory necrotic in nature. I discuss potential reasons why these results may vary, from the dispersion of the electric field to the differences intrinsic to the cancer tested.

Secondly, I review what is known about the immunomodulatory effects of IRE. I describe some of the ways that cancer and the immune system interact for form both "cold" immunosuppressive and "hot" immunogenic tumor microenvironments. I also describe how ablation by electroporation-based devices may lead to alterations of the tumor microenvironment and the presentations of tumor antigens for local and systemic immunosurveillance. I also address potential inhibitory effects such as checkpoint inhibitors that can limit immunomodulation. Then I review previous and many recent findings on the impact of IRE on the immune system.

Next, I explain my own studies with a unique model of human pancreatic cancer. We utilized a patient-derived xenograft model from primary human samples to explore the biological effects IRE can have on human pancreatic ductal adenocarcinoma. Our findings establish the model for IRE ablation studies, utilize the model to improve clinical application planning, identify cell death mechanisms resulting from IRE application, and briefly predict potential combination therapy targets and biomarkers that could enhance IRE's effects on the biological signaling of pancreatic cancer.

Finally, I explore the effects of IRE on the immune system by utilizing different *in vitro*, *ex vivo*, and *in vivo* models of murine pancreatic cancer. As mice share a similar immune system to humans, these models allowed for an array of studies that, in some cases, parallel with recent clinical findings and in others expanded our knowledge on the mechanisms and limitations behind IRE's acute and long-term effects in pancreatic cancer treatment. I also identify potential combination therapy targets to enhance and sustain the immunomodulatory effects of IRE.

While my research work focuses on IRE, there are many other electroporation-based treatments such as electrochemotherapy and nanosecond pulsed electric fields that have shown similar patterns in cell death and immunomodulation. These technologies differ in pulse parameters and combination treatments but share the same principle of electroporation-induced tumor ablation. In the reviews of cell death, inflammation, and immunomodulation I include these other modalities to compare current knowledge among these technologies and provide insight as to the future of cancer treatments.

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CHAPTER TWO

Starting a Fire without Flame: The Induction of Cell Death and Inflammation in Electroporation-Based Tumor Ablation Strategies

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ABSTRACT

New therapeutic strategies and paradigms are direly needed for the treatment of cancer. While the surgical removal of tumors is favored in most cancer treatment plans, resection options are often limited based on tumor localization. Over the last two decades, multiple tumor ablation strategies have emerged as promising stand-alone or combination therapeutic options for patients. Tumor ablation modalities focus on the *in situ* killing of the primary tumor by eliciting cell death through thermal, mechanical, or even electrical means. These strategies are often employed to treat tumors in areas where surgical resection is not possible or where chemotherapeutics have proven ineffective. The type of cell death induced by the ablation modality is a critical aspect of therapeutic success that can impact the efficacy of the treatment and systemic anti-tumor immune system responses. Thermal ablation treatments, such as radiofrequency, microwave, or cryoablation have been well-studied and rely on extreme heat or cold to induce necrosis, or lytic cell death. However, the risk of healthy tissue damage and other significant side effects associated with thermal ablation has prompted investigations into other forms of non-thermal treatments such as electroporation. Electroporation-based technologies include electrochemotherapy, irreversible electroporation, and other modalities that rely on pulsed electric fields to create microlesion tears, or pores, in cell membranes to induce cell death. Here, we review the typical types of cell death induced by electroporation-based treatments and summarize the impact of these mechanisms on cancer progression.

INTRODUCTION

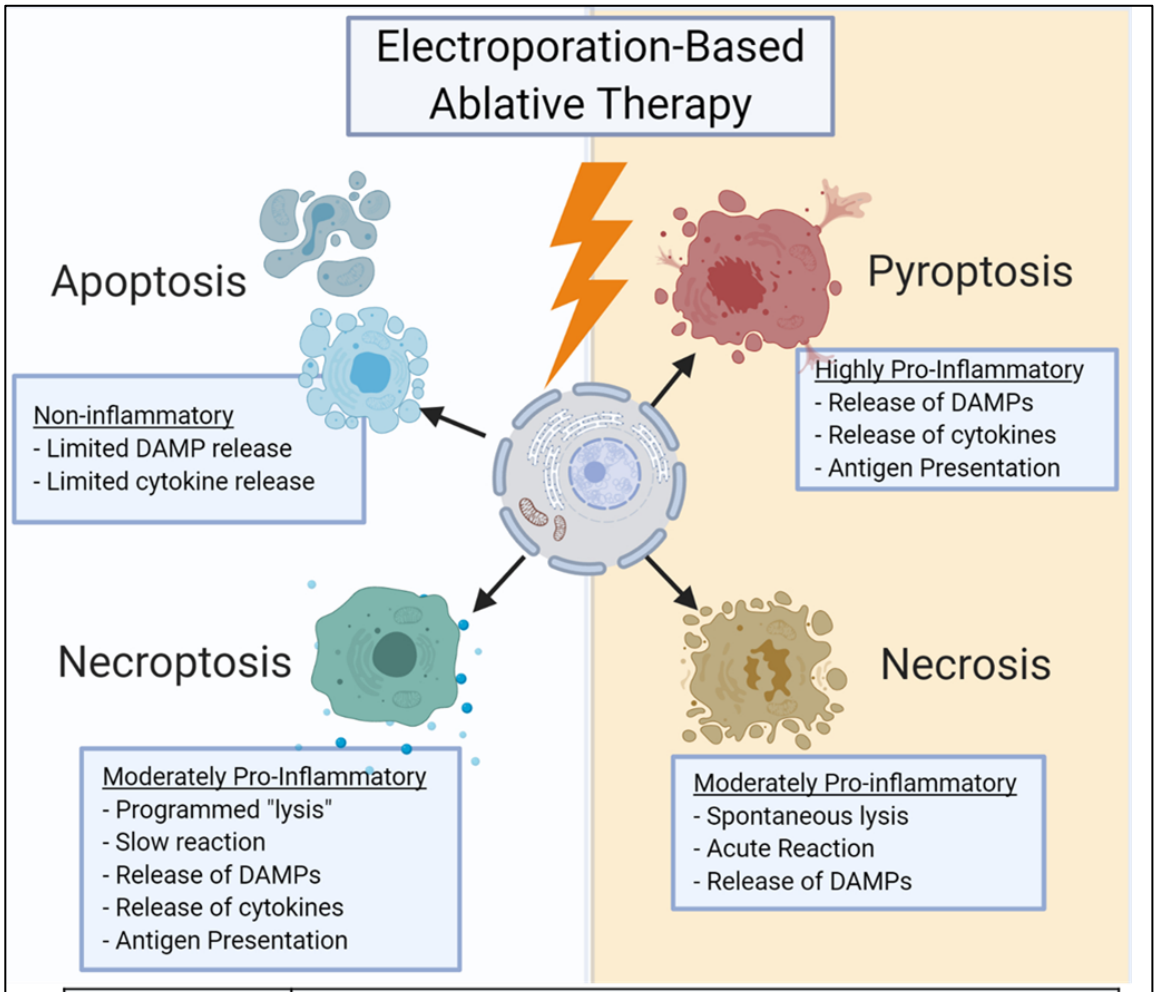
New treatment paradigms are direly needed. Minimally invasive tumor ablation treatments such as cryotherapy, laser irradiation, microwave irradiation, radiofrequency ablation, high-intensity focused ultrasound ablation, and irreversible electroporation (IRE), have shown significant promise. These modalities function through the direct or indirect induction of cell death, resulting in the destruction of tissue by thermal, mechanical, or electrical means. Thermal ablation modalities, such as microwave or radiofrequency, use intense temperatures to lyse cells and induce apoptosis in treatment margins(Saccomandi et al., 2018; Shao et al., 2019). Unfortunately, tumor location near critical structures can lead to difficulty in thermal treatment placement as hemorrhaging, heat sink effects, and healthy tissue damages can occur(Chen et al., 2005; Liang et al., 2009). Conversely, non-thermal modalities such as electroporation can overcome these treatment barriers and induce cell death through very different mechanisms that minimize the limitations associated with the thermal ablation treatments. Additionally, non-thermal ablation strategies appear to be highly effective at inducing systemic anti-tumor immune responses, which target both the local tumor microenvironment and metastatic sites(Ringel-Scaia et al., 2019; Shao et al., 2019).

Extensive investigations have explored the mechanisms underlying cell death induced by pore formation and homeostasis loss following electroporation-based tumor ablation treatments (Weaver and Chizmadzhev, 1996; Davalos et al., 2005). There is, though, significant debate in the field regarding the specific type of cell death induced by treatment. Early studies labeled the cell death as apoptosis. However, cell death is a complex and nuanced process, especially in the context of cancer. We now realize electroporation-based treatments likely induce a spectrum of cell death mechanisms ranging from apoptosis to pyroptosis and necrosis. The type of cell death induced has significant biological and physiological consequences in terms of local and systemic treatment efficacy. The death of a single cell is only the beginning of a cascade of responses that ultimately result in tumor ablation following electroporation. Here, we review the four major types of cell death currently defined following electroporation-based treatment strategies and offer insight into the biological impacts of each on tumor ablation.

IRREVERSIBLE ELECTROPORATION-INDUCED CELL DEATH MECHANISMS

Cell death can diverge into many different pathways, eliciting a large range of responses (**Figure 1**). Each pathway can be further modified by genetic, epigenetic, and regulator factors for different cell types and tissues. These pathways are not singular and can often overlap. For example, many of the same proteins and biochemical pathways are involved with multiple cell death subroutines, which can make a definitive type of cell death difficult to ascertain. There are also temporal and spatial considerations that must be considered. *In vivo*, the general consensus indicates that multiple types of cell death are occurring within the tumor following irreversible electroporation treatment. This is mediated, in part, by the proximity of any given cell to the electrodes, which impacts the

voltage each individual cell experiences during treatment(Neal et al., 2011; Faroja et al., 2013; Bhonsle et al., 2018; O'Brien et al., 2019).



Cell Death Type	Distinctive Markers
Apoptosis	Cleaved Caspase-3, phosphidylserine/annexin V binding
Necrosis	RIP-1 activation, NF-kB & TNF signaling
Pyroptosis	Cleaved Caspase-1 / -11 & Gasdermin D, Inflammasome activation
Necroptosis	RIPK3 and MLKL activation

Figure 1. Cell death types initiated by electroporation-based ablation.

In **Figure 2**, we illustrate an example of this, though the distribution and cell death would differ depending on pulse parameters and tissue-type. Inducing the optimum type of

cell death is critical for effective tumor treatment as cell death can influence both local and systemic effects that significantly impact cancer recurrence, inflammation, and

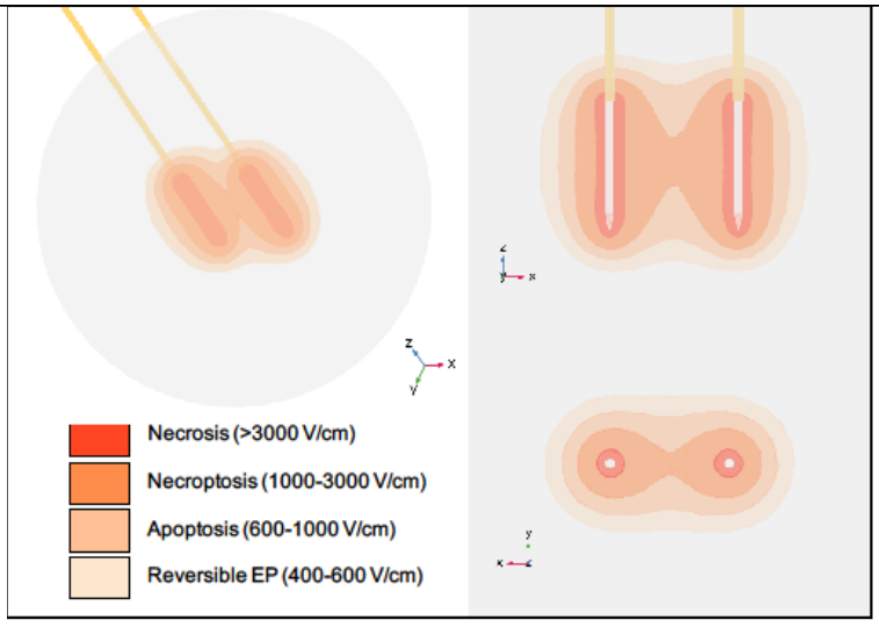


Figure 2. Regions of cell death within a typical IRE treatment zone vary spatially. Necrosis is thought to occur in close proximity to the electrodes during pulse delivery, while necroptosis, apoptosis, and reversible electroporation are thought to occur with increasing distances from the electrodes. It should be noted that this diagram is intended for illustrative purposes only. Specific volumes and thresholds will vary with many parameters, including electrode spacing and exposure, voltage applied, tissue type, number of pulses applied, and time after treatment. The electric field distribution for a single IRE pulse was simulated using COMSOL Multiphysics software (version 5.5, Burlington, MA). Two stainless steel electrodes with insulating holders were inserted into a spherical tissue domain, which was assigned a dynamic electrical conductivity corresponding with normal human pancreatic tissue(Beitel-White et al., 2018). Electrodes were placed 1.5 cm apart with 1.5 cm exposure, and a voltage of 3000 V was applied.

autoimmunity(Hanahan and Weinberg, 2011; Baroja-Mazo et al., 2014; Zhang et al., 2018; Aizawa et al., 2020; Khoury et al., 2020).

Apoptosis

Apoptosis is one of the most commonly mentioned forms of cell death in electroporation-based ablations(Hofmann et al., 1999; Vernier et al., 2003; Lee et al., 2010; Faroja et al., 2013). Generally considered non-inflammatory, apoptosis is a programmed form of cell death required for normal maintenance of tissues such as intestinal epithelium or epidermis where cells are regularly replaced to avoid the accumulation of cellular damage or mutations(Kerr et al., 1972; Johnstone et al., 2002; Berghe et al., 2006). This pathway is commonly dysregulated in cancers as the cells lose the ability to respond to internal signals due to mutations in apoptosis regulatory pathways or key genes such as Bcl2 or P53(Hoffman et al., 2002; Koff et al., 2015). The induction of apoptosis a implies

a “quiet cell death” with little immune involvement beyond dead tissue clearance. Hallmark features include the cleaving and activation of Caspase 3 and 7 along with the expression of phosphatidylserine on the cellular surface that binds Annexin V(van Engeland et al., 1996; Julien and Wells, 2017; Galluzzi et al., 2018). In the context of tumor ablation, apoptosis can be considered beneficial due to the reduced potential for inflammation-driven damage to nearby healthy tissues. Apoptosis also minimizes the systemic effects that may occur in inflammatory cell death forms such as pyroptosis or necrosis(Matsukawa et al., 1997). While apoptosis can be highly effective in ablating primary tumors, the lack of innate and adaptive immune system activation is sub-optimal for inducing systemic anti-tumor immune responses. This can negatively impact the potential to induce an abscopal effect to target metastatic lesions and may create a permissive niche for tumor reoccurrence once the apoptotic pressure is removed(Scaffidi et al., 2002; Labi and Erlacher, 2015).

Necrosis

Necrosis lies opposite of apoptosis and is characterized by rapid cell death. Necrosis is typically induced by sudden loss in cell homeostasis, such as rapid osmolarity or temperature changes, influx of calcium into the cell or mitochondrial spaces, or mechanical tissue damages that can lead to autolysis(Golstein and Kroemer, 2007). It should be noted that this section refers to cellular necrosis, which significantly differs from tissue necrosis, the presence of dead tissue often described in histology based on acellularity or tissue morphology that can be induced by any manner of cell death.

Necrosis is often referred to as accidental or lytic cell death and is characterized by the breakdown of the cell membrane and the release of large amounts of damage-associated molecular patterns (DAMPs)(Scaffidi et al., 2002; Festjens et al., 2006). Further investigation into necrosis’s mechanisms show that contrary to the chaotic description, its mechanism may also be regulated by specific programs similar to apoptosis(Festjens et al., 2006). For example, the serine/threonine kinase RIP1 appears to play a pivotal role as a central initiator of necrosis(Festjens et al., 2006). Activation of RIP1 results in NF-κB and transient MAPK signaling, directly inducing the production of pro-inflammatory mediators(Ting et al., 1996; Devin et al., 2000; Devin et al., 2003). RIP1 may also play a role in the TNF signaling and the generation of ceramide during necrosis(Thon et al., 2005). Likewise, calcium and reactive oxygen species (ROS) signaling cascades lead to the propagation and execution phases of cell death. Ultimately, these result in damaged proteins, lipids, and DNA that drive disruptions in organelle and cell membranes(England and Cotter, 2005; Festjens et al., 2006). The production of DAMPs stimulate neighboring cells to activate the innate immune system, resulting in inflammation(Cocco and Ucker, 2001). Unfortunately, inflammatory signaling recruits immune cells to the lesion that may be polarized to facilitate regeneration and repair which the tumor can reprogram to assist the tumor in grow, repair, and create a more favorable niche for progression(Vakkila and Lotze, 2004; Jacobson et al., 2013). Necrosis may also induce tumor lysis syndrome (TLS) as large numbers of DAMPs acutely released into the blood stream can lead to systemic inflammation(Matsukawa et al., 1997; Zhang et al., 2010; Sahay et al., 2016). Any tumor ablation modality that induces high DAMP production should focus on optimizing

targeting and minimizing the treatment area. It should be noted, though, that TLS has not be reported in electroporation-based treatment clinical trials.

Pyroptosis

Pyroptosis is one of twelve identified subclasses of regulated cell death and displays high local inflammatory responses distinct from necrosis.(Galluzzi et al., 2018) Traditionally, pyroptosis has been defined and characterized as being associated with the host innate immune response to viral and bacterial pathogens (Willingham et al., 2009; Jorgensen and Miao, 2015; Shi et al., 2015; Ma et al., 2018). Pyroptosis is an extremely specific form of inflammatory programmed cell death and is characterized by the cleavage and activation of Caspase-1 and Caspase 11(Shi et al., 2015; Vande Walle and Lamkanfi, 2016). Caspase-1 activation results in the cleavage and processing of IL-1β and IL-18, potent proinflammatory cytokines. These caspases also cleave gasdermin D, generating an N-terminal cleavage product that drives pyroptosis.(McKenzie et al., 2018) In addition to IL-1β and IL-18, pyroptosis produces a significant amount of DAMPs, including HMGB1, ATP, and ROS to further stimulate the innate immune system (He et al., 2015; Bortolotti et al., 2018; Ma et al., 2018). This high signaling state leads to rapid responses from the body with recruitment of immune cells to the local area as well as increase systemic signaling to enhance immunosurveillance and heighten antigen presentation potential that could be beneficial for cancer treatment(Walsh et al., 2013). While this can be ideal for allowing the immune system to recognize tumor cells in the body and create immune memory, the heightened inflammatory state can lead to severe side effects such as fever and autoimmunity(Zhang et al., 2010; Baroja-Mazo et al., 2014).

Necroptosis

In addition to pyroptosis, a fourth major regulated cell death routine termed necroptosis has also been described following irreversible electroporation. Necroptosis is also termed programmed necrosis or alternative necrosis and has features characteristic to both apoptosis and necrosis. Necroptosis is triggered by perturbations of extracellular or intracellular homeostasis and does not induce a quick, automatic lysis of the cell(Dhuriya and Sharma, 2018). Rather, the cell produces low levels of DAMPS and proinflammatory cytokines that drive moderate levels of inflammation compared to much more inflammatory mechanisms associated with pyroptosis(Frank and Vince, 2019). Necroptosis critically depends on the pseudo-kinase mixed lineage kinase domain-like (MLKL), which is phosphorylated by the kinase RIPK3(Yoon et al., 2017).While mechanistically less clear, RIPK1 has also been suggested to mediate necroptosis under some conditions and the activation of an inflammasome has been suggested to underlie inflammatory cytokine production(Lawlor et al., 2015; Galluzzi et al., 2018). This can make it somewhat challenging to discern from necrosis. However, due to the temporal nature of necroptosis and the attenuated production of local cytokines, necroptosis is less likely to induce TLS but still retains the potential of inducing inflammation that can promote an anti-tumor microenvironment and improve antigen presentation.

ELECTROPORATION-BASED ABLATION MODALITIES

Electrochemotherapy (ECT) and Calcium-based Electroporation

ECT applications represent the first electroporation-based modalities to progress to clinical trials(Belehradek et al., 1993; Heller et al., 1996). ECT is commonly combined with chemotherapeutics such as bleomycin and utilizes pulsed electric fields (PEFs) of micro- to nanosecond pulse durations for short periods that allow for the internal delivery of chemotherapeutic reagents to the site of treatment that can induce local cell death (Mir et al., 1988; Heller et al., 1996). Many chemotherapeutic agents can have off-target effects, difficulty penetrating beyond the surface of the tumor, and the potential for chemoresistance. Thus, ECT can facilitate the disruption of these barriers and promote cellular uptake of the chemotherapeutics in the treatment zone.

Expanding on these earlier ECT strategies, the use of calcium represents a significant refinement in this treatment strategy. The use of calcium in electroporation enhances necrosis in the treatment zone and overcomes several barriers of ECT, such as the ability to forgo or reduce the use of chemotherapeutics and minimize the overall cost of the procedure(Frandsen et al., 2012; Falk et al., 2017). The induction of necrosis, rather than relying on apoptosis and avoiding chemotherapeutic interference with mitochondrial function or inflammatory cell death associated with ATP depletion, is a significant advantage of calcium-based electroporation strategies(Rimessi et al., 2008; Frandsen et al., 2012). Furthermore, there is potential selectivity in calcium electroporation as malignant cells are more likely to die from the procedure than healthy or benign cells(Hansen et al., 2015; Frandsen et al., 2017). This may be due to the modification many cancers develop, ironically to avoid cell death, that make them more susceptible to calcium imbalances(Danese et al., 2017; Ji et al., 2018). Calcium-driven cell death is also being investigated to complement other electroporation-based modalities such as high frequency irreversible electroporation and nanosecond pulsed electric fields(Novickij et al., 2019; Wasson et al., 2020) .

Irreversible Electroporation

IRE utilizes microsecond pulse electric fields, similar to ECT, but for a higher pulse count. This higher pulse count results in pores and tears forming in the cell membrane that stabilize, leading to loss of cell homeostasis and initiating cell death processes(Davalos et al., 2005; Miller et al., 2005). The use of IRE in difficult-to-treat malignancies, such as pancreatic and hepatocellular tumors, is currently becoming more widespread and has led to numerous clinical trials showing inspiringly high success rates(Belfiore et al., 2015; Martin et al., 2015; Sugimoto et al., 2015; Scheffer et al., 2016; Sutter et al., 2017; Leen et al., 2018; Kalra et al., 2019). IRE-induced cell death was originally considered to be apoptotic(Hofmann et al., 1999; Lee et al., 2007; Lee et al., 2010; Faroja et al., 2013). However, a majority of these studies did not fully consider alternative types of cell death. While apoptosis is certainly occurring in some cells within the treatment zone, increasing data suggests that there are likely multiple types of cell death mechanisms occurring. Specifically, the type of cell death varies with increasing distance from the electrodes

(**Figure 2**), and the size and shape of the regions in which each type is experienced may vary between clinical treatments due to differences in pulsing parameters, tissue type, and treatment time(Beitel-White et al., 2018). While directly near the electrodes may be temperature dependent, cell death becomes temperature independent in other regions based on minimum heating effects seen *in vitro* at comparable electric field magnitudes(Davalos et al., 2015). Multiple studies argued that there may be more than one type of cell death mechanism at play, from necrotic cell death to apoptotic-like non-apoptotic cell death(Piñero et al., 1997; Tekle et al., 2008; Vogel et al., 2019; Mercadal et al., 2020). These responses could come from differing tissues being predisposed to specific types of cell death depending on the electric field strength applied(Ben-David et al., 2013). Likewise, for cells at the margins of the treatment areas, the response may actually be survival signaling to reversible electroporation. In theory, this could be taken advantage of and combined with chemotherapy treatments to increase drug delivery, tumor penetration, and treatment of remnant cancer cells (Bhutiani et al., 2016; Belfiore et al., 2017; Kodama et al., 2019).

High Frequency Irreversible Electroporation

Building upon the advantages of IRE, a novel technology termed high-frequency irreversible electroporation (H-FIRE) utilizes 1-10 microsecond bipolar bursts applied in a pattern with interpulse delay when switching poles. These parameters are highly effective in non-thermally ablating tissues without causing the muscle contractions and heart dysrhythmia associated with the long-duration monophasic pulses of IRE (Arena et al., 2011; Sano et al., 2018; Lorenzo et al., 2019; Ringel-Scaia et al., 2019; Partridge et al., 2020). Its use in pre-clinical models has progressed without the need for cardiac sinks as well(Partridge et al., 2020). Cell death following H-FIRE is considered to be highly similar to IRE. However, pre-clinical studies show that H-FIRE may be eliciting immunologic cell death and pyroptosis in addition to apoptosis and necrosis(Ringel-Scaia et al., 2019; Mercadal et al., 2020). Intriguingly, the use of H-FIRE may allow tuning of cell death with calcium similar to ECT. In *in vitro* hydrogel studies, H-FIRE applied in calcium-rich media showed a significant shift towards necrotic cell death with higher lesion areas and fewer survival signals(Wasson et al., 2020). These data suggest that H-FIRE effects could be modified by injecting calcium or sucrose near the treatment site to allow for controlled applications in difficult-to-treat malignancies or tissue locations.

