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ORIGINAL ARTICLE – PANCREATIC TUMORS

Evaluating the Regulatory Immunomodulation Effect of Irreversible Electroporation (IRE) in Pancreatic Adenocarcinoma

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ABSTRACT

Background. Irreversible electroporation (IRE) has been demonstrated as an effective local method for locally advanced (stage 3) pancreatic adenocarcinoma. Immune regulatory T cells (Tregs) induce immunosuppression of tumors by inhibiting patients' anti-tumor adaptive immune response. This study aimed to evaluate the immunomodulation effect of IRE to identify an ideal time point for potential adjuvant immunotherapy.

Methods. This study prospectively evaluated an institutional review board-approved study of patients undergoing either in situ IRE or pancreatectomy. Patient blood samples were collected at different time points (before surgery [preOP] and on postoperative day [POD] 1, POD3, and POD5). Peripheral blood mononuclear cells (PBMCs) were isolated and evaluated for three different CD4 + Treg subsets (CD25 + CD4 +, CD4 + CD25 + FoxP3 +, CD4 + CD25 + FoxP3 −) by flow cytometry and analyzed for median fold change (MFC) between each two consecutive time points (MFC = log2(T2/T1)).

Results. The study analyzed 15 patients with in situ IRE (n = 11) or pancreatectomy (PAN) (n = 4). In both groups, CD25 + CD4 + Tregs decreased on POD1 followed by a steady increase in pancreatectomy, whereas the trend in the IRE group reversed between D3 and D5 (MFC: IRE [− 0.01], PAN [+ 0.39]). For each period, CD4 + CD25 + FoxP3 + Tregs showed the most dramatic inverse effect, with D3 to D5 showing the most change (MFC: IRE [− 0.18], PAN [+ 0.39]). Also, CD4 + CD25 + FoxP3 − Tregs showed an inverse effect between D3 and D5 (MFC: IRE [− 0.25], PAN [+ 0.49]). Altogether, the Treg trend was inversely affected by the in situ IRE procedure, with the greatest cumulative significant change for all three Treg subsets between D3 and D5 (MFC ± SEM: IRE [− 0.24 ± 0.05], PAN [+ 0.37 ± 0.02]; p = 0.016).

Conclusions. The study data suggest that in situ IRE procedure-mediated Treg attenuation between POD3 and POD5 can provide a clinical window of opportunity for potentiating clinical efficacy in combination with immunotherapy.

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Pancreatic cancer is the fifth leading cause of death in the United States, with an age-adjusted death rate of 10.5% and a 5-year overall survival rate reported to be 6%.[1,2] Among many treatment methods, surgical resection is the only proven curative method, but only 15% to 20% of patients presenting with resectable tumors qualify for this treatment.

Approximately 45% of patients with newly diagnosed disease present with locally advanced (stage 3) pancreatic cancer (LAPC), which is nonmetastatic but still unresectable due to invasion of the celiac trunk, superior

mesenteric artery/vein, or unreconstructable portal vein.[3] Currently, LAPC remains a clinical challenge with optimism for finding the ideal treatment method for patients.[4]

Irreversible electroporation (IRE) is an established local ablation therapy for LAPC with promising results for increasing overall survival (OS) to 25 months from 9 to 12 months.[5] The IRE procedure uses high-voltage, low-energy DC electrical pulses delivered at very short pulse lengths around the tumor to damage the permeability of cell membranes permanently, causing cells to die by inducing apoptosis.[6,7] This is a non-thermal energy-based method that allows ablation to be used adjacent to vital structures.[8,9] Unfortunately, recurrent systemic disease is seen after local treatment with all ablation techniques (radiation and irreversible electroporation).

Controlling systemic recurrence remains the greatest unmet need in the management of stages 2a, 2b, and 3 pancreatic adenocarcinoma. Therefore, this study aimed primarily to evaluate the immunologic effects of surgical resection and irreversible electroporation on therapy and recurrence in pancreatic adenocarcinoma.

