Am J Cancer Res 2022;12(1):165-175
www.ajcr.us /ISSN:2156-6976/ajcr0139556

Original Article

Perioperative systemic immunophenotype following irreversible electroporation (IRE) predicts recurrence

Conor H O'Neill[1,2*], Min Tan[1,2*], Jun Yan[1,2*], Yan Li[1,2*], Robert C G Martin[1,2*]

[1]Division of Surgical Oncology, Price Surgical Research Institute, Hiram Polk Department of Surgery, University of Louisville, Louisville, KY 40202, USA; [2]Division of Immunotherapy, Hiram Polk Department of Surgery, University of Louisville, Louisville, KY 40202, USA. [*]Equal contributors.

Received October 2, 2021; Accepted November 19, 2021; Epub January 15, 2022; Published January 30, 2022

Abstract: Comprehensive understanding of the immunophenotypic response to local therapy will likely be required to improve outcomes for pancreatic ductal adenocarcinoma (PDAC). While the desmoplastic stroma has rendered PDAC resistant to immunotherapies, irreversible electroporation (IRE), a non-thermal method of tumor ablation, can overcome some of this resistance and immune suppression. We studied the systemic immunophenotype of patients following local treatment of PDAC. Stored lymphocytes from peripheral blood collected pre- and post-operatively for patients with PDAC who underwent surgical treatment from 12/2018 until 12/2019 were prepared for mass cytometry and a 30-marker panel identifying 37 immune-cell clusters were analyzed and compared to all clinical parameters. Stored lymphocytes from patient samples were collected pre-operatively postoperatively (Day 1, 3, 5 and 14) and during surveillance (Month 3, 6, 9 and 12). Thirty patients with locally advanced pancreatic cancer (LAPC) who underwent IRE were evaluated prospectively for changes in their immunophenotype. No significant differences in baseline demographics or tumor markers were identified. CA19-9 levels were significantly higher among patients who developed a recurrence (P=0.03). In the early perioperative period, CD4 and CD8 central memory cells were significantly higher among patients who did not recur (P=0.02 and 0.009 respectively). These findings were maintained in the late (>3 month) surveillance period. Early natural killer (NK) cells were significantly higher among those who did not recur (P=0.004) in the early postoperative period. The early immune-cell populations of CD4 and CD8 central memory cells and early NK cells were significantly higher among populations who did not recur following IRE for PDAC during the study period, with maintenance of the CD4 and CD8 central memory populations during later surveillance. Monitoring the early immunophenotype may offer opportunities to augment the immune response following tumor-disruptive IRE for PDAC.

Keywords: Immunophenotype, pancreatic cancer, recurrence, irreversible electroporation

Introduction

Pancreatic ductal adenocarcinoma remains one of the most lethal forms of cancer. It accounts for approximately 2.5% of cancers worldwide; however, it is responsible for close to 10% of cancer deaths [1]. In the U.S., PDAC is the fourth-leading cause of cancer death in 2020, with an estimated 47,050 new deaths alone with only 57,600 new diagnoses [2]. Unfortunately, PDAC incidence is increasing, with current projections forecasting PDAC death rates to surpass breast cancer worldwide [1].

Surgical resection remains the best opportunity for long-term survival, however only 10-20% of patients are resectable at time of presentation, while around 35% have locally advanced disease and 50% are metastatic at presentation [3]. Cytotoxic chemotherapy is the backbone of systemic treatment for PDAC but is limited in efficacy in part by the cumulative toxicity of therapy and its immunosuppressive effects. Newer treatments are needed, but PDAC typically fails to respond to immune checkpoint blockade. The dense peri-tumoral stroma, limited neo-antigen expression and generally unfavorable local tumoral environment have rendered PDAC resistant to checkpoint therapy [4-6], with perhaps modest response rates seen only in MSI-H PDAC [7].

Strategies to overcome the resistance to systemic cytotoxic and immunotherapy are critical

to improve survival. One such strategy, irreversible electroporation (IRE), utilizes short electrical pulses across targeted tissue. IRE disrupts cell-wall homeostasis and cellular apoptosis ensues. IRE has demonstrated promise in locally advanced PDAC due to its efficacy around critical vascular structures, with improved median survival in selected patients [8]. This non-thermal tumor disruptive therapy also disrupts the peri-tumoral milieu [9, 10]. We have previously demonstrated that IRE induces PD-L1 expression [11], and have shown systemic immunophenotypic changes following IRE with reduction of CD4+ T-regulatory cells and significant increases in proportions of CD4+ effector memory T cells in the immediate postoperative period [11, 12].

Examination of the local tumor milieu over time is not clinically feasible in pancreatic cancer, given the inaccuracies of dynamic imaging and the invasiveness of repeat biopsies every 3 months. Surrogate attempts through evaluation of a patient's systemic immunophenotype are attractive to detect clinically actionable data points to augment the immunologic response to the tumor. In this study, mass cytometry was utilized to evaluate the serum of surgically treated patients with PDAC. We hypothesized that the immunophenotype immediately following and sustained after IRE may predict recurrence-free survival. We sought to compare the immunophenotype between patients with known recurrence to those without evidence of disease using clinical correlation.