Nanosecond Pulse Electric Fields

Nanosecond pulse electric fields (nsPEFs) are characterized by PEFs with short nanosecond pulse durations and high electric field. Originally, nsPEFs were thought not to permeabilize the cell membrane but rather induce cell death by interfering with molecular patterns inside the cell, disrupting processes in the mitochondria and other intracellular membrane-bound organelles(Schoenbach et al., 2001). However, later studies have found that the cell membrane and the membranes of internal cellular structures are permeabilized, albeit with smaller tears than those of microsecond PEFs(Beebe et al., 2002; Vernier et al., 2003; Ford et al., 2010; Beebe et al., 2013). nsPEFs have been noted to induce both

apoptotic and potentially caspase-independent apoptotic-like cell death similar to necroptosis(Ford et al., 2010; Beebe et al., 2013). Recent *in vivo* findings provide greater mechanistic insight into the types of cell death induced by nsPEFs(Guo et al., 2018a; Guo et al., 2018b; Novickij et al., 2019). Based on the inflammatory and immunomodulatory conditions, these studies reveal that nsPEFs may induce programmed necrosis or necroptotic cell death in breast, melanoma, and pancreatic cancers(Guo et al., 2018a; Guo et al., 2018b; Novickij et al., 2019).

DISCUSSION

As electroporation ablation modalities become more mainstream and progress from preclinical studies to clinical applications, the characterization of specific cell death mechanisms associated with treatment will become more relevant. The type of cell death associated with therapy has significant consequences for the patient's treatment outcomes, immune system activation, regeneration and repair processes, and co-therapy applications. Seemingly conflicting reports on cell death mechanisms ranging from apoptosis to necrosis and pyroptosis has led to confusion in the field. However, as discussed here, the consensus data suggests that cell death following electroporation occurs over a spectrum and that multiple cell death mechanisms are occurring within the treatment zone. For example, studies investigating the effects of different cell size and nucleus size would suggest that cells with larger nuclei are more likely to be affected by PEFs(Ivey et al., 2015; Rolong et al., 2017) and even reversible electroporation is said to be based on cell-type for transfection efficacy(Methods, 2006; Yarmush et al., 2014; Chicaybam et al., 2017). It would reasonable to hypothesize that different tissues and cell types, both healthy and malignant, have different responses to electroporation-based technologies and may require different dosing, treatment parameters, or co-therapies to obtained optimal effects. Further investigation into these mechanisms may help tailor electroporation-based technologies as personal treatments.

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RB, NB, RD, and IA all contributed to the writing and editing of the manuscript.

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CONFLICTS OF INTEREST

IA., NB, and RD are inventors on pending and issued patents related to the work. Authors declare no other conflicts of interest.

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CHAPTER THREE

A Zap to Action: The Role of Electroporation-Based Tumor Ablation on the Immune System

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ABSTRACT:
Over the past century, cancer has remained one of the leading causes of death, and in many ways has not seen significant improvements in treatment. This is due to the complicated characteristics of cancer, including high levels of mutation, immunosuppression, and metastatic disease. These three qualities have made chemotherapeutics, target therapies, and local surgeries limited. However, recent advancements in ablation and immunotherapies have the opportunity to make an impact. Ablation therapies include any procedures that utilize mechanical, electrical, or thermal damage to destroy a tumor without surgical resection. Of note, while ablation modalities that utilize thermal mechanisms are further in the development pipeline, recent work has shown that non-thermal techniques may potentially offer more benefits with fewer side effects. Electroporation-based non-thermal modalities use short, high-voltage electrical pulses and have high probability of translating into broad clinical use. Here we review the immunostimulatory effects of electroporation-based treatments for cancer. Beyond tumor debulking, evidence is growing to indicate that electroporation-based therapies can induce inflammation and alteration of tumor microenvironments, which can greatly affect cancer progression post-treatment. We also discuss potential combinational therapy treatments for these modalities based on their immunological impacts that could enhance clinical application and patient survival.

INTRODUCTION:

The advances of cancer treatments have been staggering in the last 50 years. From advancing chemotherapeutics to cytokine and antibody treatments, many cancer patients have seen a significant swell in survival rates. However, some cancers have remained relatively unaffected by these new treatments(Khalil et al., 2016; Whiteside et al., 2016; Aroldi and Zaniboni, 2017). Dense tissue in later-stage cancers, as well as several other factors, can limit tumor penetration and response of drugs(Ashdown et al., 2015). Systemic application of chemotherapeutics can also show limited bioavailability and half-life but bring with it devastating side effects and, for many cancers, chemoresistance(Wang et al., 2011; Ashdown et al., 2015; Chen et al., 2015). Cancers such as pancreatic adenocarcinoma and glioblastoma continue to have severely low survival rates even with new immunotherapeutic options as their immunosuppressive tumor microenvironments shield the tumors from the immune system's attempts to identify the malignancy(Ansari et al., 2015; Ma et al., 2018).

These limitations have led to a scramble of targeting ablation modalities to circumnavigate the challenges faced by surgeons and oncologists. Many of these have used thermal effects that burn or freeze the tumor. However, these technologies can lead to adverse effects and limited application by causing off-target damages to nearby healthy tissues, thus making them unsuitable for many cancer patients(Chen et al., 2005; Liang et al., 2009). The use of thermal energy can also denature proteins, which can limit immune signaling by destroying potential damage signals and tumor antigens(Zhang et al., 2002; Hu et al., 2007). Non-thermal ablation modalities such as those utilizing electroporation to generate ablative effects without the use of heat, greatly limit damages to surrounding healthy tissues(Belehradek et al., 1993; Al-Sakere et al., 2007a). Moreover, immunogenic signaling could be preserved, allowing for damage signals and viable antigens to remain intact and functional after treatment(Ringel-Scaia et al., 2019; Shao et al., 2019). Preclinical and clinical reports of potential immunological effects to the primary treatment site and even metastatic lesions has led to many recent investigations on the impact of these electroporation-based modalities on the tumor microenvironment and the immune system.

Investigations into the immune effects of new ablation technologies could dramatically impact their clinical application. Not only will it allow for considerations on treatment side effects such as tumor lysis syndrome, organ malfunction from increased inflammation, or risks of autoimmune disease, but insight into the effects of the treatment on the tumor microenvironment and the whole body can inform therapeutic applications of chemotherapies and immunotherapies. It can also allow for further advances in modality development and strategic application. A better understanding of these mechanisms can lead to improved patient outcomes and prolonged survival.

THE ROLE OF THE IMMUNE SYSTEM IN CANCER TREATMENT

The idea of an in situ vaccine is not a new one. William Coley in the 1890s was famous for developing Coley's Toxins, inoculations of streptococcus and other bacteria that, when injected into a tumor, had the potential to reduce the tumor's size(Coley, 1891; 1936). Unfortunately, several of his patients died of sepsis or other related causes(McCarthy, 2006). While Coley thought he'd found a miracle drug to one of the

world's oldest diseases, scientists today surmise that the bacterial cocktail incited inflammation in the tumor that recruited the immune system, something unknown in Coley's time, to combat both the invading pathogens and the malignancy(Duong et al., 2019).

Tumor microenvironments can differ from cancer origin to person. However, many of the more difficult to treat malignancies fall into what are known as "cold" tumors, tumors that attempt to lower the involvement of the proinflammatory immune system by producing different factors or altering stromal cells to build a protective barrier around the tumor from invading lymphocytes (**Figure 1**).

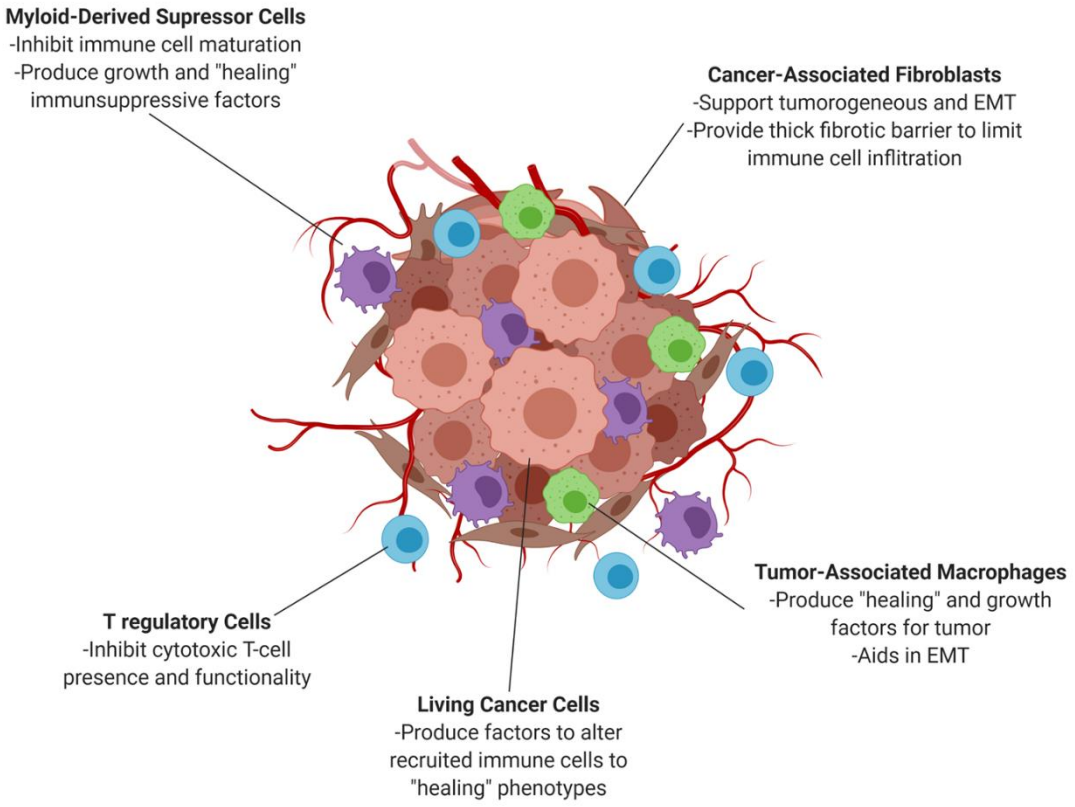

Figure 1: Immunogenically "cold" tumor microenvironment. Summary of specific pro-tumor cells located in tumor microenvironment.

Specific cell types are involved with this environment, producing a strong front against anti-tumoral immune cells and support malignant growth and disease progression(Quail and Joyce, 2013). Myeloid-derived suppressor cells (MDSCs) inhibit immune cell maturation and produce potent growth and healing-related cytokines that can block cytotoxic T lymphocytes (CTL) functionality(Almand et al., 2001; Mirza et al., 2006; Ochoa et al., 2007). Cancer-associated fibroblasts provide a thick, fibrotic physical barrier as well as producing factors such as transforming growth factor beta that promote tumorigenesis and epithelial to mesenchymal transition (EMT)(Malanchi et al., 2011; Dumont et al., 2013; Marsh et al., 2013). Tumor-associated macrophages promote tumor growth and assist with EMT, allowing cancer cells to not only metastasis but to also

become more independent in their functionality and survival(Condeelis and Pollard, 2006; Shree et al., 2011). T-regulatory cells inhibit CTL presence and function(Whiteside et al., 2012). Coley's Toxins and the first cytokine therapies relied on changing this microenvironment to allow for an increase in anti-tumor immune cell populations and recruitment to the tumor area (**Figure 2**).

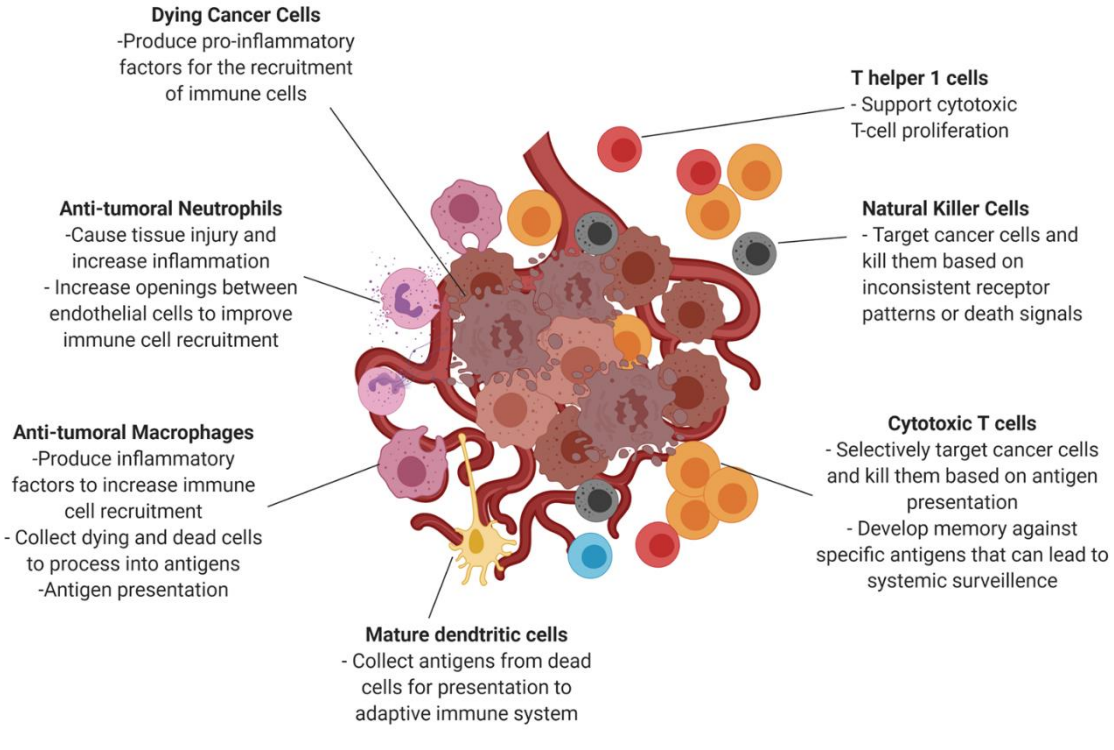

Figure 2: Inflammatory or "hot" tumor microenvironment. Summary of common anti-tumor immune cell populations recruited after injury or infection.

By improving the microenvironment, the tumor is opened to the body's natural immune defenses against cancer such as: anti-tumor neutrophils, macrophages, natural killer (NK) cells, T helper 1 cells, and CTLs(Dewan et al., 2007; Fridlender et al., 2009; Dai et al., 2018; Thorsson et al., 2018). Indeed, many cancers show an increase in survival outcomes for patients with more immune cell activation and less suppressive cell types(Bingle et al., 2002; Mirza et al., 2006; Diaz-Montero et al., 2009; Mougiakakos et al., 2010; Shree et al., 2011). These findings indicate the importance of monitoring and altering the immune activity in cancer patients to improve survival.

The first immunotherapies also lead to the discovery of the dual-edge sword of inflammation for cancer treatments. The acute activity of Coley's Toxins and early cytokine therapies resulted in undesired effects such as sepsis, swelling, and autoimmune conditions that could lead to poor quality of life, organ failure, and even death(Coley, 1936; Weber et al., 2015). The side effects of systemically delivered cytokine therapies such as interleukin-2 (IL-2) and interferon-gamma were harsh and are now avoided in treatment of most cancers due to the severe impact they can have on quality of life(Weber et al., 2015).

IL-2 also had unintended consequences to the tumor microenvironment that may have made the primary tumor more difficult to treat(Sosman et al., 1991; Mirza et al., 2006). Therefore, balance is needed on how much inflammation to induce at the treatment site. This balance is also important for the education of the immune system: too little inflammation could get only a temporary response, while high inflammation has the potential to induce off-target effects of the adaptive immune system branch responsible for targeted identification and killing. The importance of the adaptive immune system is in specific targeting of the cancer both at the primary tumor site and at distant lesions (**Figure 3**).

As many cancers readily metastasis, which lowers patient survival, the ability to induce not only a response from the primary tumor, but to also train the immune system to hunt down the metastases throughout the body, is a powerful idea. Defined by the root words *ab* as

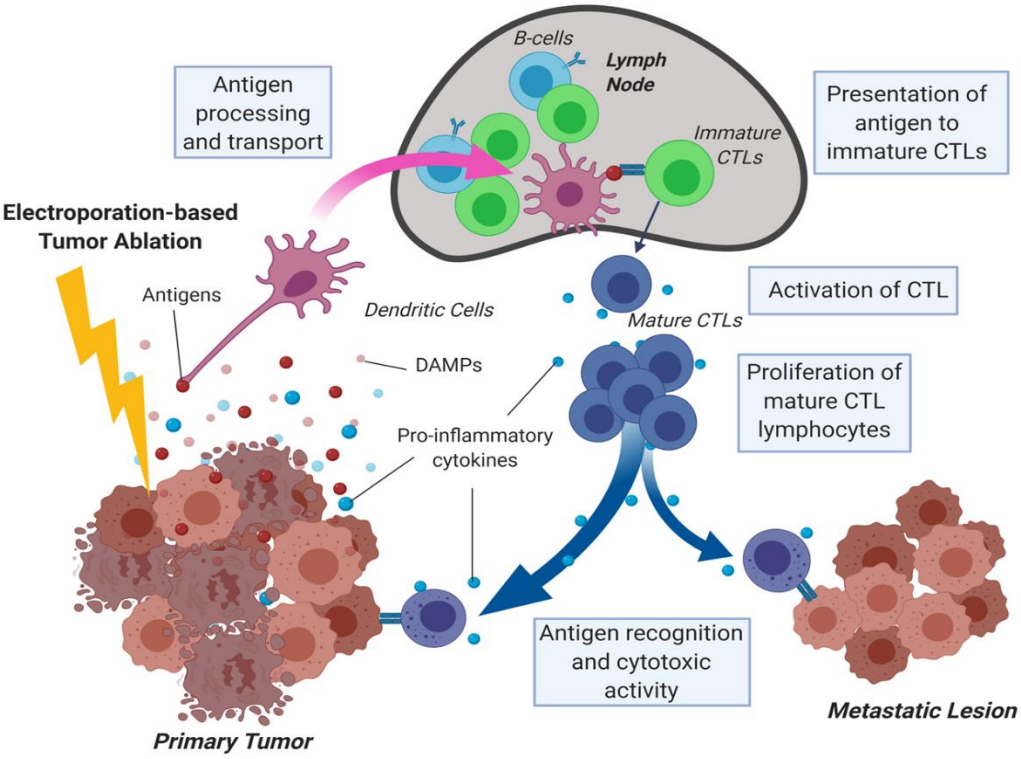

Figure 3: Adaptive immune system antigen presentation. Collection and presentation of antigens by dendritic cells to cytotoxic T-cells (CTLs) and subsequent CTL activity.

"away from" and *scopus* as "target", the abscopal effect refers to a treatment's effects being seen distant from the primary treatment site. The use of the term "abscopal" and this effect were first founded from radiotherapy patients(Mole, 1953; Ohba et al., 1998). However, the abscopal effect in radiotherapy was hotly debated in the past as there were severely limited occurrences. This was most likely due to the immunosuppressive activity that radiotherapy was rarely able to overcome(Ng and Dai, 2016).

As ever with the arms race against cancer, tumors have developed ways to avoid this adaptive onslaught. The expression of markers meant to reduce the potential for autoimmunity in response to increased inflammatory signaling can, unfortunately, also be

exploited by cancer cells. For example, checkpoint inhibitors gained recent fame from results in clinical trials and their Noble Prize award in 2018(Decatris and O'Byrne, 2016; Zang, 2018). The expansion on immunotherapy treatments for cancers has exploded in recent years due to this discovery. Checkpoint inhibitor antibodies against programmed cell death (ligand)-1 (PD/L-1) and cytotoxic T-lymphocyte antigen 4 (CTLA4) turn off these restrictive pathways to enhance the killing capabilities of cytotoxic lymphocytes (**Figure 4**).

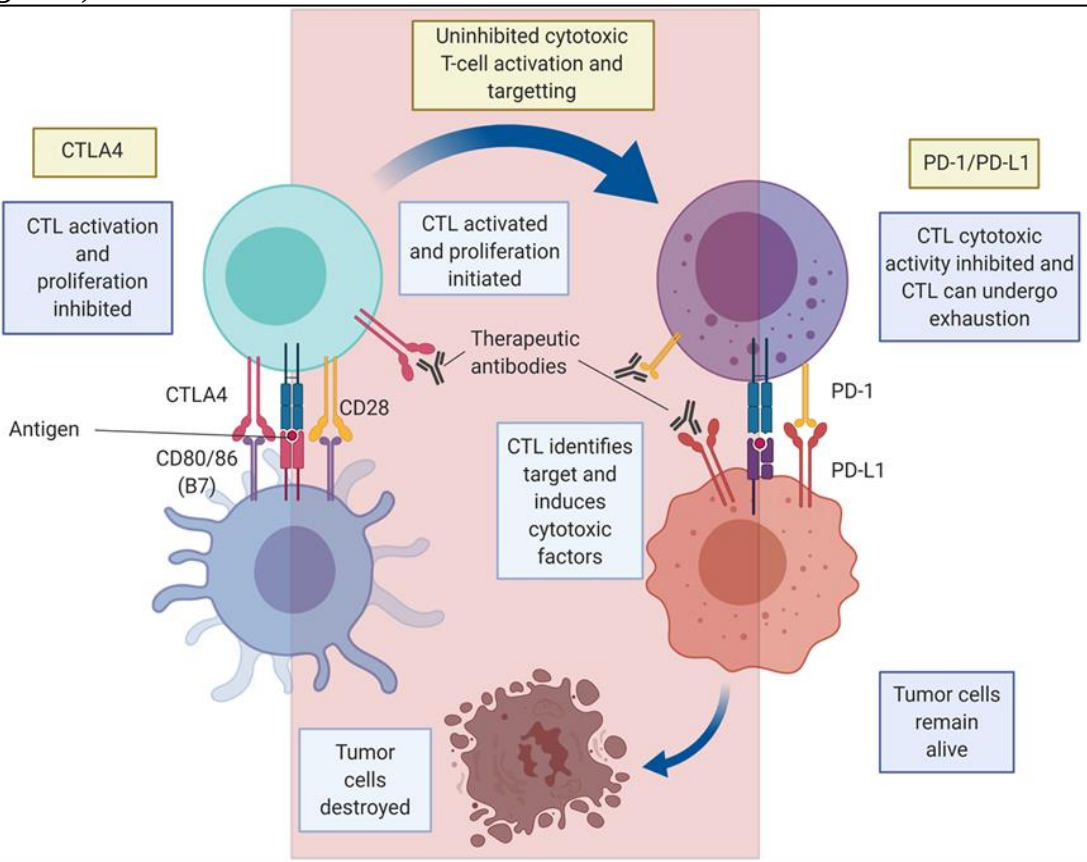

Figure 4: Checkpoint inhibitor effects on cytotoxic lymphocytes

CTLA4 limits the activation of T cells by antigen-presenting cells while PD-L1 interacts with PD-1 on the T cell surface to inhibit T cell cytotoxic actions(Freeman et al., 2000; Alegre et al., 2001). Therefore, CTLA4 inhibition results in higher amounts of CTLs while PD/L-1 inhibition makes CTLs more effective at killing target cells(Ishida et al., 1992; Krummel and Allison, 1995; 1996; Freeman et al., 2000). With these and other new immunotherapy tools at the oncologist's fingertips, the case reports of abscopal-like effects have skyrocketed, bringing high hopes for patients with cancers like advanced lung cancer or melanoma(Hodi et al., 2010; Khalil et al., 2016; Ray et al., 2016; Reck et al., 2016; Zhang et al., 2016; Brahmer et al., 2017). Unfortunately, the expression of these markers varies greatly for every cancer and patient as many cancers also rely on an immunosuppressive barrier developed by stromal cells (**Figure 1**). Because of this, "cold" cancers such as pancreatic cancer have not shown effective responses to checkpoint inhibitors or other immunotherapy options in clinic(Rossi et al., 2014; Zhang et al., 2018).

ABLATION TECHNOLOGIES AND THE IMMUNE RESPONSE

Bypassing the complexity of biologically treating cancer, ablation modalities utilizing thermal, chemical, mechanical, chemical, or electrical forces have developed for the treatment of malignancies lacking alternate therapy options. The use of these minimally to non-invasive techniques is meant to reduce the primary tumor burden and increase patient survival in cases where surgery is not feasible, and chemotherapy has shown little impact. The most studied ablation technologies are those categorized as thermal in nature and utilize intense heating or cooling to destroy tissue. These include radiofrequency, microwave, high-intensity focused ultrasound, and cryoablation among other modalities. Several of these therapies have shown immunotherapeutic and abscopal-like effects but most reporting is limited for the heat-based technologies(Wu et al., 2007; Napoletano et al., 2008). This is most likely due to the break-down in the proteins released from the treatment that does not allow for proper antigen presentation(Zhang et al., 2002; Hu et al., 2007). Cryoablation attempts to avoid this breakdown using cold instead of heat and shows the strongest potential among thermal ablation modalities for immunomodulation(Blackwood and Cooper, 1972; Bagley et al., 1974; Yakkala et al., 2019). However, cold can also lead to the degradation of the proteins(Blackwood and Cooper, 1972; Privalov, 1990). These modalities also stimulate a similar type of cell death, necrosis, that is acute and accidental, thus not ideal for the recruitment of anti-tumoral immune cell populations(Vakkila and Lotze, 2004; Jacobson et al., 2013). Moreover, thermal technologies suffer from heat-sink effects which make it difficult to fully ablate tissues and can cause nondiscriminatory damages to nearby healthy tissues.