Pancreatic cancers have failed to achieve a treatment response to immune-checkpoint therapies because of limited neo-antigen expression due to low mutation events,[10] a lack of sufficient CD4 + (helper) and CD8 + (cytotoxic) T cell responses in the tumor microenvironment,[11,12] and an immunosuppressive tumor microenvironment. Therefore, they are considered to be immunosuppressive (cold) tumors.[13,14]

Recent findings suggest that higher frequencies of CD4 + regulatory T cells (Tregs) could make immune-checkpoint therapies ineffective, and that therapeutic approaches targeting CD4 + Tregs could overcome this limitation.[15,16] As a subpopulation of CD4 + immune cells, CD4 + Tregs function primarily to discriminate foreign antigens from self, thus preventing autoimmune disease by suppressing CD8 + cytotoxic T cells against self-antigens. However, within the tumor microenvironment, CD4 + Tregs induce immunosuppression and allow the escape of tumors from immune surveillance. In the tumor microenvironment, CD4 + Tregs primarily suppress immune response by (1) inhibiting activation of CD8 + cytotoxic T cells, (2) killing tumor-specific antigen presentation by tumor-associated dendritic cells (DCs), and (3) converting helper T cells (Th) into Tregs.

Multiple studies have identified the role of CD4 + Tregs in pancreatic cancer prognosis. Circulating CD4 + CD25 + Tregs have been shown to increase pancreatic tumor progression.[17] A recent study by Jang et al.[18] identified that Tregs instruct tumor antigen-presenting DCs in the pancreatic tumor microenvironment to prevent anti-tumor immunity and that depleting Tregs induces potent anti-tumor immunity, thus suggesting that Tregs could be the master regulator of a immunomodulatory effect and outcome in pancreatic tumor. Therefore, depleting CD4 + Tregs could open the prospect of using immune-checkpoint therapies for refractory tumors such as LAPC.

The current study examined the immunomodulation effect of in situ IRE by evaluating systemic changes in CD4 + Tregs in LAPC patients. We evaluated levels of the three most clinically correlated Treg populations (CD4 + CD25 +, CD4 + CD25 + FoxP3 +, CD4 + CD25 + FoxP3 −)[16,19] before and after in situ IRE treatment and compared the response with that of pancreatectomy (PAN) patients without IRE treatment. We aimed to identify a potential window of opportunity for adjuvant immune-checkpoint therapy.[4]

METHODS

Patients

This research study was approved by the Institutional Review Board (IRB) at the University of Louisville. All potential pancreatic cancer patients undergoing either in situ irreversible electroporation (IRE) or pancreatectomy (PAN) were asked for voluntary research participation from January 2016 to July 2017. A total of 11 in situ IRE patients and 4 pancreatectomy patients consented and were enrolled in this prospective study.

To understand the possible variable changes that could occur with surgery alone, this study captured and graded all 90-day complications.[20] We used four patients undergoing pancreatectomy to provide a pilot baseline in which surgery was the first treatment. If after these four control patients we saw consistent data, we then did not believe that expanding the control population would add further value to these results. 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 anytime during the study without affecting their treatment and ongoing care. Adjunctive surgical procedures were performed at the time of IRE in accordance with previous publications and recommendations.[8,21–23]

Experimental Workflow

All patients undergoing IRE or pancreatectomy were asked for consent to participate in the study. For each time point, one vial of venous blood was drawn from enrolled patients by phlebotomy using a Na-heparin tube, transported to a laboratory for further processing to isolate peripheral blood mononuclear cells, and stored at − 80 °C by following an optimized protocol.

After thawing and staining for CD4, CD25, and FoxP3, cells were analyzed for three CD4 + Tregs (i.e., CD4 + CD25 +, CD4 + CD25 + FoxP3 +, and CD4 + CD25 + FoxP3 −). Figure 1 demonstrates the representative workflow of analysis for identifying each of the Treg subsets, which were calculated later as a percentage of CD4 cells, as shown in Fig. S1.