Non-thermal ablations include electroporation-based technologies. The advantage of non-thermal ablations are multiple: not only is there less risk of healthy tissue damage, heat-sink effects generally do not apply and the potential for alternative cell death pathways, and protein conservation make them prime candidates for potential immunomodulation(Shao et al., 2019; Sugimoto et al., 2019). They do, however, come with their own specific challenges that will need to be overcome as the technologies developed, either by redesigns or co-therapy options, to enhance their efficacy in clinic.

IMMUNE RESPONSE TO ELECTROPORATION-BASED ABLATION

Electroporation-based ablation includes many subtypes of ablative strategies, from electrochemotherapy (ECT) to nanosecond pulse electric fields (nsPEFs) (**Table 1**). These different treatments require adjustments of pulsed electric fields (PEFs) to generate the desired response of the treatment. By adjusting the polarity, duration, applied voltage, and count of pulses applied, electroporation can temporarily or permanently permeabilize cell membranes (**Figure 5**).

Technology	Pulse length	Polarity	Voltage	Pulse Count
ECT	Milli-nanoseconds	Mono	700-1200V/cm	4-10
GET	Milliseconds	Mono	600-1500V/cm	5-10
IRE	75-100 microseconds	Mono	1500-3000V/cm	80-300
HFIRE	10-1 microseconds	Bipolar	1500-4000V/cm	80-300 (bursts)
nsPEFs	Nanoseconds	Mono	25-50,000V/cm	600-1200

Table 1: Summary of PEF parameters in electroporation-based ablation modalities. Value ranges based on commonly reported parameters in literature for in vivo preclinical and clinical application. These ranges are not definite and application outside of these ranges can occur.

Reversible Electroporation

ECT is one of the first electroporation-based technologies to be used in clinical application for tumor ablation(Belehradek et al., 1993; Heller et al., 1996). ECT utilizes the opening of the cell membrane to allow for better access of chemotherapeutic agents. As many chemotherapeutics face a bioavailability challenge, this opening can not only increase the opening of the tumor to the agent but also allow for quicker transference that may avoid chemoresistance mechanisms and breakdown(Frandsen et al., 2012; Falk et al., 2017). Bleomycin has been one of the most used chemotherapeutics paired with ECT(Belehradek et al., 1993; Heller et al., 1996; Gothelf et al., 2003; Matthiessen et al., 2018). Bleomycin's mechanism of action inhibits DNA synthesis and stimulates high reactive oxygen species formation to create DNA breaks that are able to induce apoptosis, but the drug has very poor cell membrane penetration ability(Tounekti et al., 1993; Claussen and Long, 1999). ECT extends the penetration of bleomycin into the tumor, increasing uptake to allow for tumor cell death(Orlowski et al., 1988; Probst et al., 2018). The added injury to the treatment site may also stimulate damage-associated molecular patterns (DAMPs) and pro-inflammatory cytokines to stimulate an immune response to the area(Probst et al., 2018). Enhancing the potentially proinflammatory effects of ECT, the use of high calcium reagents instead or along with chemotherapeutics has shown an increase in necrotic cell death(Frandsen et al., 2012). By increasing pro-inflammatory signaling to the treatment area, this could allow for recruitment of anti-tumoral immune cells to attack remaining cells and potentially prevent recurrence. However, few studies so far have investigated the immunological impact of calcium ECT.

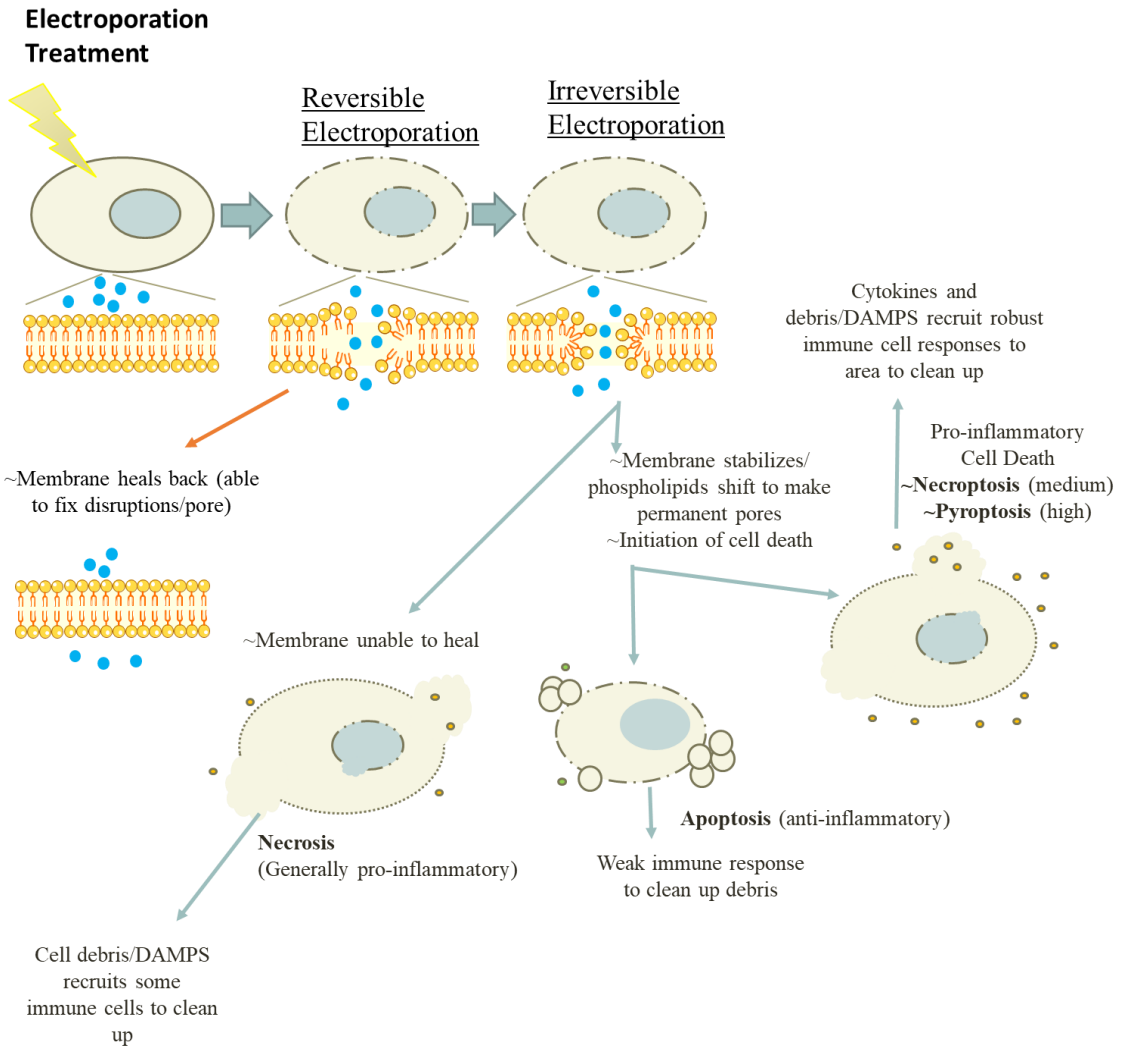

Figure 5: Mechanisms of reversible and irreversible electroporation of cell membranes and cell death.

Gene electrotherapy (GET, also known as electrogenetherapy) builds upon this use of electroporation by applying similar pulse parameters for the transfection of gene plasmids coding for the overexpression of proteins such as IL-12 into the tumor area(Kamensek et al., 2018). This can increase the transfer of these plasmids to cancer cells and allows for controlled delivery to the treatment site rather than depending on highly mutable viral targeting systems such as adenoviruses(Lohr et al., 2001; Lee et al., 2017).

As the first mass-used immunotherapies included cytokine therapies, particularly interleukins (ILs), it's well known that the increase of pro-inflammatory signaling to the treatment area can reduce tumor burden(Sim and Radvanyi, 2014; Wrangle et al., 2018). However, these systemic treatments often came with serious auto-immunity side effects that greatly impact patient standard of living(Leonard et al., 1997; Sim and Radvanyi, 2014). GET allows for more targeted delivery meant to mitigate these side effects but still provide a robust change to the treated tumor's microenvironment(Heller et al., 2006; Daud et al., 2008). Preclinical and early clinical testing indicates that the use of proinflammatory cytokine plasmids can raise proinflammatory signaling and recruit immune cells to the

tumor area(Lucas et al., 2002; Daud et al., 2008; Canton et al., 2017; Kamensek et al., 2018) GET with tumor necrosis factor and IL-12 showed immune cell infiltration to the treatment area, and increased CLT populations at tumor site and nearby lymph nodes in murine melanoma(Kamensek et al., 2018).

Most application of ECT and GET have been done to cutaneous or subcutaneous tumors that are easy to access. It is generally applied with a multi-needle fixed electrode applicator that allows for thorough coverage of the tumor and easy repositioning(Gehl et al., 2018). Current ranges of application do not limit muscle contraction and more involved or internal treatment areas may require strong paralytics and separated electrode use to adequately cover the tumor area without damages to local healthy tissues. Preclinical studies are currently underway to determine the feasibility of ECT and GET application for liver cancer and other malignancies(Girelli et al., 2015; Izzo et al., 2020).

Enhancement of ECT and GET are still needed to provide prolonged systemic immunity and increase patient survival. By combining this treatment with another, this barrier may be able to be overcome. Utilizing GET with plasmids for antibody production against immunosuppressive mechanisms such as PD-1/PD-L1 is one possibility(Jacobs et al., 2020). In the interest of DNA vaccines, the targeting of specific biomarkers may also extend GET's ablative effects(Bråve et al., 2009; Bosnjak et al., 2015). Utilizing plasmid with a "dummy" antigen and in combination in specially designed chimeric antigen receptor (CAR) T-cells may also be a potential future venue.

Irreversible Electroporation

In recent years, irreversible electroporation (IRE) has been integral to many clinical trials targeting notoriously difficult to treat malignancies included liver and pancreatic cancers.(Cannon et al., 2013; Belfiore et al., 2015; Martin et al., 2015; Sugimoto et al., 2019) The significant results of recent clinical trials have pushed IRE into Phase III clinical trials in pancreatic cancer (clinicaltrials.gov, ID: NCT03899636). IRE utilized microsecond pulsed electric fields. Unlike ECT, IRE increases the number of applied pulses so that the opening of pores on cell membranes remain permanent and induces cell death through a disruption in homeostasis (**Figure 5, Table 1**)(Davalos and Rubinsky, 2012). IRE is often compared to thermal ablation treatments but is normally applied non-thermally, reducing the risk of healthy tissue damages(Al-Sakere et al., 2007a). Furthermore, it has been shown to spare critical structures(Lee et al., 2010; Cannon et al., 2013).

Despite its lengthy time in clinical trials, until recently immunological assessment of IRE has been limited(Al-Sakere et al., 2007b; Neal II et al., 2013). In the last few years, studies investigating different aspects of the immune system after IRE have helped expand our understanding of the immunological impacts of the treatment. There is evidence of a decrease in anti-inflammatory immune cells, such as MDSC and T-regulatory populations, from assessment of the peripheral blood of pancreatic cancer patients(He et al., 2019; Pandit et al., 2019; Scheffer et al., 2019). There are also increases in CTLs and other anti-tumor immune cells in the periphery(Bulvik et al., 2016; White et al., 2018; He et al., 2019; Narayanan et al., 2019; Scheffer et al., 2019). This is, however, only one part of the immune response but is a vital piece that may be used as biomarker for treatment success. Studies *in vitro* and in preclinical models have shown increases in antigen availability in melanoma and an increase in macrophage recruitment with pro-inflammatory cytokine signaling in

liver cancer (Chen et al., 2017; Shao et al., 2019). This shows the potential of IRE to produce a pro-inflammatory tumor microenvironment that could be beneficial for immunomodulation and patient survival.

Further investigation into the mechanisms behind IRE-induced local and systemic immunity and how to increase treatment efficacy are still needed in order to determine optimal combination therapy options. This is particularly in pancreatic cancer which is well known for being unresponsive to most all new treatment options including immunotherapies(Rossi et al., 2014). IRE's clinical success in pancreatic malignancies makes it a hopeful candidate for combination. It's already shown success over and when combined with chemotherapeutics in pancreatic cancers(Hong et al., 2018; Scheffer et al., 2019). In fact, studies are investigating the use of IRE to have a similar effect as ECT and improve chemotherapeutic bioavailability in tumors(Bhutiani et al., 2016). Recent findings on the upregulation of PD-1 expression on peripheral CTLs in clinical studies combined with its reversal in checkpoint inhibitor activity in preclinical models also make IRE a prime candidate for checkpoint inhibitor combination(Scheffer et al., 2019; Zhao et al., 2019). A clinical trial combining IRE with allogeneic NK cell immunotherapy also showed an increase in survival in liver patients(Yang et al., 2019). Timing of combination therapy, especially those with short half-lives, is important as well, as delivery of a treatment before the tumor microenvironment is sufficiently inflamed may be defeated by the programmed cell death mechanisms IRE employs(cite martin's chemo before paper). A recent study highlighted the decrease in T-regulatory cells 3-5 days postoperative IRE treatment(Pandit et al., 2019). This data suggests that this timepoint may be critical in increasing clinical efficacy and introducing immunostimulatory combinational immunotherapies.

Currently, most clinical IRE treatments have been employed in single sessions. Multiple IRE treatments may become an area of investigation to achieve either an increased area of ablation or to stimulate an immune system response once initial inflammatory responses decline. However, application of IRE requires strong paralytics and cardiac synchronization to limit injury to patient due to the electrical pulses(Ball et al., 2010; Nielsen et al., 2014). This can greatly limit candidacy and potential for adverse events, especially with repeated application. In addition, multiple applications may lead to resistance in remaining cells, requiring alteration in subsequent treatment parameters(Shao et al., 2018).

High-Frequency Irreversible Electroporation

To overcome the application limitations of IRE, High-Frequency IRE (H-FIRE) is being developed. H-FIRE is thought to follow similar mechanisms as IRE but with several distinct differences. The first is in the use of bipolar rather than monopolar bursts. This may allow for more even, controlled application of treatment than monopolar IRE, though it does require a higher voltage in application(Arena et al., 2011b). The second is in the use of smaller pulse widths. While IRE is generally applied with pulses of 100us duration, H-FIRE utilizes 1-10us pulse widths (cite). This limits the propagation of action potentials responsible for muscle contraction in IRE and reduces the risk of cardiac arrhythmia, allowing for safer use with weaker paralytics and less need for cardiac synchronization(Arena et al., 2011a; Partridge et al., 2020). It has been involved with several veterinary pre-clinical trials(Arena et al., 2011a; Latouche et al., 2018; Partridge et al., 2020).

As predicted of a similar technology, H-FIRE has shown similar traits to IRE in terms of biological impact. Studies suggest that cell death is mostly necrosis with the potential for pyroptosis, a highly immunogenic form of cell death(Ringel-Scaia et al., 2019; Mercadal et al., 2020). This may also be adjusted by calcium, similar to ECT, which may mean that clinical application could be guided using calcium and protective agent injections for delicate areas of treatment(Wasson et al., 2020). And while the shortened pulses require a higher voltage for cancer cells to reach the lethal threshold, there is evidence that healthy or benign cells have a higher threshold than malignant cells, which may make it more advantageous for tumors integrated with sensitive areas(Ivey et al., 2017). The reason for this selectivity is currently unknown but is speculated to be due to the size of the nucleus(Ivey et al., 2015; Rolong et al., 2017). Selective inflammatory cell death can predispose the available antigen to more likely be from highly malignant cells rather than from nearby healthy tissue and may allow for strong antigen presentation potential.

Several preclinical studies have already found evidence of inducing a local immunological response and the potential for immune memory(Ringel-Scaia et al., 2019; Partridge et al., 2020). However, as this is a newer technology, further studies are needed to ascertain the differences in the biological and immunological responses to IRE and H-FIRE and identify optimal combination therapy options.

Nanosecond Pulsed Electric Fields

With the shortest of the pulse-widths used in electroporation, nsPEFs (also known as nanosecond pulsed stimulation) use nanosecond pulses applied at high voltage to irreversibly permeabilize cell membranes. Originally, nsPEFs were believed not to affect cell permeability but rather disrupt molecular pathways and membrane-bound organelles inside the cell to induce cell death(Schoenbach et al., 2001). Recent studies, however, have shown there are tears produced by nsPEFs, though they are smaller than those produced by microsecond pulses like IRE and H-FIRE(Vernier et al., 2003; Ford et al., 2010). Even so, the tears are able to disrupt cell homeostasis and induce apoptotic to pro-inflammatory cell death mechanisms(Ford et al., 2010; Beebe et al., 2013; Skeate et al., 2018). The intracellular affects should not be overlooked as the mitochondria of cells are also membrane-bound and can play a major role in both cancer hallmarks and potentially the cell death mechanisms associated with electroporation(Lemasters et al., 2009; Dejean et al., 2010; Wallace, 2012). Indeed, nsPEFs combined with increased calcium could enhance ablation application and necrotic cell death similarly to calcium ECT and calcium H-FIRE(Morotomi-Yano et al., 2014).

Recent preclinical mouse studies show nsPEFs altering the tumor microenvironment to one that is more pro-inflammatory in triple-negative breast cancer(Guo et al., 2018b). There have also be anti-tumoral effects seen in pancreatic cancer, liver cancer, and melanoma models treated with nsPEFs with a decrease in immunosuppressive immune cell subtypes and inhibition of secondary tumor engraftment after treatment(Nuccitelli et al., 2012; Guo et al., 2018a; Lassiter et al., 2018). Unfortunately, there seems to be a limitation current on the treatments, depending on the cancer, in producing memory against cancer recurrence(Guo et al., 2018a). Many questions as to the immunogenic potential of nsPEFs still remain but it is possible that CTLA-4 or

PD-1/PD-L1 checkpoint inhibitors could strengthen long-term immunomodulation and help with the induction of immune memory in more immunosuppressive tumor types.

DISCUSSION:

The immune system is a complex system responsible for the health and protection of the body from many diseases, including cancer. However, many cancers have evolved to utilize the immune system to their own needs, reprogramming cells to immunosuppressive subtypes and expressing immunoregulatory markers such as PD-L1 and CTLA-4 to inhibit anti-tumoral immunity. Because of this, many treatments that have been found to work *in vitro* are found to be inefficient in immunocompetent preclinical and clinical applications. Understanding of the tumor microenvironment and the processes in which anti-tumor immunity can be re-obtained is vital for the development of new tumor treatments.
Electroporation-based techniques have recently begun to investigate this intricate web of ablative cell death and tumor micro-environmental shifts. These investigations, though still early in their development, are vital for informing on combination therapy options that can enhance treatment efficacy and patient survival.

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RB, AZ, and IA all contributed to the writing and editing of the manuscript. RB wrote the original draft and incorporated all suggested edits.

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CONFLICTS OF INTERESTS

IA is an inventor on pending and issued patents related to the work. Authors declare no other conflicts of interest.

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CHAPTER FOUR

Patient derived xenografts expand human primary pancreatic tumor tissue availability for ex vivo irreversible electroporation testing

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Abstract

New methods of tumor ablation have shown exciting efficacy in pre-clinical models but often demonstrate limited success in the clinic. Due to a lack of quality or quantity in primary malignant tissue specimens, therapeutic development and optimization studies are typically conducted on healthy tissue or cell-line derived rodent tumors that don't allow for high resolution modeling of mechanical, chemical, and biological properties. These surrogates do not accurately recapitulate many critical components of the tumor microenvironment that can impact *in situ* treatment success. Here, we propose utilizing patient-derived xenograft (PDX) models to propagate clinically relevant tumor specimens for the optimization and development of novel tumor ablation modalities. Specimens from three individual pancreatic ductal adenocarcinoma (PDAC) patients were utilized to generate PDX models. This process generated 15-18 tumors that were allowed to expand to 1.5 cm in diameter over the course of 50-70 days. The PDX tumors were morphologically and pathologically identical to primary tumor tissue. Likewise, the PDX tumors were also found to be physiologically superior to other *in vitro* and *ex vivo* models based on immortalized cell lines. We utilized the PDX tumors to refine and optimize irreversible electroporation (IRE) treatment parameters. IRE, a novel, non-thermal tumor ablation modality, is being evaluated in a diverse range of cancer clinical trials including pancreatic cancer. The PDX tumors were compared against either Pan02 mouse derived tumors or resected tissue from human PDAC patients. The PDX tumors demonstrated similar changes in electrical conductivity and Joule heating following IRE treatment. Computational modeling revealed a high similarity in the predicted ablation size of the

PDX tumors that closely correlate with the data generated with the primary human pancreatic tumor tissue. Gene expression analysis revealed that IRE treatment resulted in an increase in biological pathway signaling associated with interferon gamma signaling, necrosis and mitochondria dysfunction, suggesting potential co-therapy targets. Together, these findings highlight the utility of the PDX system and capability to improve tumor ablation modeling for IRE to increase clinical application efficacy. It is also feasible that the use of PDX models may benefit other ablation modality testing.

INTRODUCTION

While prevention and early diagnosis are key to reducing cancer-related mortality, lack of treatments for many types of cancers, such as pancreatic cancer, has led to a stagnation in patient survival rates. New treatment options are vital to increasing the survival of these patients. Current progress in ablation modalities has shown success in clinical trials by improving patient morbidity and mortality, crossing barriers impassable for surgery and chemotherapy. However, the treatment parameters for these ablation modalities often derive from modeling data generated from *in vitro* or *ex vivo* studies using the mechanical or electrical properties of healthy tissue or cell line data from rodents. With only 15% of pancreatic cancer patients eligible for surgical resection, the amount of direct human tumor tissue available for testing is severely limited (Ryan et al., 2014). Additionally, tumor tissue integrity declines over time once excised, leading to degradation of tissue mechanical and electrical properties that influence the accuracy of the *in vitro* and *ex vivo* modeling results compared to clinical application (Herman P Schwan, 1956).

Beyond human applications, tumor ablation is also an emerging therapeutic strategy in the veterinary clinic, where canine and other large animal patients are often used in comparative oncology studies. While this offers several advantages in terms of access to sufficient malignant animal tissues from spontaneous tumors for analysis and modeling, these studies are often limited due to cost and a general paucity of validated reagents available to assess biological responses to treatment (J.L. Shepps, 1980). Therefore, databases for tissue properties are often used (Chiang et al., 2013; Hasgall et al., 2018). However, this limits modeling for newer modalities and databases, in general, have been generated using healthy rather than malignant tissues, which can further complicate modeling accuracy (Cheng and Fu, 2018). Immortalized cancer cell lines can also be utilized but are highly homogeneous and lack the secondary structures and biological complexity of the *in situ* tumor, resulting in significant deviations between the models and clinical observations(Willey et al., 2015).

To combat these limitations, we propose incorporating patient-derived xenograft (PDX) models to evaluate tumor ablation efficacy. PDX rodent models involve the engraftment of cancerous tissue from patients into immunocompromised animals, typically NOD *scid* gamma (NSG) mice. Over time, a small cancer biopsy will proliferate into a tumor that closely matches the biological complexity of the original patient's tumor. This tumor can then be excised and sub-cultured into exponentially greater numbers of mice to further propagate the tumor (**Figure 1A**). This process enables robust, high power modeling that is not possible utilizing direct from patient human specimens. While not yet widely utilized in the biomedical device development, PDX models have proven to be highly valuable tools in the pharmaceutical industry to determine individual patient responses to newly developed drugs (Koga and Ochiai, 2019). Thus, we foresee similar

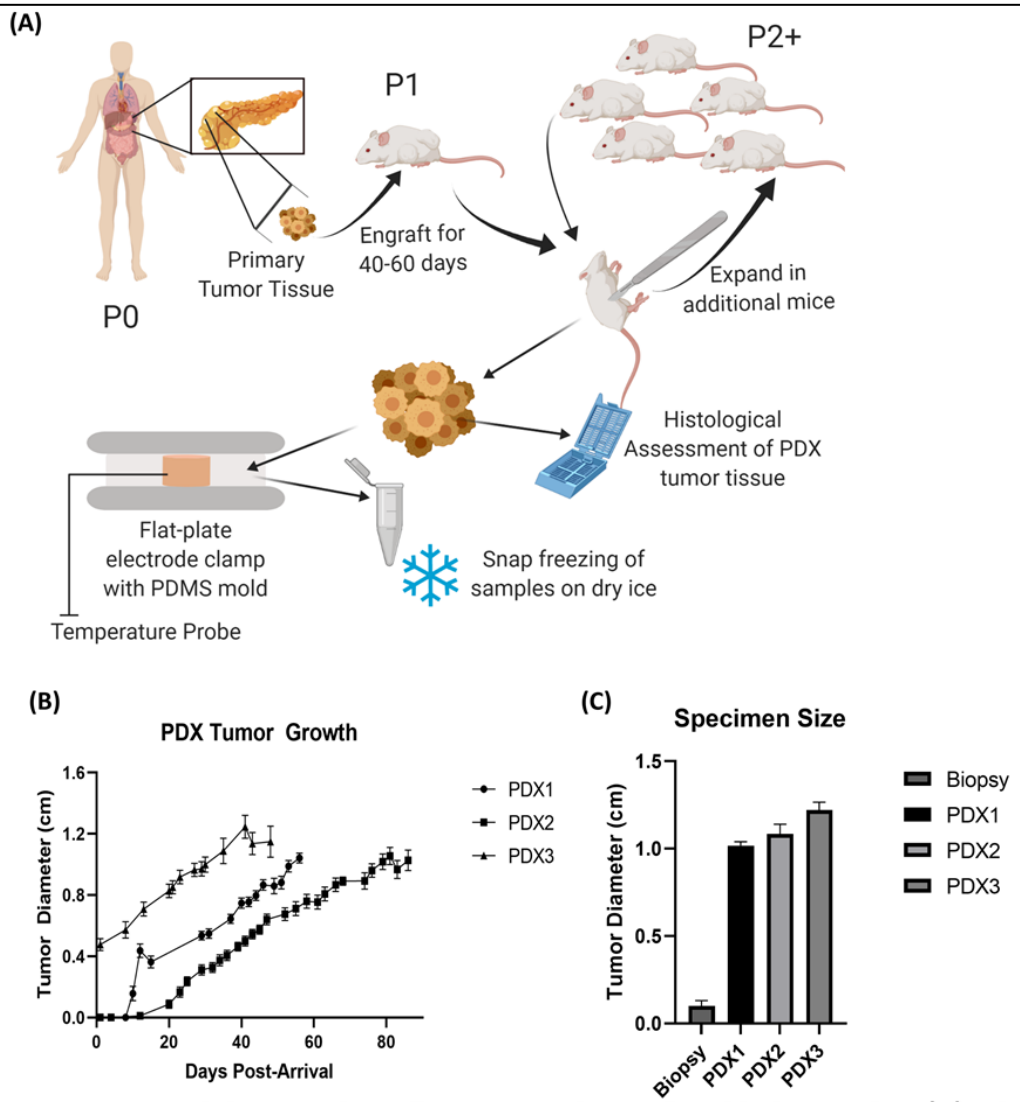


Figure 1. PDX models expand small tumor specimens for ablation testing. (A) Schematic of pancreatic cancer patient-derived xenograft model. Primary human pancreatic tumor tissue was implanted into an NSG (Passage 1) and allowed to progress, excised, and expanded into larger cohort of mice (Passage 2), and then collected for histological assessment and *ex vivo* testing. **(B)** Tumor growth curve from PDX model mice. SEM, n=16-18 mice for each model. **(C)** Average maximum specimen size, SEM, n=13-16.

applications for the development of tumor ablation modalities. For the purpose of *ex vivo* tissue characterization and experimentation, the use of a flank PDX model as we demonstrate here may be more desirable than an orthotopic model. While orthotopic methods such as cell line injection models or genetic predisposition models such as KPC may lead to higher structured tumors, the amount of available tissue for testing can remain relatively small due to the size limitations in the peritoneal cavity and increase morbidity risk to the host due to metastatic lesions (Hingorani et al., 2005). A flank model also allows for easier tumor size assessment without the need for medical imaging equipment.

Here, we utilized PDX models of pancreatic cancer to evaluate tissue properties and ablation volumes following treatment with irreversible electroporation (IRE), a non-

thermal electrical ablation modality that has shown significant improvements in Stage III and Stage IV pancreatic cancer patient survival in recent and ongoing clinical trials (Kwon et al., 2014; Martin et al., 2015; Scheffer et al., 2016; Belfiore et al., 2017). Previous IRE modeling studies in the pancreas have been hindered by lack of surgical candidates and minimal tissue size, limiting data points for computational modeling of electrical properties and ablation volumes and statistical power in studies. These same limitations can be seen in other electroporation-based technologies as well as microwave ablation, which also relies on dielectric properties and the use of such databases for treatment planning (Chiang et al., 2013; Cheng and Fu, 2018). Our results show that the PDX models retain the physiological and biological characteristics of the original patients' tumors. Likewise, the increased volume and quality of tumor specimens significantly improved the accuracy of ablation modeling. Further mechanistic studies were also possible, revealing several hallmarks of pancreatic cancer that were significantly impacted by IRE treatment. Together, these data support the integration of PDX models in tumor ablation studies and provide additional data to better define the mechanisms by which IRE treatment results in significantly prolonged survival in pancreatic cancer patients.

METHODS AND MATERIALS

	PDX Specimens			Primary Human Specimen					
Patient	1	2	3	A	B	C	D	E	F
Model ID	J000077960	J000096053	TM01212	n/a	n/a	n/a	n/a	n/a	n/a
Primary Site	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas
Diagnosis	PDAC	PDAC	PDAC	Neuro-endocrine	PDAC	PDAC	PDAC	PDAC	PDAC
Tumor Site	Pancreas	Lung	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas	Pancreas
Tumor Type	Primary	Metastatic	Primary	Primary	Primary	Primary	Primary	Primary	Primary
AJCC Stage/Grade	IV/2	IV/2	Unspecified	Low-Grade NET, 5cm	IIb	III	IIa	III	III
Sex	F	M	M	M	F	F	F	F	F
Treatment	Naive	Naive	Naive	Chemo	Chemo	Chemo / XRT	Naïve	Chemo	Chemo
Age	68	64	Unspecified	52	67	77	72	49	63

Table 1. Human patient characteristics. Human patient specimen characteristics used in the PDX models and primary human patient specimen characteristics for comparison tissues used *ex vivo* assessments.

Experimental animals

All experiments were conducted under institutional IACUC approval and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Murine Pan02 cells (NCI) were cultured with RPMI 1640 (ATCC) supplemented with 10% FBS (Atlanta Biologicals). Female NSG and C57Bl/6J mice were anesthetized and injected subcutaneously in right flank with $6x10^6$ cells in 100 µL of Matrigel (Corning, n=5). Female NSGs engrafted with patient derived pancreatic cancer were generated by The Jackson Laboratory (detailed in **Table 1**). Mice were engrafted subcutaneously in right flank. Mice cohorts were confirmed by Jackson to have palpable masses before shipping to our facility and were received carrying passage 2 tumors (n=16-18 mice per patient). Therefore, variance on beginning tumor size is expected between cohorts. All NSG mice were housed under immunocompromised conditions with autoclaved cages and water and

irradiated chow. All mice were house in SPF conditions with ad libre chow. Mice were monitored three times weekly until experimental endpoints were reached, tumors reached 1-1.6 cm in diameter calculated by the square root of the product of cross diameter measurements, or if considered clinically moribund.

PDX tissue collection

Mice were euthanized according to IACUC protocol by carbon dioxide fixation followed by cervical dislocation. Tumor tissue was harvested post-euthanasia. A thin (2mm) slice was taken laterally for histological assessment and remaining tissue stored in phosphate-buffered saline (PBS) during transport. All tissue was collected in groups of 3-5 mice and used for *ex vivo* testing within two hours from excision to maintain tissue integrity.

Human patient specimen collection

This research study was approved by the Institutional Review Board (IRB) at the University of Louisville (02.0496). All potential pancreatic cancer patients undergoing either in situ IRE or pancreatectomy were asked for voluntary research participation from January 2016 to July 2018. A total of 6 pancreatectomy patients consented and were enrolled in this prospective study (detailed in **Table 1**). Research participation did not affect the treatment options of patients or inpatient care. All the participating patients were well-informed that they could withdraw their consent at any time during the study without affecting their treatment and ongoing care. The human patient data included in this manuscript is a subgroup of a larger cohort shown in a prior conference paper (Beitel-White et al., 2018).

IRE ex vivo application and tissue properties

Fresh tumor tissue was cut into 2-3 cylindrical sections and placed into a polydimethylsiloxane (PDMS) mold to retain a cylindrical shape factor (thickness (t) = 0.56 cm, diameter = 0.6 cm). A cylindrical shape ensures a known shape factor enabling simple calculation of the electrical conductivity with the following equation:

$$\sigma = \frac{(I \cdot t)}{(V \cdot A_c)}$$

where I is induced current, V is applied voltage, and A_c is cross-sectional area. The tissue-containing mold was placed between stainless steel, parallel-plate electrodes (Harvard Apparatus) connected to a BTX pulse generator (Harvard Apparatus).

A fiber optic probe (Luxtron m3300, LumaSense) was inserted to measure temperature at a frequency of 2 Hz during treatment. Parallel-plate electrodes ensure a uniform electric field is applied across the tissue sample. Prior to IRE pulsing, a 25 V, 100 µs pre-pulse was delivered in order to establish initial conductivity. A total of 100 IRE pulses were applied to the sample, with 100 µs pulse width and electric fields between 0-3000 V/cm. Changes in conductivity during electroporation were assumed to take place primarily during the first pulse. Thus, the average current value recorded during the last 5 µs of the first IRE pulse was used to calculate a single conductivity value for each sample.

IRE in vivo application

Mice were anesthetized with isoflurane inhalant via nose-cone during treatment. Tumors were treated at day 7-14 post-injection, once tumors reached 0.5cm in size. Paired needle acupuncture electrodes (0.4mm diameter) were inserted into either side of the tumor with a 5mm gap and 2000V/cm electric field was applied with an irreversible electroporation generator (Harvard Apparatus) for a total of 100 pulses of 100μs duration. Control mice were anesthetized in the same manner with electrodes inserted in similar fashion without an applied electrical current. All animals recovered fully from the inhalant and were monitored for potential health issues or treatment complications.

Numerical investigation of tissue conductivity response

A numerical model was constructed in COMSOL Multiphysics v5.4 (COMSOL Inc., Burlington, Massachusetts) to approximate the electric field distribution prior to (static) and during IRE (PDX1 and primary) in a two-needle electrode configuration (1.5 cm spacing/exposure, 100 pulses and 100 μs duration). Electric potential boundary conditions were set to maintain a voltage-to-distance ratio of 1,750 V/cm with all remaining external boundaries assigned as electrically insulating. The dynamic response to IRE was incorporated by applying the electrical conductivity curves to a tissue domain of dimension 10x8x8 cm. A "finer" mesh setting was selected and resulted in a mesh with 134,057 tetrahedral elements. The IRE ablation was estimated to occur at electric field values greater than 500 V/cm, which is a previously reported threshold determined *in vitro* from primary murine pancreatic cancer cells.(Arena et al., 2012) Associated Joule heating and thermal dissipation effects were modeled using a modified Bioheat equation and Joule heating term. A more detailed methodology can be found in previous work (Neal II et al., 2012; Lorenzo et al., 2019).

Histopathology

Tumor tissue sections were fixed in 10% formalin for at least 24 hours, embedded in paraffin, and mounted on slides in 5μm sections. Slides were stained with hematoxylin and eosin. Primary human patient specimen histopathology photomicrographs were provided by D.J.G from Virginia Tech Carilion School of Medicine. Histopathology analysis of all tissues was evaluated by a board-certified veterinary pathologist (S.C.O.).

Gene Expression Evaluation

Tissue specimens were collected and snap frozen within 15 minutes post-treatment *ex vivo* and after 24hrs *in vivo*. RNA was extracted from each sample via AllPrep DNA/RNA/Protein kit (Qiagen) and quantified via Nanodrop (Thermofisher). RNA was pooled equally for each electric field magnitude with 3-5 samples per treatment for a total of 540 ug RNA and converted into cDNA via RT_2 First Strand (Qiagen). cDNA was plated on RT_2 Profiler Human Cell Death Pathway, Human Cancer Pathway Finder, Mouse Innate and Adaptive Immunity, and Mouse Cancer Pathway Finder arrays (Qiagen) and run on ABI 7500 Fast Block (Thermofisher). RT_2 Profiler plate results were normalized to individual untreated tumor tissues and plate housekeepers. ΔΔCT and fold regulation calculated via Qiagen Data Analysis Center. Gene expression data was analyzed by Ingenuity Pathway Analysis (Qiagen) and compared between individual patients and treatment dose. Examples of assay/program generated gene groupings are shown in **Supplemental Figure S1**. Heatmaps illustrating gene expression were generated using the web-based Heatmapper platform.

Statistical Analysis

A Student's two-tailed t-test was utilized for comparisons between two experimental groups. Multiple comparisons were conducted using one-way and two-way ANOVA where appropriate followed by Mann-Whitney or Tukey post-test for multiple pairwise examinations. Statistical significance was defined as $p<0.05$. All data are represented as the mean ± SEM.

RESULTS

PDX Tumors Are Superior Models and Faithfully Recapitulate the *In Situ* Tumor Microenvironment Compared to Cell Line Based Models of Pancreatic Cancer.

To evaluate the potential of the PDX model to function as a surrogate for human *ex vivo* pancreatic cancer tissue in tumor ablation modality studies, we utilized xenografts from three separate human patients (**Table 1**). All patients were diagnosed with pancreatic ductal adenocarcinoma (PDAC) and tissue was collected from either the primary tumor mass (Patients 1 and 3) or a metastatic lung lesion (Patient 2). Xenografts were passaged (P2) in a total of 16-18 mice for each patient (**Figure 1A**). Each tumor was allowed to progress to at least 1cm in diameter for each mouse (**Figure 1B**). The size of PDX derived tumor tissue available for downstream applications is significantly increased (1.11cm ± 0.04 cm in diameter) in comparison to 16G human tissue specimen biopsies (0.1cm ± 0.03cm in diameter) that the original engraftment consisted of (**Figure 1C**). In addition to size advantages, the PDX tumor specimens were immediately available for downstream assessments following mouse necropsy, compared to typical delays in the range of hours for the direct from patient human specimens. Previous studies have shown significant changes in tissue electrical properties that occur approximately 1-hour post-harvest (Herman P Schwan, 1956). Thus, this immediate availability is a critical advantage of the PDX model for assessments of irreversible electroporation (IRE). Together, the increased size and quality of PDX specimens allowed for more robust testing of IRE treatment parameters and improved accuracy in electric property modeling.

To evaluate the histopathological features of the P2 tumors generated in the PDX model, specimens were collected at necropsy, fixed, and processed for hematoxylin and eosin (H&E) staining. These specimens were compared to the following: 1) the P0 donor histological reports provided with each PDX mouse; 2) PDAC pathology from reference sources; 3) PDAC pathology comparisons with specimens directly from human patients; and 4) Cell line (Pan02) derived tumor tissue from NSG mouse flank injections. Specimens were evaluated by either a board-certified veterinary (S.C.O.) or human (D.J.G.) pathologist. The analysis revealed that the PDAC tumors, in general, faithfully recapitulated the common histopathologic features and biological complexity of the patient's original tumors and were highly consistent with PDAC pathology. PDX tumors exhibit irregularly round cells that often form prominent ductular structures with lumens containing necrotic debris, sloughed cells, or small amounts of mucinous secretion (**Figure 2A**). Neoplastic cells exhibit significant differences in cell size and shape across the tumor cell population which is consistent with malignancy (**Figure 2A**). Individual tumor cells have abundant eosinophilic cytoplasm (**Figure 2A**). An identifiable but not prominent fibrovascular tumor stroma is also present (**Figure 2A**). All of these features were also readily observed in specimens collected from the original donors (available from the

Mouse Tumor Biology Database) and primary human PDAC tumor samples (**Figure 2B**). However, in the Pan02 tumors, neoplastic cells are elongated and spindle-shaped with no evidence of glandular formation and exhibit fewer cytological criteria of malignancy with uniform size and shape (**Figure 2C**). They form vague streams with minimal fibrous connective tissue stroma. Mitotic figures are prominent but are not as bizarre as in PDX

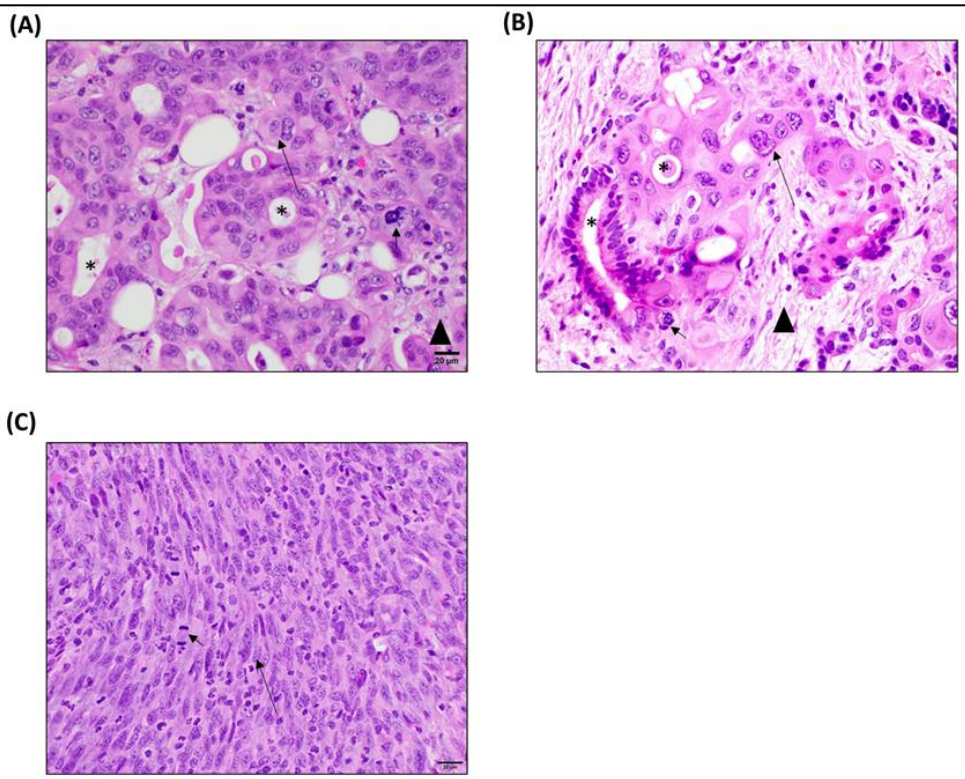


Figure 2. PDX tumors better recapitulate human PDAC histopathology and complexity compared to cell line based Pan02 models. (A) Representative image of PDX pancreatic tumor taken from Patient 1. Tumors from the PDX model exhibit similar characteristics as human patie samples including the formation of ductular structures and a similar tumor cell morphology. The have an identifiable collagenous stroma but not as robust as in patient samples. Mitotic figures are often abnormal. **(B)** Tumor cells from human patients often form ductular structures with lumens. Moderate to large amounts of fibrous connective tissue stroma separates tumo r cells. Individual tumor cells are irregularly round with abundant amounts of eosinophilic cytoplasm ar irregularly round nuclei. Mitotic figures are often abnormal **(C)** Representative image of a Pan02 tumor expanded in a NSG mouse. Tumors derived from PanO2 cells lack the formation of ducts. Tumor cells are arranged in vague streams. They are more spindle in shape with less cytoplasm and elongated nuclei. The tumor stroma is scant. Mitotic figures are of normal morphology. Short arrows indicate mitotic figures, asterisks indicate ductal structures, and long arrows indicate elongated nuclei, and arrowhead indicates tumor stroma tissue. All images are H&E stain and were taken at 40x magnification.

model and human specimens (**Figure 2C**). Individual cells contain significantly less cytoplasm than PDX tumors and are more densely packed (**Figure 2C**). Thus, based on histopathology, these data indicate that the PDX tumors faithfully recapitulate the human

pancreatic tumor microenvironment and are more physiologically accurate compared to cell line derived Pan02 tumor models.

Irreversible Electroporation (IRE) Electric Field Distributions are Consistent Between PDX and Human Tissue Specimens

In an effort to improve computational modeling and refine patient treatment algorithms utilized in IRE treatment, we utilized specimens from the PDX tumors to evaluate and refine electric field distribution simulations. The overwhelming majority of tumor ablation modalities, including IRE, has been developed and modeled utilizing either healthy tissue or specimens collected from cell line derived tumors. Moreover, these collections have been without measuring the effect of IRE itself on tissue conductivity, utilizing a "static" model that does not incorporate dynamic conductivity changes over treatment time. Based on the findings from the histopathology assessments, we hypothesized that PDX tumors would provide a more accurate model system that better recapitulates the electrical properties of patient's tumors both for initial dielectric properties and dynamic changes. To evaluate this hypothesis, PDX tumors from 3 individual patients, and primary human pancreatic tumor tissue from 6 individual patients collected during surgery (**Table 1**)(Beitel-White et al., 2018) were subjected to *ex vivo* IRE applications utilizing parallel-plate electrodes and compared (**Figure 3A)**. Individual tumors from PDX patients were observed to have different raw conductivities, with PDX3 differing significantly from primary human tissues at several electric fields tested (**Figure 3B**). However, when evaluated as a percent change in conductivity by considering the different initial base-line conductivities of the tissue prior to IRE application, these differences in conductivity were reduced between the 3 PDX patients to closer to that of the primary tissues for most electric fields tested, although PDX tumors from patient 1 and 3 exhibited higher raw conductivity overall than PDX patient 2 (**Figure 3B-C**). As expected, only minimal changes in temperature were observed in all tissues at conditions below 1000 V/cm, with Joule heating more severe at higher electric field magnitudes (**Figure 3D-E**).

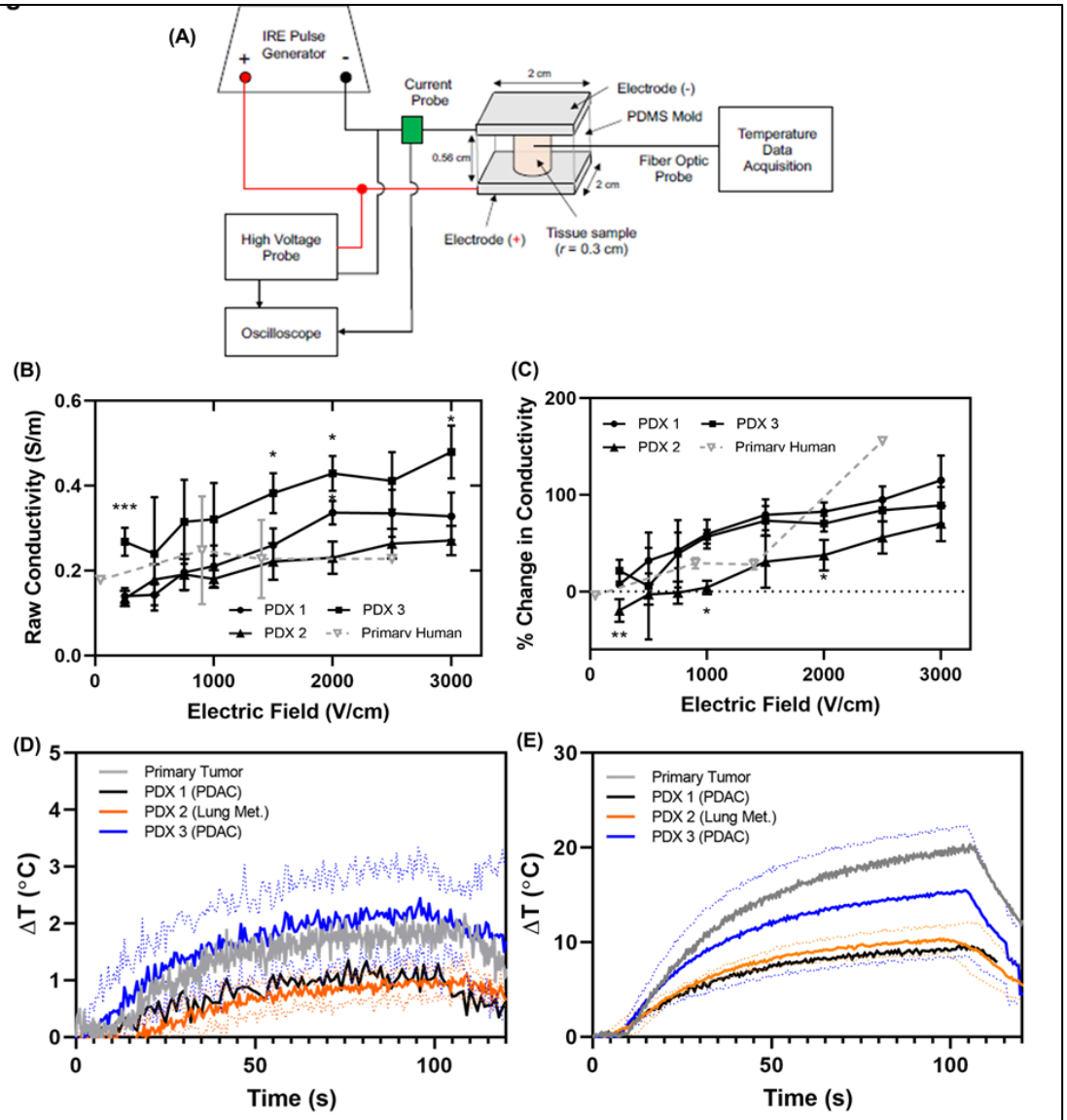


Figure 3. PDX and primary human tissues exhibit similar conductivity and temperature changes during IRE application. (A) Schematic of IRE treatment and tissue characteristic assessment *ex vivo*. **(B)** Raw and **(C)** percent change in conductivity for PDX (n=3-6), and primary human tissues (n=2-3) for each electric field were collected at electric field magnitudes ranging from 0-3000V/cm during IRE application. Conductivity values were calculated based on the average current recorded during the last 5 µs of the first IRE pulse. SEM, 2-way ANOVA, p-value *<0.05, **<0.01 ***<0.001. Change in temperature induced by IRE at **(D)** 1000 V/cm and **(E)** 2500 V/cm were recorded throughout pulse application.