Blood Collection, PBMC Isolation, and Cryopreservation

Whole venous blood was collected using Na-heparin plasma tubes (green-top tubes) from enrolled patients before surgery (preOP) and then on days 1, 3, and 5 after surgery (i.e., postoperative day [POD]1, POD3, and POD5). Whole-blood specimens were brought to the lab within 2 h and immediately processed for isolation of PBMCs using the Immuno-Lyse (Beckman Coulter, Brea, CA) red blood cell (RBC) lysis method (lysis without permeabilization) with hypotonic ACK Lysing Buffer (Gibco, Waltham, MA) consisting of 150 mmol NH_4Cl, 10 mmol $KHCO_3$, and 0.1 mmol Na_2-EDTA (pH 7.2–7.4)0.[24] After the RBC lysis from 300 µL of whole blood, PBMCs were collected and stored in RPMI with 10% HAS w/v and 10% dimethyl sulfoxide (DMSO) v/v. Using a cryogenic container (CryoCooler, VWR), the PBMCs were frozen to − 80 °C facilitating a 1 °C/min rate.

Flow Cytometry Analysis

Frozen PBMCs were thawed from − 80 °C in a 37 °C water bath with continuous mixing. This is a critical step to ensure fast thawing while maintaining good consistency and viability. Thawed PBMCs were transferred into 5-mL polypropylene centrifuge tubes (Cat # MCT-500-C-T; Axygen Scientific, New York, NY) and centrifuged at 300 RCF (relative centrifugal force) for 5 min to remove RPMI/HSA/DMSO media, and the PBMC pallet was resuspended in 2 mL of buffer (phosphate-buffered saline [PBS], pH 7.4, 0.5% BSA, and 2 mmol of ethylenediaminetetraacetic acid [EDTA]). The PBMCs were counted using a hemocytometer and appropriately diluted to achieve 100,000 cells/100 µL of buffer.

For staining, we used the FoxP3 Staining Buffer Set (Cat # 130-093-142; Miltenyi Biotec, Bergisch Gladbach, Germany) and performed the staining procedure per manufacturer's instructions. Briefly, surface-marker staining was performed first, followed by Fc-blocking and later by intracellular staining.