The PDX electrical conductivity data were utilized in COMSOL simulations and modeling to predict ablation sizes and tissue damage contributions. Analysis of IRE with a two monopolar configuration (1.5 cm spacing/exposure, 1750 V/cm, 100 pulses and 100 µs duration) determined that electric field distributions did not significantly differ between the PDX tumors and primary tumor tissues from patients (**Figure 4**). For example, using the PDX tumor from patient 1 (PDX-1) as a representative tumor, there is a large discrepancy in predicted ablation volume and geometry between the static case and

incorporating dynamic conductivity measured from PDX-1 and primary tumor tissue (**Figure 4A**). Thermal damage volumes were obtained by applying the Arrhenius equation as described previously(Garcia et al., 2011). Using an IRE threshold of 500 V/cm and a thermal damage threshold of Ω=1.0, the total ablation areas for static case, PDX-1, and primary tumor consisted of non-thermal IRE volumes accounting for 95.5%, 88.0%, and 94.1% of the total ablation volume (**Figure 4B**). The thermal damage accounted for 4.5%, 12%, and 5.9% of the total ablation volume, respectively (**Figure 4B**). These data have direct translational implications and suggest that further optimization of our treatment parameters (such as lower on-time [90 µs], lower voltage, or thermal mitigation strategies) are possible and could decrease the potential for thermal damage (O'Brien et al., 2019).

IRE Treatment Impacts Critical Hallmarks of Pancreatic Cancer

To complement the electrical property assessments, we also utilized the PDX models to

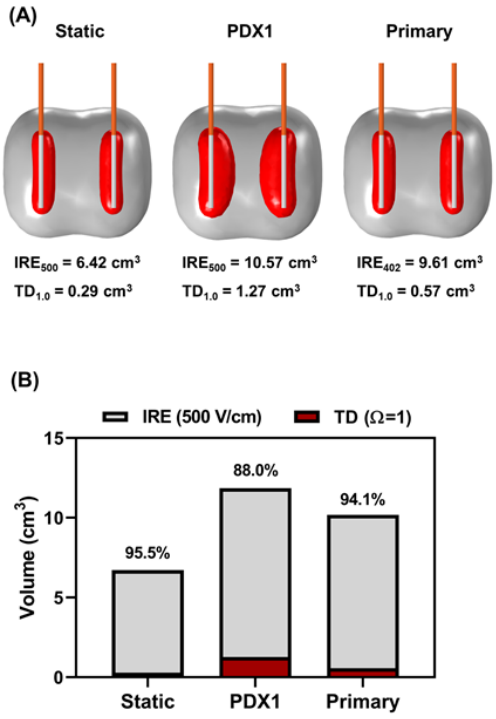


Figure 4. Modeling of PDX and primary human tissues results in similar predicted ablation sizes and damage contributions. (A) COMSOL model depiction of the predicted ablation area at 500 V/cm (grey) and thermal damage area at Ω=1.0 (red) resulting from 100 pulses, 100 µs on time, and 1,750 V/cm voltage to distance ratio modelling clinical IRE. **(B)** Quantification of COMSOL model of predicted contributions of IRE and thermal damage to tumor ablation model.

identify biological signaling networks associated with pancreatic cancer that are impacted by IRE treatment. Gene expression profiling was utilized to identify genes dysregulated by treatment and Ingenuity Pathway Analysis (IPA) utilized these data to predict the biological functions significantly impacted by IRE as previously described (Hazy et al., 2019; Ringel-Scaia et al., 2019; Wasson et al., 2020). We identified 150 individual genes associated with cancer hallmarks and cell death were predominantly up-regulated in untreated PDX tumor specimens (**Figure 5A**). Indeed, we observed similar expression patterns for these genes in all three PDX specimens (**Figure 5A**). Following IRE treatment

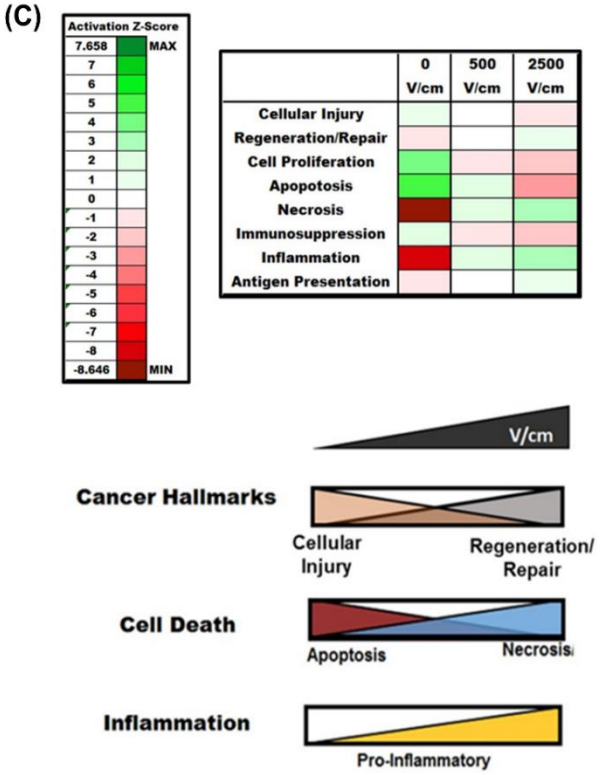
with 500 V/cm, we observed a general downregulation in in these the majority of these genes; however, there was a wide disparity between the individual genes down-regulated per patient. Patient 1 had the greatest number of genes significantly downregulated (80/150; 53%), followed by Patient 3 (45/150; 30%). Patient 2 also had a significant number of genes down-regulated following IRE treatment (29/150; 19.3%). However, these were significantly less compared to the PDX tumors from Patient's 1 and 3. Clustering analysis revealed significant similarities in gene transcription patterns in treated PDX tumors from Patient's 1 and 3, whereas the treated tumors from Patient 2 clustered separately (**Figure 5A**). This observation is potentially due to the tumors from Patient's 1 and 3 being primary PDAC, compared to the metastatic lung PDAC tumor from Patient 2 (**Table 1**). This could indicate differences in the biological responses to IRE between primary and metastatic tumors at lower V/cm. At the higher 2500 V/cm, we observed and even greater down-regulation in individual gene expression associated from all 3 PDX patient specimens. As with the 500 V/cm specimens from Patient 1 and 3 clustered together, while the specimen from Patient 2 clustered separately (**Figure 5A**). However, at the higher V/cm, the differences were due to an increased number of genes significantly down regulated in the PDX tumors from patient 2 (132/150; 88%), compared to the number down regulated from Patient 1 (88/150; 58.7%) and Patient 3 (74/150; 49.3%)(**Figure 5A**).

IPA analysis of the gene expression data identified 8 pathways significantly dysregulated in the pancreatic cancer PDX tumors as well as urine Pan02 tumors following IRE treatment (**Figure 5B**). These pathways were grouped as either canonical pathways, disease and biological function pathways, toxicity pathways, or regulator effects networks and included the following: death receptor signaling; apoptosis signaling; organismal injury, cancer, necrosis, decreased transmembrane potential of mitochondrial membrane, pro-apoptosis, and activation of antigen presenting cells (**Figure 5B**). Many of these pathways agreed between PDX and Pan02 groups with several overlapping, such as necrosis and organismal injury. Several of these pathways were identified as having the largest change in global gene expression from baseline (0 V/cm) to maximum treatment (2500 V/cm) and could be grouped even further into 3 functional categories: cancer hallmarks, cell death, and inflammation (**Figure 5C**). Intriguingly, we identified several counterbalancing trends in gene expression patterns for each category. For cancer hallmarks, cellular injury and regeneration/repair were the dominant functions impacted by treatment. At baseline, there was increased cellular injury signaling in the PDX tumors, which dose dependently declined following IRE treatment **(Figure 5C)**. Conversely, we observed an increase in regeneration and repair signaling with increased IRE dosage (**Figure 5C**). Similar data was observed for cell death where apoptosis signaling was increased at baseline and decreased with higher dosages of IRE; whereas, necrosis

Figure 5

(B)

Top Pathways Affected Post-IRE Treatment	
PDX Tissues	Pan02 Tissues
Canonical Pathways	
Death Receptor Signaling	Bacterial and Viral PRRs
Apoptosis Signaling	Apoptosis Signaling
Disease and Biological Function	
Organismal Injury	Organismal Injury
Cancer	Inflammation
Toxicity Pathways	
Necrosis	Necrosis
Decreased Transmembrane Potential of Mitochondrial Membrane	Increased LDH, ALT, Creatine, and AST
Pro-Apoptosis	Fibrosis
Regulatory Effects Networks	
Activation of Antigen-Presenting Cells	Il1-Beta



signaling was lower at baseline and increased with higher dosages of IRE (**Figure 5C**). Pancreatic tumors are typically immunosuppressive (**Figure 5C)**(Ryan et al., 2014); however, following IRE, we observed a dose-dependent increase in pro-inflammatory inflammation signaling and potential for antigen presentation in the PDX specimens. IPA

Figure 5. IRE induces patient- and dose-dependent gene expression changes in PDX pancreatic tumors. (A) Gene expression arrays were utilized to evaluate changes in the expression of genes associated with cancer and cell death following IRE treatments at 0, 500, and 2500V/cm. A heatmap of the expression data was generated (z-score ranking ±3). n= 3-6 specimens in each group. **(B)** Summary table of dominate biological pathways affected by IRE in pancreatic tumor tissue from human PDX samples and from murine Pan02 samples. **(C)** IRE significantly alters cancer hallmark and immunosuppressive biological pathways in PDX pancreatic tumor models. IPA analysis of affected biological pathways assigned Z-scores based on predicted impact from individual gene expression changes. 0, 500, and 2500V/cm IRE treated tissues were compared and showed significantly increased necrosis, regeneration/repair, and inflammation signaling. Diagram of dose-dependent effect of IRE on biological pathways involved in cancer, cell death, and inflammation.

analysis also identified several general biology signaling pathways that were significantly impacted by IRE treatment, including decreased transmembrane potential of mitochondria (**Figure 5B**). While we did not observe a dose dependent trend in this pathway, its finding is intriguing and is consistent with reduced tumor viability following IRE treatment.

While a diverse range of biological signaling mechanisms can be significantly dysregulated during pancreatic cancer, there are 12 distinct core signaling pathways found dysregulated in 60-100% of human clinical cases(Jones et al., 2008). We specifically evaluated these pathways in the PDX tumors to determine if any were specifically altered by IRE treatment at 2500 V/cm. Our analysis revealed that most of these pathways were unaltered. However, IPA revealed that the changes in gene transcription identified in our core signaling analysis were most likely associated with decreased signaling downstream of EGFR and K-RAS (**Figure 6A**). Specifically, IPA revealed significant decreases in AKT, JAK, NF-κB, VEGF, and STAT1/3 signaling downstream of EGFR (**Figure 6A**). We also identified significant decreases in MEK1/2, JNK, and ERK1/2 signaling downstream of K-RAS (**Figure 6A**). Intriguingly, even though TGF-β signaling is also upstream of most of these signaling mechanisms and one of the core signaling pathways in pancreatic cancer, the gene expression data did not identify a significant impact of IRE treatment on TGF-β signaling (**Figure 6A**). Furthermore, IPA predicted that the downregulation of these specific pathways would result in the reduction of a variety of biological functions critical to pancreatic cancer progression (**Figure 6A**). For example, the reduced EGFR signaling pathways were predicted to result in reduced tissue invasion, tumor growth, tumor metastasis, G0-G1 phase transition, and gene expression (**Figure 6A**). Likewise, the reduced K-RAS signaling is predicted to result in reduced cell proliferation, anti-apoptosis signaling, cell proliferation, tumor metastasis, and G0-G1 phase transition

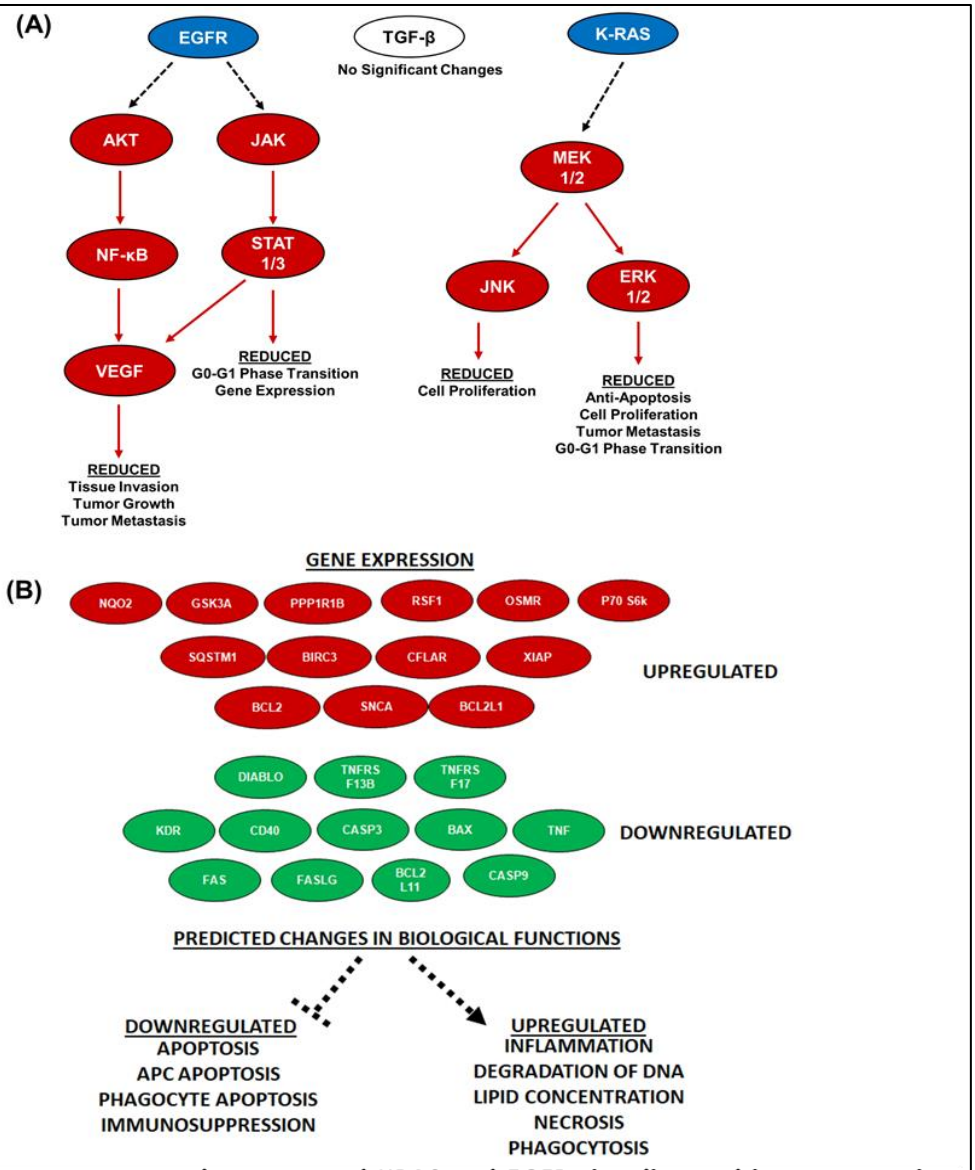


Figure 6. IRE treatment potently attenuated KRAS and EGFR signaling and increases antigen presentation. (A) Specific pancreatic cancer-related pathways were analyzed via IPA and showed significant alternations in gene expression of KRAS and EGFR pathway signaling molecules following IRE treatment at 2500 V/cm. n = 3-6 specimens in each group. **(B)** Antigen presentation pathways are significantly increased in PDX tumors following IRE treatment. Twenty-five genes were identified as being key regulators associated with the increase in antigen presentation signaling following IRE treatment (red is up-regulated; green is down-regulated). These genes are predicted to impact the function of 9 key drivers of antigen presentation and impact the biological functions shown at the bottom of the schematic, all predicted to result in increased antigen presentation.

(**Figure 6A**). The identification of specific pathways altered by IRE treatment provides insight regarding potential biomarkers to monitor for treatment progress or evaluate for treatment failure, patient selection criteria, and combination therapeutic strategies.

Several recent studies have revealed that IRE and other electroporation-based tumor ablation modalities significantly alters the tumor microenvironment and immune

system activation (Goswami et al., 2017; Guo et al., 2018a; Guo et al., 2018b; Beitel-White et al., 2019; Ringel-Scaia et al., 2019; Shao et al., 2019). In general, these studies show that irreversible electroporation results in a shift in the immunosuppressive tumor microenvironment to one that is more pro-inflammatory and anti-tumorigenic. Likewise, several of these studies reveal an increase in the systemic anti-tumor immune response and improved engagement of the adaptive immune system targeting metastatic cells (Guo et al., 2018b; Ringel-Scaia et al., 2019). However, these findings have yet to be applied to pancreatic cancer. Our IPA analysis revealed that IRE treatment induced gene expression patterns that were consistent with an up-regulation of antigen presentation in the PDX tumor specimens (**Figure 5**). This increase in antigen presentation was due to the significant up-regulation of 13 genes and down-regulation of 12 genes identified or predicted by the IPA analysis to be associated with this biological function (**Figure 6B**). Globally, the changes identified in these genes are predicted to significantly down-regulate apoptosis, antigen presenting cell apoptosis, phagocyte apoptosis, and immunosuppression (**Figure 6B**). Conversely, the changes in these genes are predicted to increase inflammation, degradation of DNA, lipid concentration, necrosis, and phagocytosis (**Figure 6B**). It is important to note that the PDX model is devoid of most immune system components. Thus, the changes shown reflect the direct effects of IRE on the tumor cells, without the confounding effects of the immune system.

DISCUSSION

Tumor specimens from human patients have the most clinical and physiological relevance for modeling tumor ablation technologies. However, the lack of high-quality and low-quantity patient tissue for robust modeling has impacted the field. Other methods, such as cell lines (i.e. immortalized cells maintained as monoculture colonies), are useful for understanding basic mechanistic insight related to cancer biology and mechanisms of tumor ablation. However, prolonged propagation and maintenance of these lines has led to the loss of many of the biological characteristics associated with the original tumor. Their behavior following treatment often does not recapitulate the responses observed in the patient tumor. Even cell line co-culture and organoid models have significant limitations. To circumvent this for IRE ablation, we utilized PDX derived tumors that faithfully recapitulates the morphological features of the patient's pancreatic cancer and is highly effective in generating abundant volumes of tumor specimens for electrical property and ablation modeling. Histopathological analysis shows similar morphological features in the PDX model tumors as compared to donor tissue that are of higher complexity than immortalized cell line-generated tumors. In terms of electrical conductivity, the PDX tumor data was fairly consistent with the available data from the primary human patient specimens, allowing for the development of a predictable model for clinical application. Thermal response differences in the PDX models were also minimal and may be due to tissue necrosis that has been known to occur in patient tumor tissue (Mitsunaga et al., 2005). This implies that PDX tissues are effective surrogates for human primary tissues in terms of electrical and thermal responses to electroporation pulses and potentially other targeted ablation modalities.

The PDX tumors evaluated in the current study included 2 primary PDAC specimens from the pancreas and 1 metastatic PDAC specimen from the lung. This allowed us to robustly evaluate 8 different electric fields (ranging from 0 – 3000V/cm) using 3-6

specimens for each parameter and patient. The resulting data were then utilized to improve the accuracy of our predicted ablation area and thermal damage area modeling. Together, these data will ultimately be incorporated into the IRE treatment planning algorithms to improve patient treatment outcomes in the clinic. While we did observe differences in the raw conductivity and change in temperature between patients, the percent changes were not statistically different between patient samples or tumor origin for most electric field magnitudes. We originally hypothesized that individual differences in the tumor microenvironment, genetic differences between patients, or differences in tissue derived from metastatic sites compared to primary tumors may have different physiological or electrical properties that could impact IRE treatment. However, based on the findings here for the specimens and treatment parameters evaluated, we did not observe any significant differences between individual patient's PDX tumors.

Gene expression analysis on the treated PDX tissues gives insight to the mechanism behind IRE's ablative ability. The biological pathways and functions between the 3 different PDX models were consistent prior to treatment and, in general, had similar changes post-treatment. Our analysis revealed a strong shift from apoptosis to necrosis following treatment, which is consistent with previous findings in pre-clinical mouse studies (Ringel-Scaia et al., 2019). As pancreatic tumors are typically classified as "cold" or non-immunogenic, a more pro-inflammatory type of cell death could lead to tumor microenvironmental changes that make it susceptible to co-immunotherapy options, such as checkpoint inhibitors (Zhao et al., 2019). Clinically, pancreatic cancer has had a lack of response to most individually-applied immunotherapeutics (Zhang et al., 2018). IRE may improve immunotherapy efficacy as it triggers a shift from an inherently immunosuppressive microenvironment to one that is more pro-inflammatory and subsequently anti-tumor (Ringel-Scaia et al., 2019; Zhao et al., 2019). Lastly, the impact of IRE on downstream KRAS and EGFR signaling could prove vital for determining treatment strategies. These pathways are commonly dysregulated in pancreatic cancer patients (Thomas et al., 2007; Ryan et al., 2014; Awasthi et al., 2017). Our data indicates that these pathways are highly down-regulated following IRE. This downregulation can alter several relevant biological functions for cancer biology, including proliferation, cell death, invasion, and metastasis. Interestingly, we did not observe significant changes in other pathways commonly associated with pancreatic cancer, such as TGF-β signaling, which is highly involved in pancreatic cancer pathophysiology (Shen et al., 2017). Thus, it is tempting to speculate that components of the KRAS and EGFR signaling pathway may prove to be therapeutic targets post-IRE or effective biomarkers to gauge treatment efficacy. Likewise, these data may suggest that pancreatic cancer patients with underlying mutations in genes associated with TGF-β signaling may have reduced responses to IRE based therapeutic strategies.

While PDX models have become an essential tool in drug discovery applications, these models have yet to be widely adopted in biomedical engineering and device development. The data presented here supports their further incorporation in these fields and demonstrates their utility in expanding malignant tissues that retain morphological and clinically relevant properties. Their use allows for the robust investigation of potential treatment associated biomarkers and co-therapy options. In the context of IRE, increased use of PDX models are anticipated to improve our understanding of tissue electrical properties in both primary and metastatic tumors and these data will improve ablation zone

predictions, ultimately leading to more precise and predictable clinical applications in the pancreas.

CONFLICTS OF INTEREST

IA, ML, NW, and RD are inventors on pending and issued patents related to the work. NM is employed by AngioDynamics. The work described in this manuscript was supported, in part, through sponsored research funding provided by AngioDynamics adhering to institutional conflict of interest guidelines. Authors declare no additional conflicts of interest.

AUTHOR CONTRIBUTIONS

IA, RD, and NM contributed to the concept and design of the study; RB and VR monitored animals and collected animal tissues. NB performed *ex vivo* IRE and tissue property assessments. SC and DG assessed histology and provided photomicrographs. ML provided COMSOL modelling. RB performed tissue processing and RNA evaluation. IA analysed IPA results. NB, ML, SC, and IA wrote sections of the manuscript. RB wrote the original draft of the manuscript and final formatting.

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DATA AVAILABILITY STATEMENT

Data that supports the findings in this paper is available upon reasonable request from IA.

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Supplemental Figure S1: Example List of Genes and Groupings Utilized for Expression Profiling and Pathways Analysis.

Angiogenesis:
ANGPT1, ANGPT2, CCL2 (MCP-1), FGF2 (BFGF), FLT1 (VEGFR1), KDR (VEGFR3), PGF, SERPINF1, TEK (TIE-2, TIE2), VEGFC.

Cell Death (general):
APAF1, BCL2L11, BIRC3 (c-IAP2), CASP2, CASP7, CASP9, CFLAR (Casper), FASLG (TNFSF6), NOL3, XIAP (BIRC4).

Pro-Apoptotic: ABL1, APAF1, ATP6V1G2, BAX, BCL2L11, BIRC2 (c-IAP1), CASP1 (ICE), CASP3, CASP6, CASP7, CASP9, CD40 (TNFRSF5), CD40LG (TNFSF5), CFLAR (Casper), CYLD, DFFA, FAS (TNFRSF6), FASLG (TNFSF6), GADD45A, NOL3, SPATA2, SYCP2, TNF, TNFRSF1A (TNFR1), TNFRSF10A (TRAIL-R), TP53 (p53).

Anti-Apoptotic: AKT1, BCL2, BCL2A1 (BFL1), BCL2L1 (BCLXL), BIRC3 (c-IAP2), CASP2, IGF1R, MCL1, TNFRSF11B (OPG), TRAF2, XIAP (BIRC4).

Autophagy: AKT1, APP, ATG12, ATG16L1, ATG3, ATG5, ATG7, BAX, BCL2, BCL2L1 (BCLXL), BECN1, CASP3, CTSB, CTSS, ESR1 (ERa), FAS (TNFRSF6), GAA, HTT, IFNG, IGF1, INS, IRGM, MAP1LC3A, MAPK8 (JNK1), NFKB1, PIK3C3 (Vps34), RPS6KB1, SNCA, SQSTM1, TNF, TP53 (p53), ULK1.

Necrosis: ATP6V1G2, BMF, C1orf159, CCDC103, COMMD4, CYLD, DEFB1, DENND4A, DPYSL4, EIF5B, FOXI1, GALNT5, GRB2, HSPBAP1, JPH3, KCNIP1, MAG, OR10J3, PARP1 (ADPRT1), PARP2, PVR, RAB25, S100A7A, SPATA2, SYCP2, TMEM57, TNFRSF1A (TNFR1), TXNL4B.

Epithelial-to-Mesenchymal Transition (EMT): CDH2 (N-Cadherin), DSP, FOXC2, GSC, KRT14, OCLN, SNAI1 (SNAIL), SNAI2, SNAI3, SOX10.

Hypoxia Signaling: ADM, ARNT, CA9, EPO, HMOX1, LDHA, SLC2A1.

Metabolism: ACLY, ACSL4, ATP5A1, COX5A, CPT2, G6PD, GPD2, LPL, PFKL, UQCRFS1.

Telomeres & Telomerase: DKC1, PINX1, TEP1, TERF1, TERF2IP, TINF2, TNKS (TIN1), TNKS2.

CHAPTER FIVE
Irreversible electroporation induces a pro-inflammatory tumor microenvironment that allows for antigen presentation in pancreatic cancer

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ABSTRACT

Pancreatic cancer is often a late-diagnosed disease with few treatment options and extremely low survival rates, making it the third leading cause of cancer-related deaths in the US each year. Its localization near critical structures limits surgical candidacy to only 15% of patients and a strong immunosuppressive barrier has shown staunch resistance to new immunotherapeutics. Irreversible electroporation (IRE), a novel form of tumor non-thermal ablation via electric pulse application to disrupt tumor cell homeostasis and initiate cell death, has had strong success in clinical trials for pancreatic cancer patients. Avoiding critical structures, the application of IRE in late-stage patients has been able to increase survival substantially compared to standard of care and other ablation modalities with low to moderate adverse effects. However, little is known on the effect of IRE beyond the initiation of cell death in the tumor tissue. We hypothesize that IRE can induce a pro-inflammatory type of cell death in pancreatic cancer that can promote inflammation and anti-tumor immune system responses. We utilized *in vitro, ex vivo,* and immunocompetent *in vivo* murine models to investigate changes to pancreatic cancer and the tumor microenvironment. Our findings show programmed necrotic cell death, increased pro-inflammatory cytokine production, and shifts in immune cell populations from a pro-tumor (such as MDSCs and T regulatory cells) to a proinflammatory composition containing cytotoxic lymphocytes and neutrophils after IRE application. Progression-free survival was significantly extended for treated mice. However, by 14 days these populations skew towards pretreatment composition and tumor recurrence occurs. Interestingly, IRE increases Ifn-γ signaling and produces viable antigens for presentation to the adaptive immune system but also increases PDL-1 expression. Our findings propose potential therapeutic targets for use in combination with IRE, such as gemcitabine or PD-1/PD-L1 inhibitors, to improve patient survival.

INTRODUCTION

Pancreatic cancer has historically been challenging to treat with a survival rate of less than 8% that has remained relatively unchanged in the last 50 years. The localization of the primary tumor in a complex area next to or even around critical structures such as the mesenteric artery leaves 85% of patients unable to undergo surgical resection(Ansari et al., 2015). Furthermore, the metastatic rate of pancreatic cancer leads to increased rates of organ failure and reoccurrence for patients even at a diagnosis of Grade I disease(Ansari et al., 2015). Other treatment options include chemotherapy, which normally extend life expectancy by just several weeks, or radiation therapy, which has shown no improvement to life expectancy(Ryan et al., 2014). Immunotherapies have also proven fruit-less as pancreatic cancer is known to be highly immunosuppressive, blocking proinflammatory immune cells from penetrating the established tumor area(Zhang et al., 2018).

Electroporation-based technologies, notably irreversible electroporation (IRE), have made strides to overcome the barriers of pancreatic tumor treatment. IRE is a generally non-thermal application of short, high voltage monophasic pulses that disrupts cell membranes and induces cell death(Weaver and Chizmadzhev, 1996; Davalos et al., 2005). The induction of cell death in the tumor has be shown to reduce tumor burden and increase progression-free survival in clinical trials(Belfiore et al., 2015; Martin et al., 2015; Scheffer et al., 2016). However, this increased survival can be limited in some patient populations and investigations into cotherapy options have begun to determine how best to improve patient outcomes(Bhutiani et al., 2016; Leen et al., 2018; Scheffer et al., 2019). Studies into the biological and immunological effects of IRE on pancreatic malignancies have been limited but findings in several groups hint that treatment of cancers with IRE could stimulate a necrosis-like programmed cell death that may initiate an anti-tumor immune response(Scheffer et al., 2019; Shao et al., 2019; Zhao et al., 2019).

We hypothesize that IRE induces pro-inflammatory cell death in pancreatic tumors that can alter the tumor microenvironment, increase anti-tumor immune cell populations, and allow for the generation of antigens that can be utilized by the adaptive immune system to reduce disease burden. We utilized *in vitro*, *ex vivo*, and *in vivo* models of pancreatic cancer along with primary immune cells to study cell death induction, tumor microenvironment changes, and immunomodulatory potential of IRE on pancreatic cancer. These findings will identify potential co-therapy options for IRE that may increase pancreatic cancer patient survival.

METHODS AND MATERIALS

2.1 In Vitro IRE Treatments

Murine Pan02 cells (Jackson Labs) were cultured in RPMI 1640 (ATCC) with 10%FBS and 1x Penicillin/Streptomycin. Pan02s were suspended in low-conductivity dielectrophoresis (DEP) buffer(Ringel-Scaia et al., 2019) at 6x10^6 cells/mL and treated with IRE in 4mm electroporation cuvettes (Fisher Scientific) at a volume of 500µL in a safety stand (Harvard Apparatus) with an irreversible electroporation generator (Harvard Apparatus) and a fiberoptic temperature probe. Treatment was given in 4 sets of 25 pulses at 100µs duration with 30 second delay between sets. Cells rested for 45min post-treatment

on ice before samples were collected for cell viability via Trypan Blue, then divided among three wells (density of 1x10^6) to dilute DEP buffer 1:4 with complete growth media. Cells were harvested at 8, 24, and 48hr. Supernatant was collected for LDH assessment (Thermofisher Pierce) and ELISA (BD Biosciences) and cell pellets were processed for either RNA in TRIzol (Sigma Aldrich) or protein in a cell lysis buffer collection buffer containing 1x protease inhibitor (Thermofisher), 2% SDS, 10mM Tris HCl, 100mM NaCl in 10mL molecular-grade water. Each timepoint was performed in triplicate for each electric field assessed and was biologically replicated three times for a total n=9 for each group.

2.2 Experimental Animals

All experiments were conducted under institutional IACUC approval and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. 8-12-week age-matched male and female C57Bl6 mice (Jackson Laboratory) were injected subcutaneously with 6x10^6 Pan02 cells suspended in Matrigel (Corning) in the right flank. Mice were monitored for health and tumor size three times a week or more often based on health condition. Tumor size was assessed by Vernier calipers as the square root of the product of two perpendicular diameter measurements as has been performed in previous studies(Ringel-Scaia et al., 2019). Mice were euthanized at experimental timepoints or at end of study once tumors reached 1.6cm in diameter or if considered clinically moribund. Mice were house in SPF conditions with ad libre chow.

2.3 In vivo IRE application

Mice were anesthetized with isoflurane inhalant via nose-cone during treatment. Paired needle electrodes (~21 gauge) were inserted into the tumor in four separate directions sequentially (See **Supplemental Figure 1**) and 2000V/cm electric field was applied with an irreversible electroporation generator (Harvard Apparatus). Application was for four sets of 100µs (25 pulses a set, one set per direction, total of 100 pulses) to ensure tumor coverage and mimic clinical application. Control mice were anesthetized in the same manner with electrodes inserted in similar fashion without an applied electrical current. All animals recovered fully from the inhalant and were monitored for potential health issues or treatment complications.

2.4 Tissue Collection and Processing

Mice were euthanized via carbon dioxide asphyxiation followed by heart blood collection from cardiac puncture or cervical dislocation according to IACUC guidelines. Tumor was excised then sliced longways through the center and shortly perpendicularly to allow for maximum tumor margin coverage for histological assessment. Thoracic cavity was opened, the heart perfused with phosphate-buffered saline, and lungs remove. The lungs were excised and inflated with 10% formalin, and then placed with tumor slices in cassettes in 10% formalin. Remaining tumor and lung tissue were snap frozen on dry ice for later RNA and protein analysis.

2.5 Histopathology

Fixed tissues were embedded in paraffin and sliced and mounted in 5µm sections. Slides were stained with H&E and graded blindly by a board-certified veterinary pathologist (SC) for extent of necrotic tissue in primary tumor and number of metastatic lesions identified per lung.

2.7 Gene expression analysis

Pan02 tumor tissue RNA was isolated via RNeasy (Qiagen) and assessed by Nanodrop 2000 (Thermofisher). Samples were equally pooled in each group for cDNA via RT2 First Strand (Qiagen). Samples were run on RT^2 profiler arrays PAMM-033Z and PAMM-052Z (Qiagen) on ABI 7500 Fast Block. A total of 171 unique genes were assessed. PCR results were analyzed using Qiagen Data Analysis browser service to normalize data and calculate fold regulation based on array housekeepers. Individual sample gene expression was verified with TaqMan primer probes (Thermoscientific) for targeted genes.

2.8 Gene Pathway analysis

Gene expression data from RT2 Profiler arrays were assessed using Ingenuity Pathway Analysis software (IPA, Qiagen). IPA utilizes fold regulation of gene expression and publicly available databases to predict changes to biological pathways. Z-scores are calculated by the IPA software to determine the predicted upregulation or downregulation of a pathway based on the number and strength of gene expression changes found to be involved with the pathway.

2.9 Protein analysis

Blood sera were assessed via enzyme-linked immunoabsorbance assay (ELISA, BD Biosciences) for Ifn-γ and Il-2. Cell pellets from *in vitro* studies and Pan02 tumor specimens were processed after snap freezing with protein lysis buffer . Western blotting was performed for cleaved caspase-3, PARP, and β-actin (Cell Signaling Technologies). Blots were assessed with iBright CL1500 Imaging System and iBright Analysis (Thermofisher) and normalized to β-actin.

2.10 Flow cytometry

Pan02 tumor tissue were harvested, mechanically digested, and cells diluted in complete RPMI 1640 (ATCC). Cells were fixed with fixation buffer (eBiosciences) and stored in 4ºC. For cell surface staining, cells were incubated with anti-CD16/32 (Fc block) in FACS buffer followed by staining for 30min in the dark with desired antibodies. Cells were then permeabilized with True-Nuclear Transcription Factor Buffer Set (Biolegend) following manufacturer's guidelines for use with FoxP3 binding. Cell staining and population identification can be found in **Supplementary Table 1**. Cell populations were identified with BD FACSAria Fusion flow cytometer (BD Biosciences). Downstream analysis was performed with FlowJo.

2.11 Cell Transfection

5x10^6 Pan02 cells were transfected with hemagglutinin (HA)-GFP-FLAG plasmid (Addgene 22612) via reversible electroporation (450V, 10 pulses, 10µg plasmid) and cultured for 10 days under antibiotic selection. Cells were verified by fluorescent microscopy on EVOS M5000 for GFP and Western blot for HA (Cell Signaling Technologies).

2.12 Antigen Presentation

5x10^6 Pan02-HA cells were treated with various electric field magnitudes of IRE for four sets of 25 pulses each with a 30 second set delay to mimic clinical application. Cells were incubated for 24hr and the lysate collected. Bone marrow-derived dendritic cells (DCs) were harvested from C57Bl6 mice and cultured for 8 days with GM-CSF (20ng/mL). DCs were stimulated with rIl-4 (20ng/mL) and exposed to lysate for 24hrs. Cd8+ T-cells were isolated from C57Bl6 spleens using MojoSort mouse Cd8+ negative selection kit (Biolegend). T-cells were co-cultured with DCs for up to 4 days. Cells were collected, fixed, and then stored in PBS until they were assessed. T-cell proliferation was tracked by CFSE (Fisher Scientific) using the BD Accuri C6 Plus flow cytometer (BD Biosciences) on Days 2-4 post-introduction. Downstream analysis was performed with FlowJo.

2.13 Statistical Analysis

Data were analyzed using GraphPad Prism, version 8. A Student's two-tailed t-test was utilized for comparisons between two experimental groups. Multiple comparisons were conducted using one-way and two-way ANOVA where appropriate followed by Mann-Whitney or Tukey post-test for multiple pairwise examinations. Statistical significance was defined as $p \leq 0.05$. All data are represented as the mean ±SEM.

RESULTS

3.1 IRE induces proinflammatory cell death in vitro

Pan02 murine pancreatic cancer cells were tested *in vitro* with IRE and incubated for various timepoints to determine cell death and cytokine production (**Figure 1**). It should be noted that the conductivity of the solution differs from that of a tissue and can lead to higher heat generation than would be seen *in situ*. The morphology of cells in suspension versus in a tissue may also increase the lethal threshold of the cells(Arena et al., 2012). Experiments were done in suspension to limit the effects of processing that would be required to obtain RNA and protein samples from hydrogel models, similar to those done in our previous studies(Goswami et al., 2017; Ringel-Scaia et al., 2019). Long-term culture in DEP buffer solutions can lead to cell toxicity and requires the removal or dilution of the media for studies requiring more than 6 hours(Khoshmanesh et al., 2011). Centrifugation was avoided to limit unnecessary mechanical forces on recovering cells. Instead, cell solutions were divided among wells and diluted with growth media to non-toxic levels of DEP suspension solution based on preliminary toxicity tests (not shown). Treatment *in vitro* showed little to no increase in temperature throughout application until reaching

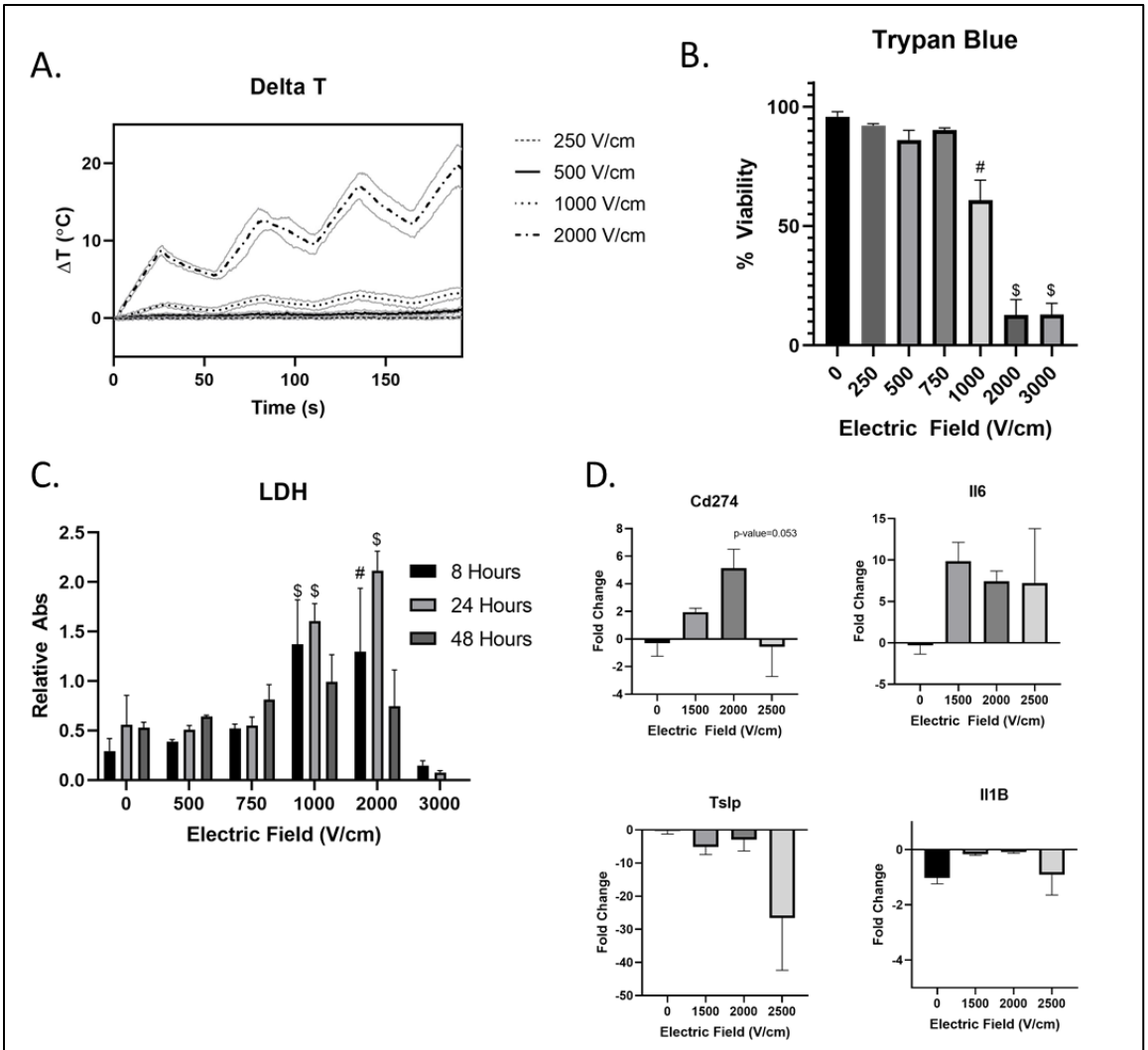

Figure 1: IRE induces programed pro-inflammatory cell death *in vitro*. Murine pancreatic Pan02 cells were treated in suspension with IRE at various electric field strengths. A) Temperature was measured via fiberoptic probe throughout treatment. n=11, SD shown. B) Trypan Blue exclusion was used to measure acute cell death after IRE application. C) LDH release into the supernatant was measured at various timepoints as an indicator of cell death over time. n=9, combination of three biological replicates, SEM shown. P-value $^{\#}$<0.001, $^{\$}$<0.0001 D) RT-PCR was performed for proinflammatory (Il6, Il1-β) and anti-inflammatory markers (Tslp, Pdl1). N=3, SEM shown.

2000V/cm, the extreme end of clinical application in amperage (**Figure 1A**). As electric field increased, acute cell viability decreased (**Figure 1B).** LDH levels increased over time after IRE application, indicating an increase in cell death as cells that were electroporated underwent extended programmed cell death (**Figure 1C**) rather than a simple instantaneous cell death from treatment until 3000V/cm, where cell death would be caused by thermal damage rather than electroporation (**Figure 1C**). RNA expression of cells 24 hours post-IRE treatment showed elevated levels of Il6, unchanged levels of Il1β, a potential decrease in Tslp, and an increase in Cd274 (F**igure 1D**).

3.2 IRE induces proinflammatory cell death and limits disease progression in vivo

The engraftment of a sub-dose ($1.2x10^6$ cells) of murine Pan02 cells in wildtype C57Bl6 (WT) and NOD-scid-gamma (NSG) showed a significant increase in the progression of tumor growth in the immunocompromised animals (**Supplementary Figure 2**). Metastatic lesions also developed more quickly in the NSG mice (**Supplementary Figure 2)**. This indicates that the involvement of a competent immune system is necessary to limit tumor growth and metastatic lesion formation.

To study pancreatic cancer in an immunocompetent mouse model, murine Pan02 cells were injected subcutaneously into wildtype C57Bl/6 mice at full-dose and allowed to grow for 7-14 days or until tumors reached ~0.5cm in diameter. The subcutaneous placement allowed for simple monitoring of the tumor size and condition and easy IRE application with inhalant isoflurane rather than extensive surgery, paralytics, and analgesics. IRE application mimicked clinical application by being performed with similar

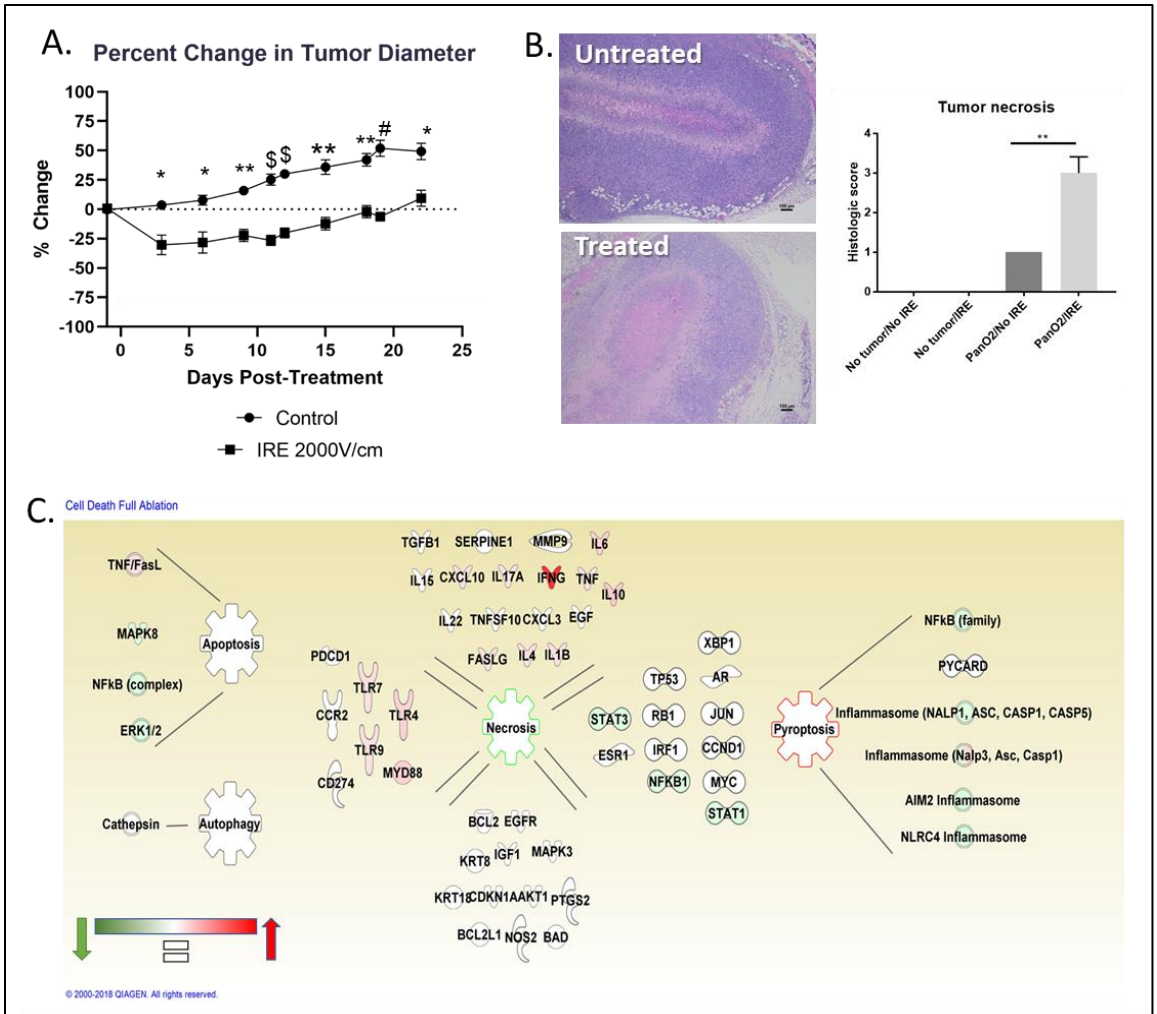

Figure 2: IRE ablates pancreatic tumors and induces proinflammatory programmed cell death *in vivo.* PanO2 cells were injected subcutaneously in immunocompetent mice and treated with IRE once they reach 0.5cm in size. Tumor tissue was evaluated for tissue necrosis and cell death 24hrs post-treatment. A) Tumor diameter post-treatment showed a significant decrease of around 30% post-IRE treatment and a 12-day delay in tumor growth compared to untreated control tumors. B) H&E staining of tumors and grading of necrosis (1=low-grade necrosis, 4= few to no detectable cancer cells). n=5, SEM shown. p-value *<0.05, **<0.01, $^{\#}$<0.001, $^{\$}$<0.0001 C) Gene expression evaluated by IPA predicts necrosis cell-death patterns rather than apoptosis.

parameters in altering pulse sets to ensure coverage of the three dimensional shape and a 30 second delay between sets to reduce Joule heating. IRE significantly reduced tumor size and progression in the model, increasing areas of necrosis in the tumors (**Figure 2A-B**). Gene expression analysis for cancer and innate and adaptive immunity pathways showed an increase in necrosis signaling and proinflammatory cytokines and a reduction in apoptotic signaling within 24hrs of treatment (**Figure 2C**).