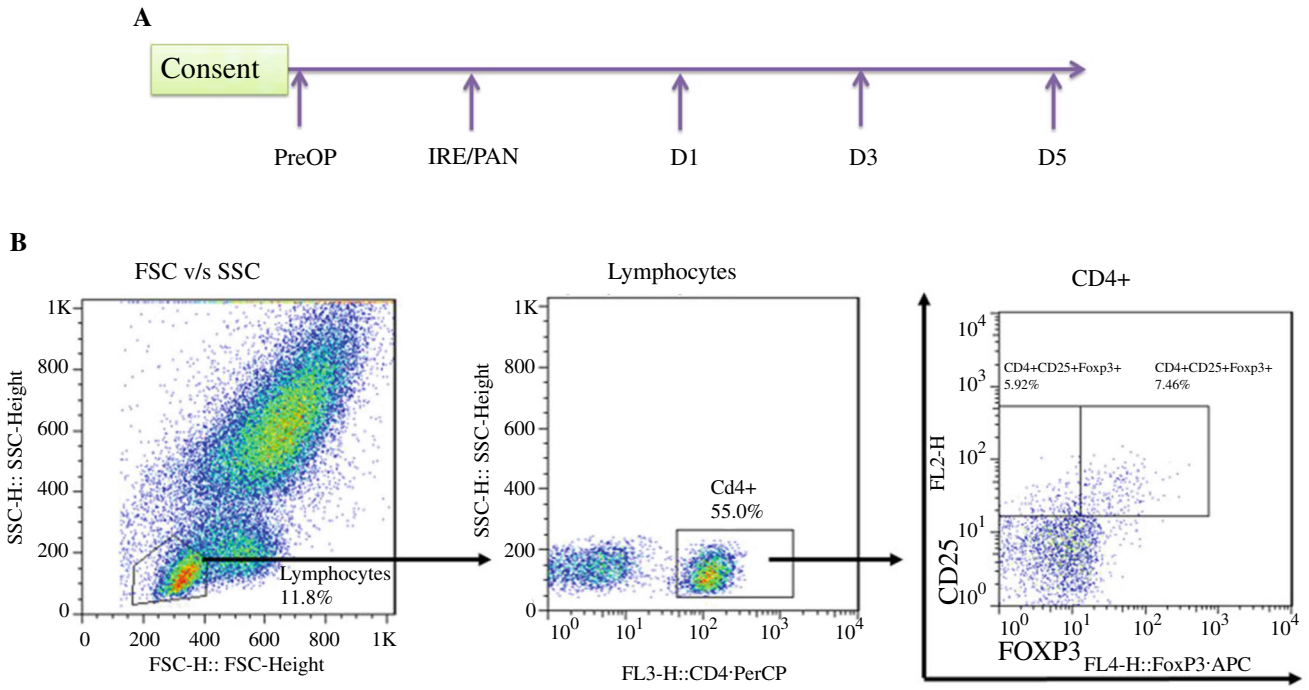

FIG. 1 Experimental workflow. Total in situ irreversible electroporation (IRE) patients (n = 11) and pancreatectomy (PAN) patients (n = 4) consented to participate in the institutional review board (IRB)-approved prospective study. **a** Whole blood was collected in Na-heparin tubes for isolation of peripheral blood mononuclear cells (PBMCs) at different time points. Postoperative days D1, D3, and D5 correspond to blood collection time points. **b** Representative workflow to identify regulatory T cells (Tregs) from PBMCs. First CD4 + lymphocytes were identified, which were gated further to identify CD4 + CD25 +, CD4 + CD25 + FoxP3 +, and CD4 + CD25 + FoxP3 − Tregs. preOP, before surgery

Finally, cells were analyzed after appropriate gating to identify the CD4 + lymphocyte population using BD FACSCalibur (BD Biosciences, San Jose, CA), and the data were recorded. For each patient, all blood collection time-point specimens (preOP, POD1, POD3, POD5) were analyzed simultaneously to avoid experimental (sample-to-sample) variation.

IRE Generator and Treg Correlation

We extracted IRE generator data for four IRE patients (randomly selected) from the generator. The raw IRE data (electrical pulse, change in range of current, and voltage) were exported as an XML file for each patient. Corresponding Treg levels (% of CD4 +) of pre-IRE and 24-h post-IRE for each patient were obtained. These data were blinded and sent to a collaborator at an external institution for correlation analysis.

Data and Statistical Analysis

Descriptive statistics were used for this study because the overall effect of either surgical stress or IRE for pancreatic cancer has never been demonstrated. Data are presented as median ± SE of the mean (SEM) unless otherwise noted.

For each patient, samples were analyzed for median fold change (MFC) between each two consecutive time points (MFC = log2[T2/T1]). Student's *t* test with equal variance was performed between IRE and PAN for each MFC analysis. Statistical analysis and box plot preparation were performed using the Sigma Plot software suite (London, UK). A *p* value lower than 0.05 was considered statistically significant.

RESULTS

The study enrolled 15 patients, who completed all blood-sample time points. Their preoperative management, operative management, and follow-up evaluation are presented in Table 1.

TABLE 1 Patient demographics

	LAPC IRE (n = 11) n (%)	Pancreatectomy (n = 4) n (%)
Median age (years)	63	65
BMI (kg/m^2)	25.4 ± 3.5	26.8 ± 4.8
Male/female	2	2/2
Medical history		
Hypertension	3 (26)	1
Cardiac disease	1 (6)	0
Diabetes	1 (10)	2
Tobacco use	2 (18)	3
Surgical history		
Abdominal	1	1
Colon	1	0
Appendix	1	2
Tumor location: head/body	9/2	4/0
Tumor size: cm (range)	2.8 (2.2–3.2)	2.9 (2.1–4.3)
Chemotherapy		
FOLFIRINOX	9 (82)	3 (75)
Gemzar	5 (45)	2 (50)
Radiation therapy	5 (45)	0
Median time from diagnosis to IRE or resection: months (range)	4.8 (3.0–6.8)	5.2. (2.8–8.5)
Median total IRE delivery time: min (range)	49 (5–307)	
Additional surgical therapy		
Hepaticojejunostomy	8	4
Gastrojejunostomy	6	4
Cholecystectomy	5	2
Total 90-day AEs		
Complications	5 (45)	1
Grade 1	3	
Grade 2	1	
Grade 3	1	
Adjuvant therapy (%)	100	100