IRE-treated mice displayed a doubling in progression-free survival, where progression was considered 20% above treatment size (**Figure 3A**). Histological

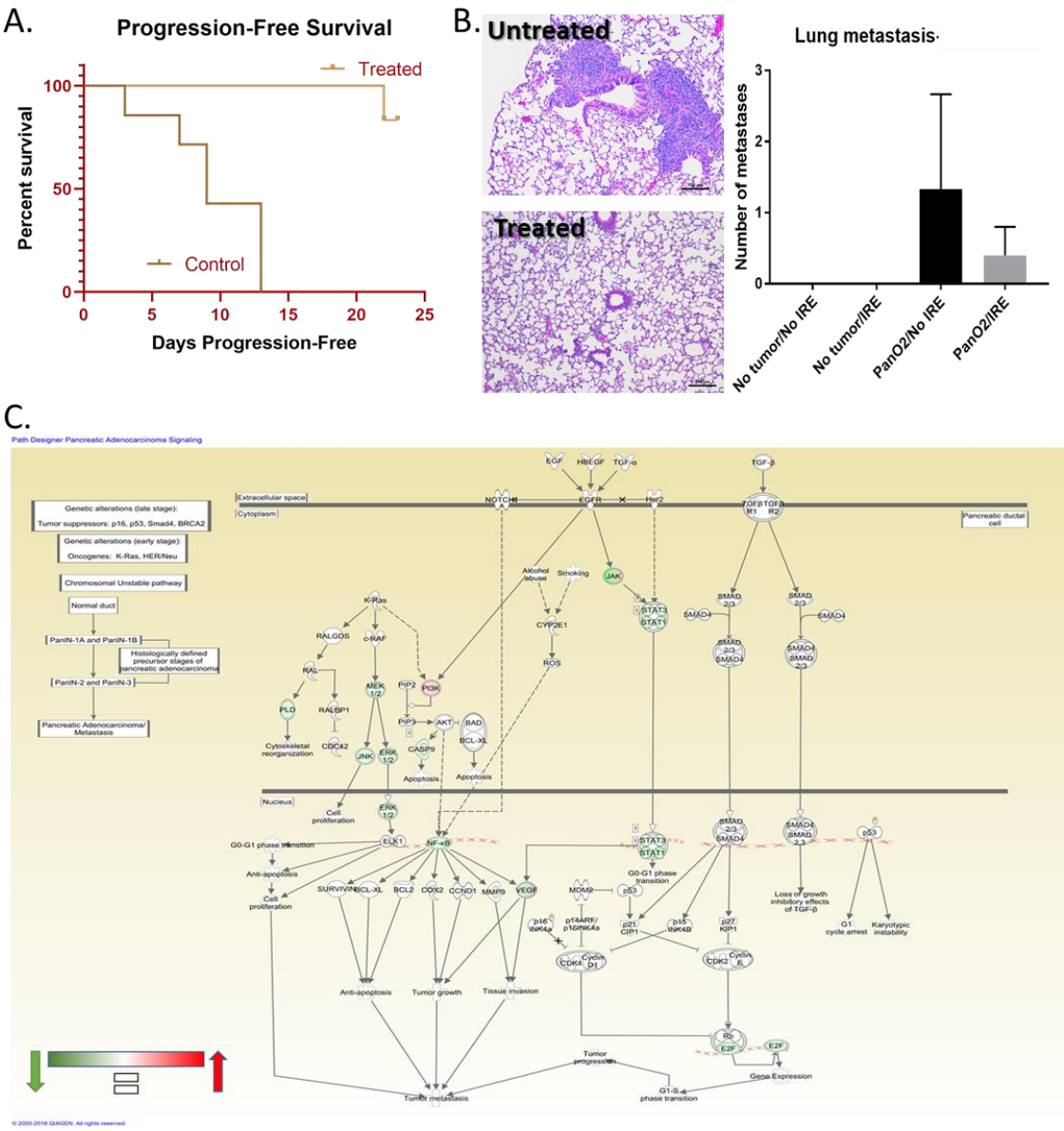

Figure 3: IRE increases progression-free survival and potential decreases metastatic burden and pancreatic cancer hallmarks. A) IRE-treated mice experienced significantly extended progression-free survival. n=7, p-value=0.0014 . B) H&E staining and counting of lung metastases by end of study (Day 40, 26 days post-IRE treatment). n=5. C) Gene expression evaluated by IPA to predict affected cancer biology pathways shows a decrease in proliferation, metastasis, and chromosomal instability.

assessment of the metastatic burden showed a potential but not significant reduction in metastatic lesion number in treated mice (**Figure 3B**). To investigate the potential of IRE in reducing disease burden by altering the biology of the tumor itself, tumor tissue was collected at multiple timepoints and genetic expression analysis via RT2 Profiler arrays was performed. Treated mice showed an increase in proinflammatory signaling and chromosomal stability regulation with a decrease in tumor metastatic potential and proliferation pathways (**Figure 3C**).

3.3 Immune cell populations are temporally altered post-IRE treatment

As pancreatic tumors are known for encouraging immunosuppressive cancer subtypes such as myeloid-derived suppressor cells (MDSCs)(Bayne et al., 2012; Rossi et al., 2014), we decided to investigate the immune cell populations located in established pancreatic tumors and their dynamics post-IRE treatment. Pan02 tumors from treated mice were digested at different timepoints and stained for flow cytometry analysis (**Supplemental**

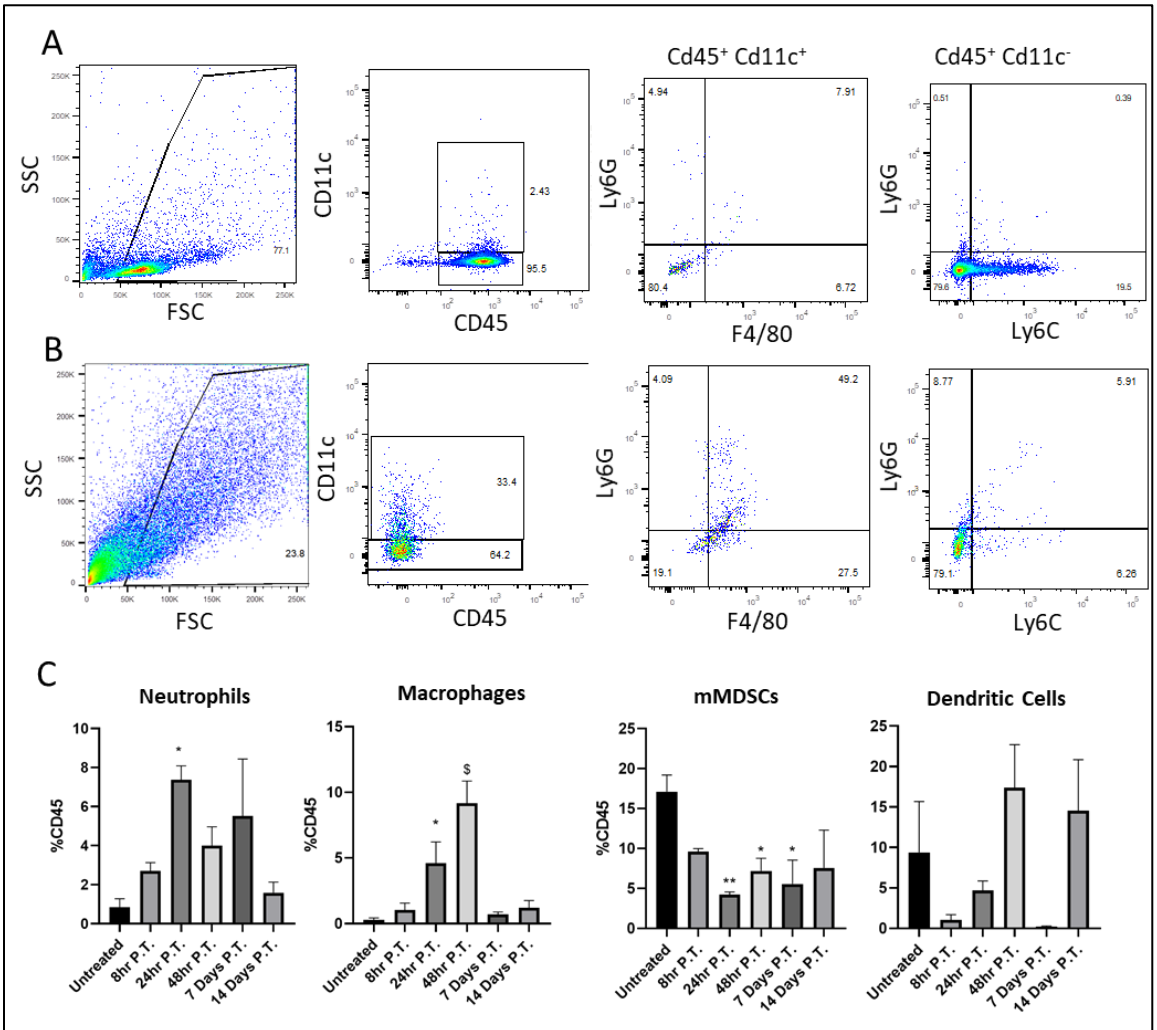

Figure 4: Innate immune cells shift over time post-treatment indicate an acute response. Tumors were collected at different points post pre-treatment and assessed by flow cytometry for innate immune cell composition. Examples of untreated (A) and treated with IRE (B) flow plots 24hr after treatment are shown. C) Summary of innate immune cell compositions over time. Analyzed by one-way ANOVA, n=4 animals for each collection point, SEM. p-value=* ≤0.05, **<0.01, #<0.001, $<0.0001.

Table 1, Figures 4 and 5). Examples of panel gating are shown for untreated (**Figure 4A, 5A**) and IRE-treated (**Figure 4B, 5B**) tumors. IRE treatment altered local tumor immune cell populations temporally, decreasing MDSCs acutely after treatment and increasing recruitment of macrophages to the area (**Figure 4C**). Over time, neutrophil levels also increased (**Figure 4C**). Damage caused by IRE was also able to acutely recruit cytotoxic CD8+ and CD4+CD8+ (Double-positive) T cells to the tumor area, while T regulatory cell populations decreased (**Figure 5C**). However, many of these changes appear to be temporal as several of these populations, such as macrophages and CD8+ T cells, returned to pre-treatment levels by Day 14. **(Figure 4C, 5C)**.

3.4 IRE produces viable antigens for adaptive immune system activation

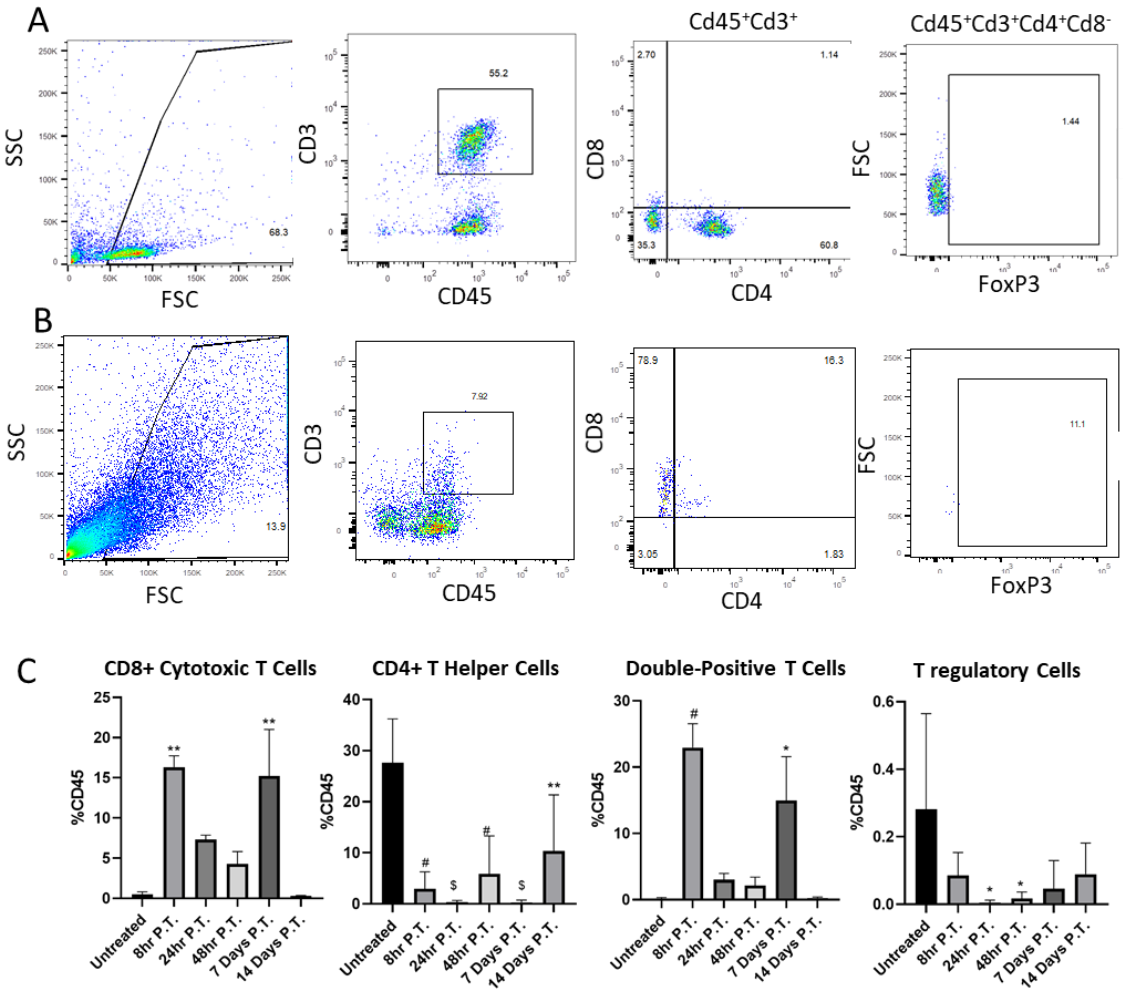

Figure 5: T-cells shift over time post-treatment indicated an acute response but limited long-term effects. Tumors were collected at different points post pre-treatment and assessed by flow cytometry for adaptive immune cell composition. Examples of untreated (A) and treated with IRE (B) flow plots 24hr after treatment are shown. C) Summary of local adaptive immune cell compositions over time. Analyzed by one-way ANOVA, n=4 animals for each collection point, SEM. p-value= *≤0.05, **<0.01, #<0.001, $<0.0001..

After confirming that pro-inflammatory immune cell recruitment occurs acutely after treatment, we next assessed the potential of IRE to produce viable antigens without destruction of the proteins, something that would normally be limited in pancreatic cancer by apoptotic cell death but increased in inflammatory cell death such as programmed necrosis. Pan02 cells were transfected with a plasmid by electroporation to express hemagglutinin (HA) (**Figure 6A**). This allowed for a known antigen that could be assessed throughout the study and compared to a recombinant HA protein as a positive control. These Pan02-HA cells were treated *in vitro* with IRE at various electric field strengths and plated for 24hrs. Supernatants were collected and cultured with stimulated primary murine dendritic cells. Primary murine naïve CD8+ T-cells were isolated from C57Bl6 mouse spleens, dyed with CFSE to track proliferation, and co-cultured with the dendritic cells. T-cells were collected 2-4 days after introduction for flow cytometry assessment. CFSE intensity determined T-cell proliferation and, therefore, antigen presentation strength. Cells treated at 1000V/cm and 2000V/cm electric fields of IRE showed a clear diluted signal indicative of cell proliferation and a decaying signal as the dyed cells became too light for the range of the flow cytometry over time (**Figure 6 B**). Ifn-γ protein expression showed increases at 1000V/cm but, interestingly, not significant increase at 2000V/cm despite the increases cell populations in diluted CFSE populations (**Figure 6C**). Il2 overall did not show a strong expression among our different treatment groups(**Figure 6C**).

3.5 *Increase in inflammation also increase anti-immune markers in pancreatic cancer*

While viable antigen was shown to be produced by IRE, the recurrence of tumor growth and reversion of local immune cell populations at later time points turned our investigations towards potential inhibitors of the inflammatory response stimulated by IRE. Tumor samples were processed for individual PCR analysis to further assess Ifn-γ, a cytokine strongly increased in the profiler array analysis, and Pdl-1, which showed an increase in the *in vitro* study (**Figure 1E**). The significant increase in Ifn-γ was confirmed at 24 hours post-IRE application (**Figure 5C**,**7A**). Blood sera also showed an increase in Ifn-γ over time. There was also an increase in PDL-1 expression 24 hours post-IRE treatment that could indicate a survival mechanism in response to damages and inflammation in the tumor area (**Figure 7B**). This gene expression decreases over time *in vivo*.

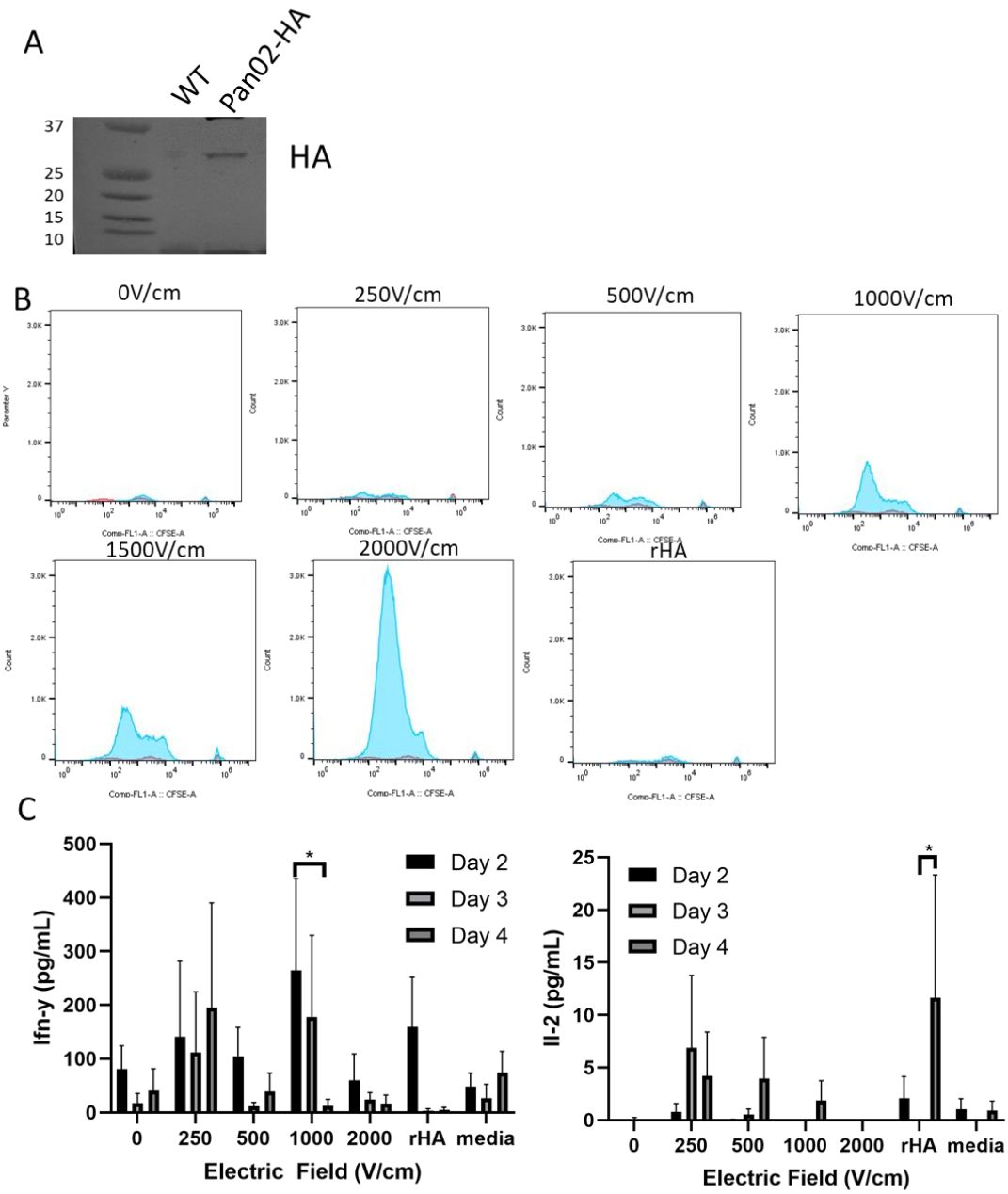

Figure 6: IRE produces viable antigens for antigen presentation *in vitro*. A) Pan02 cells were transfected with HA-GFP-FLAG plasmid for a traceable HA antigen. B) Pan02-HA cell supernatant from 24hr post-IRE treatment *in vitro* stimulated T-cell proliferation at different electric field magnitudes. Maximum CFSE expression peak overlayed in orange. C) T-cells stimulated by IRE produced Ifn-γ and Il-2 during proliferation detected by ELISA. n=3, SEM, p-value*≤0.05.

DISCUSSION

Deciphering the mechanism of immune response to IRE in pancreatic cancer begins with determining the types of cell death IRE is able to induce. IRE has a history of inducing apoptosis to necrosis-like cell death(Piñero et al., 1997; Hofmann et al., 1999; Tekle et al., 2008; Faroja et al., 2013; Mercadal et al., 2020). Our findings indicate that IRE induces

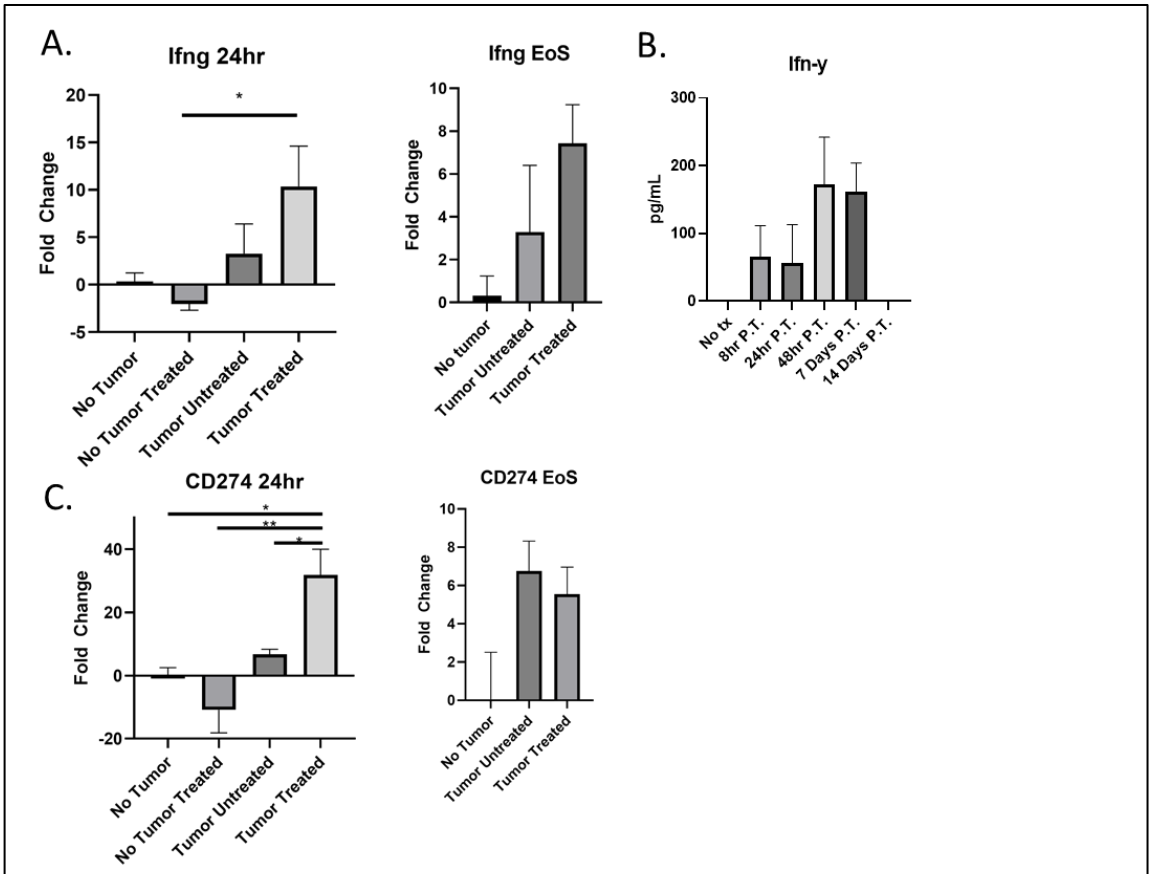

Figure 7: IRE stimulated Ifn-γ and Pdl-1 in vivo. A) RT-PCR of Ifn-γ shows increase at 24hr post-IRE and continued high expression by end of study (25 days post-IRE). n=5, SEM, p-value= *≤0.05. B) Blood sera also displays increase in Ifn-γ protein expression over time. n=4, SEM. C) RT-PCR of Cd274 shows increased gene expression 24hr post-IRE but attenuated expression by end of study. n=5, SEM, p-value= *≤0.05, **<0.01.

necroptotic-like cell death in murine pancreatic cancer. The induction of such a cell death mechanism allows for a prolonged cell death process evident in the release over time of LDH (**Figure 1C**) that can also produce pro-inflammatory cytokines (**Figure 1E, 2C**) to remodel the composition of immune cell populations at the treatment area.

Further effects on the biology of the remaining tumor cells by irreversible electroporation showed a decrease in certain cancer hallmarks such as cell proliferation, metastatic potential, and chromosomal instability (**Figure 3C**). These delays may explain the effectiveness of IRE in clinic as the cancer become less malignant until it has recovered from the treatment, extending progression-free survival. It also indicates key timing for combination therapy application or repeated IRE treatment that may extend a patient's survival. Furthermore, changes in these pathways may allow for targeted combination therapies that could take advantage of IRE's permeabilization ability. Investigations into combination therapies of IRE with chemotherapeutics such as gemcitabine, a standard of care for many pancreatic adenocarcinoma patients, are currently underway. Combination may allow for better chemotherapeutic penetration into the tumor mass and the tumor cells themselves that could have a synergistic effect to improve patient survival(Belfiore et al.,

2015; Bhutiani et al., 2016). Indeed, electrochemotherapy with reversible electroporation has already shown efficacy in advanced melanoma and head and neck cancer(Belehradek et al., 1993; Heller et al., 1996). However, the choice of chemotherapy may also effect patient outcomes; while chemotherapies such as FOLFIRINOX may halt tumor progression, long-term use in stable-disease patients had little survival difference in receiving IRE compared to gemcitabine combination treatments(Belfiore et al., 2015; Vogel et al., 2019). This may be due to FOLFIRINOX's high toxicity that may make the patient's body less able to take advantage of IRE's opening of the tumor area while gemcitabine, known for its immunostimulatory abilities, has less toxic effects but is not as strong at halting tumor progression when administered alone(Nowak et al., 2003; Conroy et al., 2011; Pei et al., 2014).