LAPC locally advanced pancreatic cancer, *IRE* irreversible electroporation, *BMI* body mass index, *AE* adverse events

Day 5 After Surgery Showed a Significant Differences in All Three Tregs for IRE Compared With Pancreatectomy

We aimed to investigate how in situ IRE influences Tregs compared with pancreatectomy (PAN). We calculated MFC for three different intervals (i.e., preOP to D1, D1 to D3, and D3 to D5) for both the IRE and PAN groups of patients. Then we compared longitudinal MFC changes between the IRE and PAN treatments. As shown in Fig. 2a, the trend of the longitudinal changes in MFC were quite distinct in the IRE group compared with the PAN group.

We next asked what the time point was when the Treg subpopulation showed the maximum difference. The CD25 + CD4 + Tregs decreased in both groups on POD1, followed by a steady increase in pancreatectomy. However, the trend in the IRE group reversed between D3 and D5 (MFC: IRE [− 0.01], PAN [+ 0.39]). The CD4 + CD25 + FoxP3 + Tregs showed the most dramatic inverse effect for each interval, with D3 to D5 showing the most change (MFC: IRE [− 0.18], PAN [+ 0.39]). The CD4 + CD25 + FoxP3 − Tregs also showed an inverse effect between D3 and D5 (MFC: IRE [− 0.25], PAN [+ 0.49]).