Our studies indicate a sharp decline in MDSCs and T regulatory cells responsible for the immunosuppressive barrier known in pancreatic tumors (**Figure 4C, 5C**). These cells may be decreasing due to the semi-specificity of IRE in cell death induction as they would be concentrated at the site of treatment and continue to decrease in number over time similarly to the increased cell death seen in Pan02 cells *in vitro* (**Figure 1C**). Other immune cell populations such as neutrophils and cytotoxic lymphocytes are increased in the area, showing a trend of recruitment of proinflammatory cell-types. Future classification of these immune cell types, such as determining tumor-associated neutrophils and macrophages from mature inflammatory cell types could provide further insight into potential targeted combination therapies. While our findings show an exciting if temporal change to the immune cell populations at the tumor site, it does not address what global immune cell populations may be occurring in a patient distant from the treatment site. However, recent clinical trials have assessed T regulatory cell populations post-treatment and have found a similar trend of decreased populations after treatment(Beitel-White et al., 2019; Scheffer et al., 2019). Reduced T-regulatory cell populations in peripheral blood or in the primary tumor have been shown to increase the prognosis for pancreatic cancer patients(Tang et al., 2014; Liu et al., 2017).

The increase in inflammation to the treatment area is not without consequence. While the immune cell populations shifted, MDSCs and T-regulatory cell populations eventually begin to repopulate the tumor area while CD8+ and double-positive T cell population decline by Day 14 post-treatment. This is a vital timepoint for the adaptive immune response. While we see an increase in Ifn-γ at 24 hours (**Figure 7A**) and would be consistent then with the increase to CD8+ T cells seen in the FACS data (**Figure 5C**), the immune response may be suppressed by the increase in Pdl1 expression on the tumor cells. PDL-1 is not commonly expressed in pancreatic tumors and immunotherapies involving PD1/PDL-1 inhibitors have thus far been ineffective for pancreatic cancer treatment due to the immunosuppressive barrier maintained by MDSCs and T regulatory cells(Zheng et al., 2013; Rossi et al., 2014). Therefore, the increase of inflammation by IRE may have a limited effect as "defense mechanisms" against inflammation induce checkpoint inhibitor expression that returns the tumor to a "cold" immunological state(Spranger et al., 2013; Qian et al., 2018). Similar to our findings, a recent clinical trial of IRE on pancreatic cancer also showed an increased in the PD1/PDL-1 dynamic: after treatment, the generation of a

higher population of PD-1 positive CD8 T-cells were observed(Scheffer et al., 2019). While disheartening to see the immunological effects of IRE reduced, this may in fact be a boon for patient treatment; the induction of tumor microenvironment changes caused by IRE may allow for better penetration of immunotherapeutics such as PD1/PDL-1 antibodies and the increase reliance on PD1/PDL-1 to reduce inflammation could allow the tumor to be more vulnerable to a co-treatment(Ribas, 2015). A recent preclinical trial on pancreatic cancer has shown beneficial effects of this combination of therapy(Zhao et al., 2019).

Ours and other's findings of IRE's ability to produce viable antigen also shows a promising future for increasing patient survival(Shao et al., 2019). The potential of IRE to trigger vaccine-like effects personalized to the patient's own tumor is very appealing. However, this also comes with the potential of IRE in co-treatment to induce autoinflammatory effects. Currently, IRE's track record in pancreatic cancer patients of inducing pancreatitis has been relatively small(Tian et al., 2018). Checkpoint inhibitors immunotherapies, on the other hand, have shown a recent history of autoimmunity(Khan and Gerber, 2019). More preclinical and pilot studies will be needed to determine if the potential benefits of combination treatments of IRE and immunotherapies will outweigh the risks but the current outlook to extend pancreatic cancer patient survival remains promising.

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CONFLICT OF INTEREST

IA, NW, and RD are inventors on pending and issued patents related to the work. NM is employed by AngioDynamics. The work described in this manuscript was supported, in part, through sponsored research funding provided by AngioDynamics adhering to institutional conflict of interest guidelines. Authors declare no additional conflicts of interest.

AUTHOR CONTRIBUTIONS

IA, RD, and NM contributed to the concept and design of the study. RB ran *in vitro* and *in vivo* models, monitored animals, and collected animal tissues. NB performed *in vitro and in vivo* IRE and temperature measurements. SC assessed histology. RB performed HA plasmid isolation, transfection of cells, tissue processing, RNA evaluations, flow staining, and analysis assessment. AH assisted with tissue processing and performed protein

evaluation. IA analysed IPA results. NB and IA wrote sections of the manuscript. RB wrote the original draft of the manuscript and final formatting.

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Western Blot is currently underway for clearer picture and housekeeper. Delayed by COVID-19.

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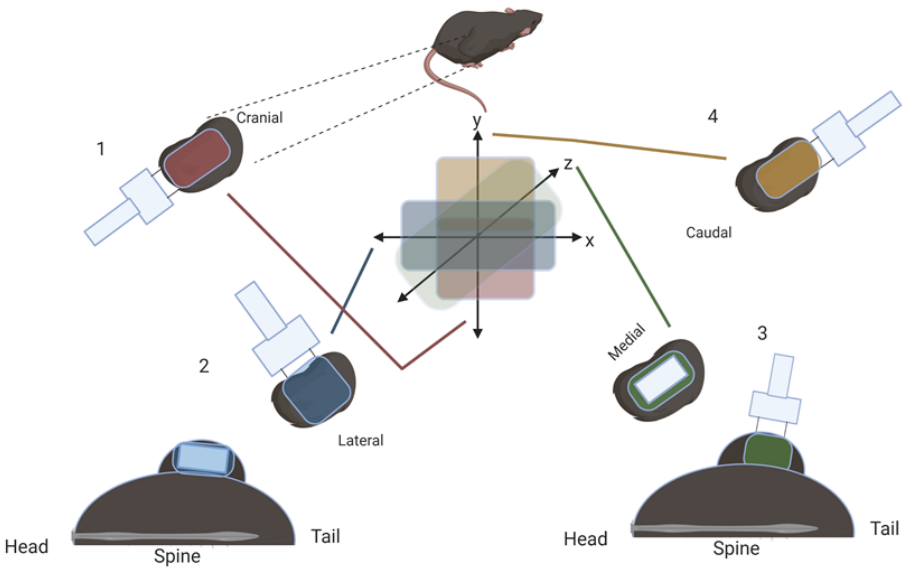
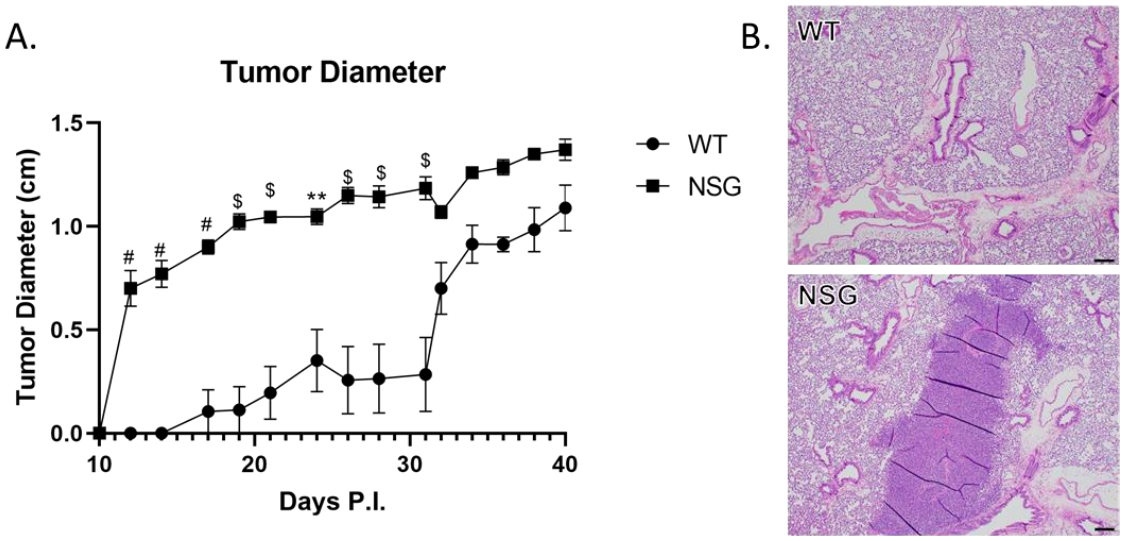
SUPPLEMENTAL FIGURES



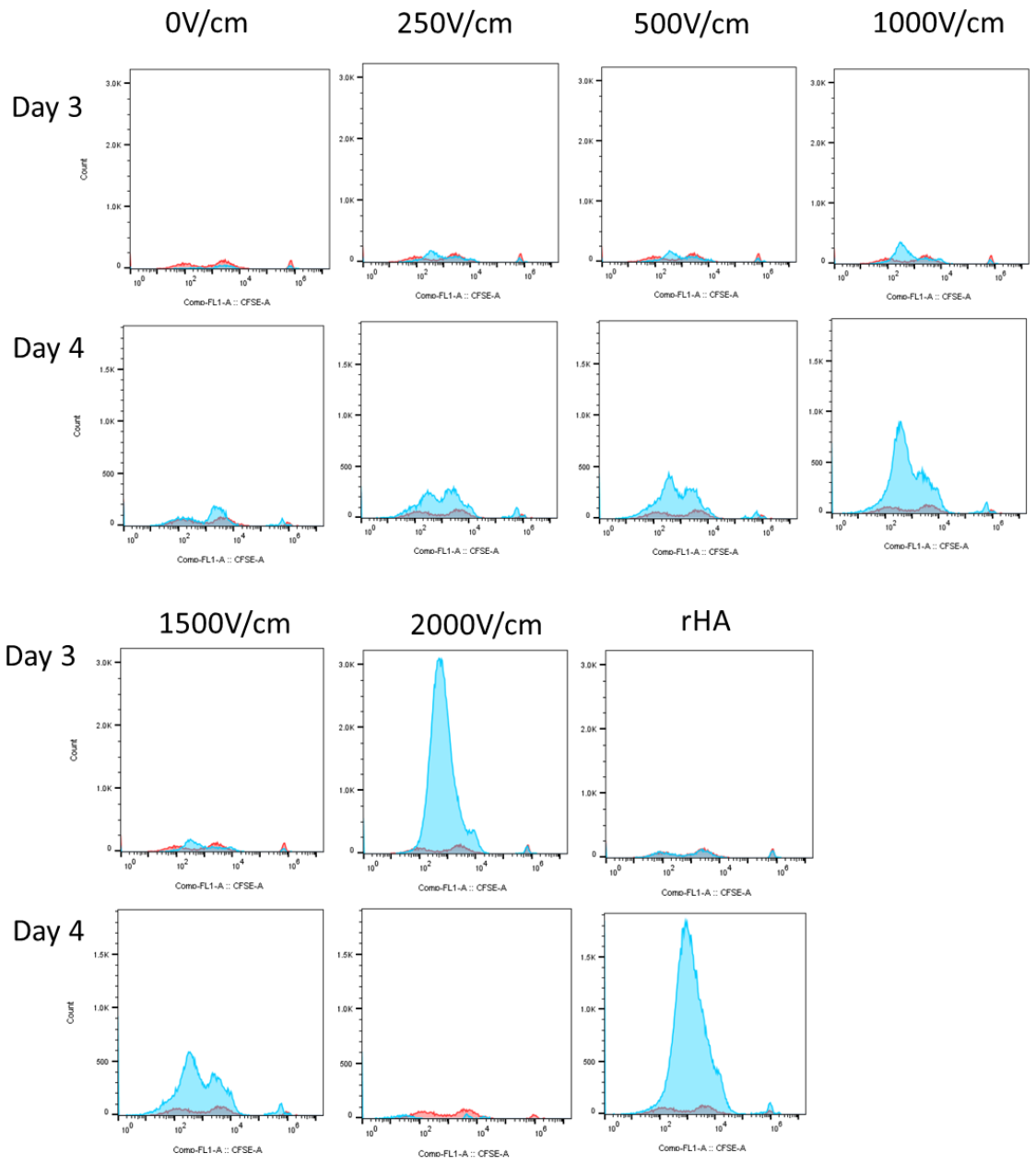
Supplemental Figure 1: Schematic of multidirectional IRE application in mouse flank model. Electrode needles were approximately 21 gauge and spaced 5mm apart. Needles were coated to avoid potential arcing, leaving exposure at tips of 5mm.

Supplemental Table 1

Cell Type	Markers
Neutrophils	Cd45+Cd11c-Ly6C-Ly6G+
Mononuclear myeloid-derived suppressor cells	Cd45+Cd11c-Ly6C+Ly6G-
Dendritic Cells	Cd45+Cd11c+F4/80-
Macrophages	Cd45+Cd11c+Ly6C-F4/80+
Cytotoxic Lymphocytes	Cd45+Cd3+Cd4-Cd8+
Double-Positive T cells	Cd45+Cd3+Cd4+Cd8+
T-helper cells	Cd45+Cd3+Cd4+Cd8-
T-regulatory Cells	Cd45+Cd3+Cd4+Cd8-FoxP3+



Supplemental Figure 2: Immune system is essential to reduce metastatic burden from Pan02 murine pancreatic cancer. NOD-scid gamma (NSG) and wildtype (WT) C57Bl/6 mice were injected subcutaneously with 1.2x10^6 Pan02 cells in Matrigel. A) Tumor diameter was measured throughout study. B) Lung metastatic lesions were observed in NSG but not in wildtype mice at the end of the study. N=3-5, two-way ANOVA, SEM. p-value *<0.05, **<0.01, $^{\#}$<0.001, $^{\$}$<0.0001.



Supplemental Figure 3. Pan02-HA cell supernatant from *in vitro* stimulated T-cell proliferation *ex vivo* at different electric field magnitudes. CFSE samples collected on Day 3 and Day 4. Representative trial out of 3 biological repeats. Maximum CFSE expression from unstimulated media-treated cells are overlayed in orange.

CHAPTER SIX
Conclusions and Future Directions

Throughout this body of work, there is a theme of the investigation on multiple levels. Both cancer and the immune system are diverse, complex, and integrate in ways. This can make studying them a difficult task without the employment of multiple model systems and assessments.

First, I established a foundation on cell death mechanisms caused by irreversible electroporation (IRE) (**Chapter Two**). Literature investigation found there to be a debate on this topic, with listings of apoptosis to necrosis to necroptosis. These mechanisms can have a huge impact on how the cancer and the immune system respond to treatment as they can incite different levels of inflammation and induce different signaling pathways (**Chapter Two Figure 1**). We discussed that applied electric fields, and therefore electroporation, is a spatial effect and, as the electrical fields weaken across the treated tissue, different forms of cell death may be possible (**Chapter Two Figure 2**). This links well to our investigation utilizing the human pancreatic patient-derived xenograft (PDX) model (**Chapter Four**). By apply IRE on *ex vivo* tissue in a uniform manner and testing multiple electric field potentials, we were able to see shifting patterns of cell death signaling related to applied dosage (**Chapter Four Figure 5**). Our trust in these results were strengthened by the establishing the strength of the model, comparing the PDX tissues to primary human tissues in terms of tissue morphology and electrical properties (**Chapter Four Figure 2-3**).

The use of a PDX model led to the investigation of affect biological pathways in pancreatic cancer (**Chapter Four Figure 6**). These studies, at this time, were limited to gene expression analysis from acute treatment, but show potential for future studies. The downregulation of KRAS and EGFR signaling pathways in pancreatic cancer are linked to multiple dysregulated pathways in pancreatic cancer including proliferative ability, evasion of cell death, and potential for metastasis. Interestingly, the TGF-β-involved pathways did not appear to be affected, which may mean that pancreatic cancer cases with highly dysregulated TFG-β signaling may be less responsive to IRE treatment. The alteration of these pathways may be why patients in clinical see significant increases to progression-free survival and may indicate vital These were, however, only acute studies. Further verification of the effects of IRE on these pathways in immunocompetent models is warranted, especially over time. Unfortunately, a standard PDX model is immunocompromised, which can limit the impact of the immune system on the biological response of the cancer and not fully portray the full biological effects of IRE on pancreatic cancer. While our work with the Pan02 immunocompetent murine model of pancreatic cancer allowed us to verify some of these signaling effects at 24hrs (**Chapter Five Figure 3**) and expand upon potential signaling differences in chromosomal stability, there are, of course, inherent differences between human and mouse cancers, subcutaneous versus orthotopic placement, cell-line versus primary tissue engraftment (**Chapter Four Figure 2, 5**). One way we are currently addressing these in the development of an immunocompromised porcine model of pancreatic cancer that will allow us study primary human tissues engrafted into a large animal, hopefully orthotopically, that can better resemble patient malignancies. Unfortunately, this is another version of a PDX model that

lacks a robust immune system. Therefore, the development of PDX models that are humanized by the introduction of human immune cells may enhance such investigations even further.

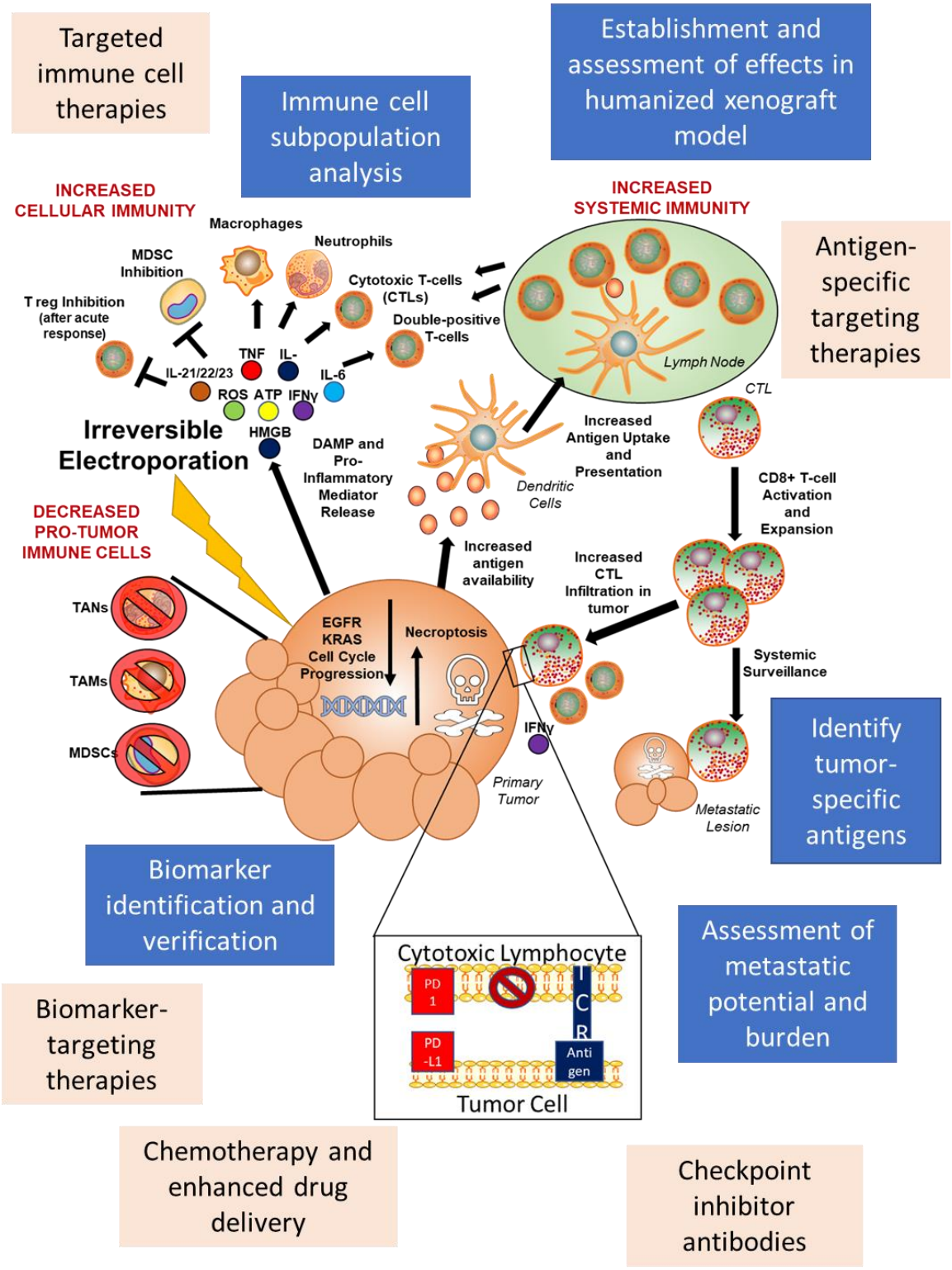
For determining the impact of IRE on the immune system, I investigation literature pertaining to evidence of IRE's ability to stimulate inflammation and alterations of immune cell populations (**Chapter Three**). Most studies fell into two categories: alterations of lymphocyte populations at the treatment site (mostly assessed by immunohistochemistry staining) and altered lymphocyte populations in peripheral blood. I explained the impact on certain cell populations on the tumor microenvironment in **Chapter Three Figures 1** and **2**. Studies within the last two years also delved into antigen presentation potential, though not in the context of pancreatic cancer, and the potential for checkpoint inhibitor combination treatment. Therefore, I included schematics explaining these mechanisms (**Chapter Three Figure 3, 4**). However, there is still a large gap in knowledge on the effects of IRE on pancreatic cancer. How are innate immune cells effected? Can IRE develop viable antigen from a cancer that is often quoted as being "highly immunosuppressive"? And why might checkpoint inhibitors such as antibodies targeting PD-1/PD-L1 be effective combination treatments?

In an attempt to answer these questions, I employed multiple models. I used *in vitro* murine pancreatic cancer models to determine how quickly pancreatic cells were dying after IRE application (**Chapter Five Figure 1**). I then used those same Pan02 cells in immunocompetent C57Bl/6 mice and applied IRE *in in vivo* to compare cell death gene expression signaling to those seen in the PDX model (**Chapter Four Figure 5, Chapter Five Figure 2**). These studies showed that IRE, in a voltage-dependent manner, induced programmed inflammatory cell death similar to necrosis or necroptosis. I used the same murine Pan02 model to evaluate immune cell populations at the tumor site after treatment over time (**Chapter Five Figure 4, 5**). I also did a small confirmation on inflammation signaling and PD-L1 expression (**Chapter Five Figure 7**). These studies showed a decrease in immunosuppressive cell types such as myeloid-derived suppressor cells and T regulatory cells as well as an increase in inflammatory signaling and potentially immunostimulatory cell types such macrophages, neutrophils, and cytotoxic T cells (**Chapter Five Figure 4, 5**) but an increase in PD-L1 expression as well. This indicates that, while we are affecting the tumor microenvironment, this effect may only be temporary. The tumor could be responding to the "hot" microenvironment by displaying immunosuppressive markers such as PD-L1 to limit immunomodulation induced by IRE. Future work may include identifying and exploring immune cell subpopulations and their potential for combination therapy targeting to reduce or enhance their recruitment and activity. Another interesting avenue would be the adaptation of this model into one with more easily traceable mestastasis. In **Chapter Five**, metastasis was measure via histopathological graded of the lungs (**Figure 3**). Unfortunately, lesions were only visible once they reached large sizes and microlesions may not have been traceable. The use of tagged Pan02 cells may enhance metastasis visualization and allow for frozen sectioning and immunohistochemistry as well as protein verification of the tag may allow for tracking of metastatic disease progression in this model.

These findings led us to speculate whether pancreatic cancer antigens had the strength to develop a strong T-cell immune response. To investigate the viability and strength of antigens produced by IRE from pancreatic cancer, I employed an *in vitro/ex*

vivo antigen presentation model (**Chapter Five Figure 6**). Not only were IRE-treated pancreatic cancer cells able to produce antigens that could induce CD8+ T-cell proliferation, these antigens did so strongly (**Chapter Five Figure 6, Supplementary Figure 3**). There were, however, limitations to this model in the use of a transgenic cell line. These Pan02 cells were genetically modified to express influenza hemagglutinin (HA), a traceable antigen for future studies. We used a recombinant HA protein as a positive control on this assay to compare responses. Future work would include investigated non-transgenic Pan02 cells treated with IRE, testing resulting T-cells against the cancer, and identifying Pan02-specific (and potentially pancreatic cancer-specific) antigens.

In conclusion, my studies have expanded upon the knowledge of how IRE can impact both biological and immunological systems in pancreatic cancer. We are using these findings and this data to determine optimal combination therapies for pancreatic cancer to expand patient survival. Future investigations could entail combining IRE with immunogenic chemotherapeutics such as gemcitabine in native and nanoparticle-encapsulated forms to enhance ablative effect in treatment margins, identifying and targeting IRE-specific biomarkers with small-molecule inhibitors, and testing the efficacy and application timing of checkpoint inhibitors such as PD-1/PD-L1-blocking antibodies after IRE ablation. For summary, I have included a figure outline the findings of this body of work and relevant potential combination therapies to be investigated in the future.



Concluding Figure. Summary of findings and future directions. Summary schematic of proposed cell death and immunomodulatory effects on pancreatic cancer. Boxed phrases considerations in blue for future studies and in orange for potential combination therapies.