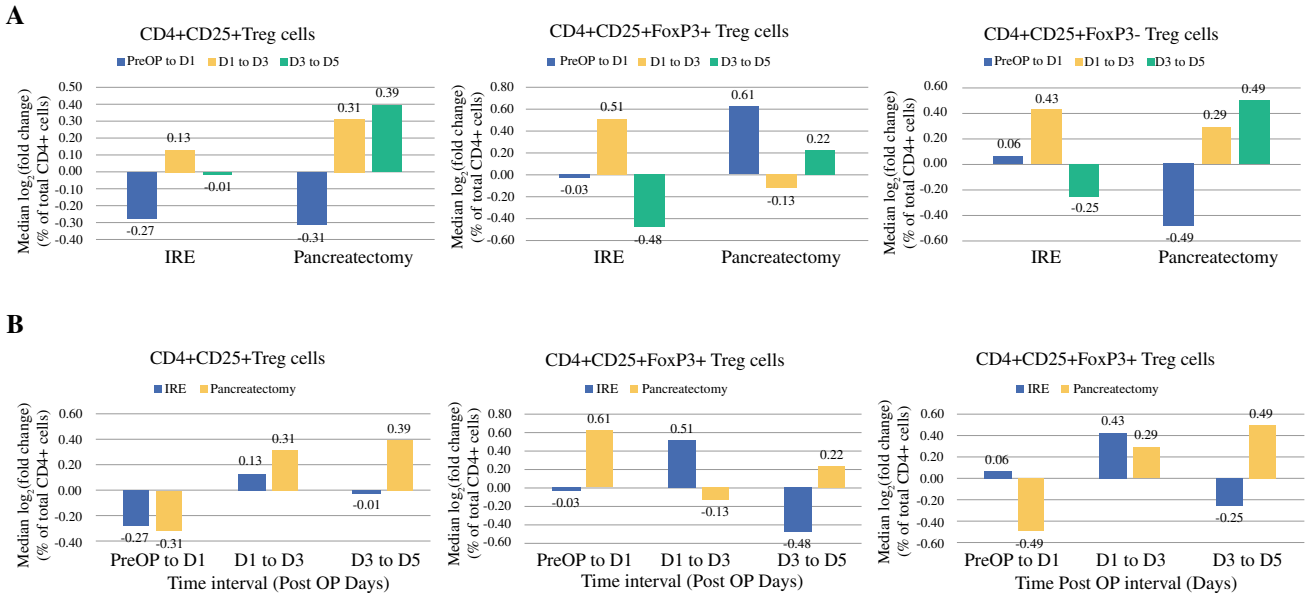

FIG. 2 Day 5 after irreversible electroporation (IRE) showed the greatest significant difference in Tregs: bar graphs show the median fold change (MFC) in %CD4 + T cells for three different intervals: preOP to D1, D1 to D3, and D3 to D5. **a** Treatment group comparison analysis showing that IRE treatment influenced all three Tregs (CD4 + CD25 +, CD4 + CD25 + FoxP3 +, and CD4 + CD25 + FoxP3–) compared with pancreatectomy (PAN). **b** IRE group MFC compared with PAN for each interval for all three Tregs. The CD4 + CD25 + FoxP3 + Treg subset showed the most dramatic inverse effect on MFC for each time interval, with D3 to D5 showing the greatest change (MFC: IRE [− 0.18], PAN [+ 0.39]). The CD4 + CD25 + FoxP3– Treg subset also showed an inverse effect between D3 and D5 (MFC: IRE [− 0.25], PAN [+ 0.49]). The greatest cumulative significant change for all three Tregs was observed between D3 and D5 (MFC ± SEM: IRE:− 0.24 ± 0.05; PAN: +0.37 ± 0.02; $p = 0.016$)

Altogether, these data suggested that rhw in situ IRE procedure influenced all three Tregs compared with pancreatectomy. The Treg trend was inversely affected by the in situ IRE procedure, with the highest cumulative significant change for all three Treg subsets between D3 and D5 (MFC ± SEM: IRE [− 0.24 ± 0.05], PAN [+ 0.37 ± 0.02]; $p = 0.016$) (Fig. 2b).

IRE Current Correlates Change in Treg Subsets After IRE Treatment

For a more complete understanding of the potential factors contributing to the depletion of systemic Treg subsets, we correlated IRE current measurements (range of output current [Amperes]) from the IRE generator with Treg levels. This evaluation was performed blinded using data from four IRE patients. The range of the output current (ampere [A]) was determined by the pancreatic tissue's inherent electroconductive property (resistance [R]), for which the basic physics principle V = IR (Ohm's law) applies. The higher the resistance (R) for pancreatic tumor/tissue, the lower the range of output current (A), so the range of output current (A) is a subjective variable. We identified that the IRE range of current (A) changes correlates with levels of CD4 + CD25 + Tregs ($R = 0.83$), CD4 + CD25 + FoxP3 + ($R = 0.94$), and CD4 + CD25 + FoxP3 − ($R = 0.94$) (Fig. 3).

DISCUSSION

This study demonstrated that IRE exhibits an immunomodulatory effect by altering systemic CD4 + Tregs in LAPC (stage 3). This is the first such clinical report to demonstrate at least a transitory immunomodulatory effect of IRE on LAPC patients. In LAPC patients, IRE treatment significantly affects all three of the most clinically relevant Tregs (i.e., CD4 + CD25 +, CD4 + CD25 + FoxP3 +, CD4 + CD25 + FoxP3 −), and Treg levels attain a maximum decrease on day 5 compared with that in patients undergoing pancreatectomy. We also identified that the clinically recommended change in current (recommended 12–15 amps from baseline) correlates with levels of Tregs for all three subsets. We demonstrated that IRE ablation could provide a transitory treatment window of opportunity by decreasing the key immunosuppressive Tregs, and that this transient decrease in Tregs could be exploited for administration of immune-checkpoint therapy.

Resistance of the pancreatic tumor decreases with time during IRE treatment because IRE-mediated irreversible pores on tumor cell membranes facilitate a higher current

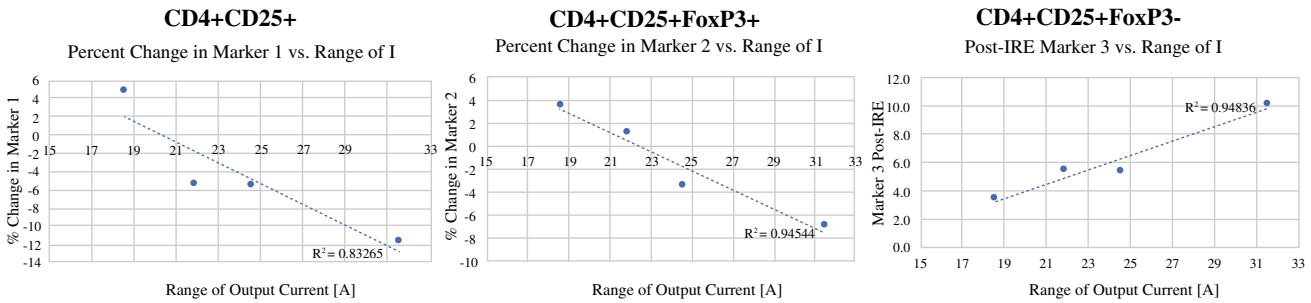

FIG. 3 The irreversible electroporation (IRE) current correlates with the change in Treg subsets after IRE treatment (24 h = 1 day). The correlation graphs show percentage change in Treg versus range of current. The IRE range of current (A) correlates with levels of CD4 + CD25 + Treg (marker 1, $R = 0.83$) and significantly with CD4 + CD25 + FoxP3 + (marker 2, $R = 0.94$) and CD4 + CD25 + FoxP3– (marker 3, $R = 0.94$)

transfer (a decrease in both voltage and resistance), thus potentially dictating IRE-mediated effects on systemic Tregs.[25] Thus, higher current, although more advantageous, cannot be achieved if the patient's tumor/pancreas conductivity (or resistance) is not favorable.[25] This indeed implies that each patient's Tregs outcome could be significantly different after IRE.

This is a first study to report systemic changes in Tregs after IRE ablation in pancreatic cancer patients. Significant efforts have been made to overcome the immunosuppressive "cold" tumor microenvironment in pancreatic cancer patients by using novel approaches and treatment methods, including combinations with currently approved drugs.[26,27]

Our current data suggest that IRE ablation, by lowering regulatory T cells, can potentially prime immunosuppressive pancreatic tumors for immune-checkpoint therapy. Many previous studies have reported the effect that IRE ablation has on immune response.[28]. In mice models, findings have shown IRE to be more effective in immunocompetent tumors than in immunocompromised tumors, and increased tumor infiltration of CD3 + cells have been reported.[29] A study using the rat model of osteosarcoma reported a significant increase in peripheral CD3 + and CD4 + and an increased ratio of CD4 +/CD8 + in the IRE group compared with surgery.[30] A recent study using a breast cancer mouse model reported the potential of IRE to change an immunosuppressive tumor microenvironment to a pro-inflammatory environment with an increase in T lymphocytes and antigen-presenting cells. A study by Al-Sakere et al.[31] however, reported no significant immunologic response in tumor tissue after IRE in a mouse model using a sarcoma cell line.[31]

Our study examined the longitudinal changes in the CD4 + Treg population. However, a few limitations still exist, and future work in this direction is encouraged. First, based on existing literature, we measured the systemic changes in Tregs, which may not necessarily represent the Treg population in the tumor microenvironment of LAPC because each patient's tumor exhibits distinct tumor infiltration properties and inflammation. Second, we followed up with patients only until POD5, and a longer follow-up period is needed to understand the long-term persistent effects of in situ IRE on the patient's immune system.

Multiple studies have shown that circulatory Tregs can predict prognosis, and more comprehensive analysis of other immune system components are encouraged to elucidate, for example, the systemic effect of IRE on CD8 + cytotoxic T cells, helper T cells, macrophages, antigen-presenting dendritic cells (DCs), and the like. Such studies would help us understand more completely the status of systemic immune suppression. As a future direction, with our promising current pilot study findings, our next IRB-approved prospective study will analyze Treg status more comprehensively.

In conclusion, IRE significantly lowered CD4 + CD25 + and CD4 + CD25 + FoxP3 + Tregs compared with pancreatectomy, and these IRE-mediated changes in Tregs were found at their maximum levels on POD5. We have initiated a clinical trial for 10 LAPC patients (ClinicalTrials.gov Identifier: NCT03080974) undergoing IRE treatment. Based on our current pilot study data, we propose POD3 to POD5 as an ideal window of opportunity for initiating potential immunotherapy after IRE in LAPC.

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CONFLICT OF INTEREST Robert C. G. Martin is a paid consultant for AngioDynamics. The remaining authors have no conflicts of interest.

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