Methods

Patient selection

An Institutional Review Board (IRB)-approved prospective cohort of preoperatively diagnosed National Comprehensive Cancer Network (NCCN) stage III LAPC of patients treated by IRE between December 2018 and December 2019 was evaluated. Patients with biopsy-proven non-metastatic PDAC amenable to surgical therapy were selected. All patients provided written informed consent. A diagnosis of LAPC disease was established by biopsy-proven adenocarcinoma of the pancreas with unreconstructable venous involvement or greater than 180° encasement of the superior mesenteric artery (SMA) or celiac artery without evidence of metastatic lesions [13-15]. Patients were further considered for inclusion in the study if the treating physician at the aforementioned participating institutions believed that ablation of their soft tissue would be feasible in the care of their disease, as has been previously described and outlined [16-18]. Staging included triple-phase computed tomographic (CT) scan with less than 1.5-mm cuts at the time of diagnosis and repeated 1-2 weeks prior to IRE [19, 20].

Irreversible electroporation

Patients found to be free of metastatic disease and without primary tumor progression on restaging were included and further received an open surgical in situ IRE based on intraoperative findings and location of the primary tumor as described previously [19]. Open IRE was performed utilizing the NanoKnife (AngioDynamics, Latham, NY, USA) system, as previously described, and were performed by surgeons in the operating room [21-23]. All participating institutions utilized the registry protocol for standardization of settings setup and delivery of energy during the IRE procedure as previously reported [16, 18, 24, 25]. In short, IRE was performed with continuous ultrasound guidance to bracket the tumor with electrodes through a transmesocolic approach for caudally oriented pancreatic head/uncinate tumors or directly for cranially oriented lesions. Patients were deeply paralyzed and electrical pulses were delivered until efficacy through changes in resistance was realized. Critical structure patency was then confirmed. Pancreatoduodenectomy was performed when possible after extensive intraoperative ultrasonography of the liver and pancreas determined feasibility for resection.

Peripheral blood was obtained preoperatively and on postoperative days 1, 3 and 5. Additional blood draws were performed at the 2-week and 1-month postoperative visits and each subsequent follow up (every 3 months from IRE) until recurrence or completion of surveillance. Four patients were selected who underwent R0 pancreatoduodenectomy as a form of baseline control. Subsequent blood draws were performed during surveillance follow up until recurrence. Patient variables and demographics were recorded. Preoperative treatment was

noted, including chemotherapy and chemoradiation. Serum cancer antigen 19-9 (CA 19-9) levels were measured during follow up visits. Adjuvant treatment details were noted, including systemic therapy and chemoradiation. Disease recurrence was noted.

Post-procedure evaluation and follow up

After IRE follow-up imaging via triple-phase CT scan was performed during the immediate postoperative period to evaluate for early complications, assess the patency of vital structures, and to establish a baseline of the post-ablation bed, as has been previously reported [15, 26, 27]. Ablation success was evaluated at 3 months post-IRE treatment via triple-phase CT scan following pancreatic imaging protocol, along with CA19-9, and PET-CT. Ablation success and recurrence have been previously defined [14]. Participating institutions standardized utilization of CT scans to avoid the difficulty encountered with cross-comparing CT scans to MRI or CT scan to PET scans in previous studies. Response and progression were evaluated using the international criteria proposed by RECIST 1.1 [28]. Serial imaging over at least two months was subsequently used to detect recurrence through study comparison in combination with clinical and serum CA19-9 studies. If equivocal findings were seen on CT then a PET was obtained to either confirm or refute local and/or regional recurrence when required.

Mass cytometry

Mass cytometry utilizes metal-conjugated antibodies rather than fluorophores for characterization of material of interest with the practical advantage of detecting substantially more parameters per cell than in typical flow cytometry without the same broad-emission contamination. We characterized the immunophenotype broadly, using the Maxpar® Direct Immune Profiling Assay (Fluidigm, South San Francisco, CA, USA) enabling 37 immune-cell populations to be characterized. These populations included major lymphocyte populations of CD3(+) T-cell subsets, B-cell subsets, and natural killer (NK) cells, as well as monocyte subsets, dendritic cells, and granulocyte subsets.

Stored frozen lymphocytes from date of collection were thawed rapidly and assessed for viability using Trypan blue. Samples with at least 3×10^6 cells with >80% viability were selected. Cells were washed with staining buffer and incubated with FcR block for 10 minutes. Cells were fixed with 16% formaldehyde and then permeabilized and stained with the manufacturer's proprietary buffers. Cells were incubated overnight at 4C. On day 2, cells were washed and suspended in staining buffer for data acquisition using the Cytof® mass cytometry and Helios® software (Fluidigm, CA, USA). Data acquisition reports were exported from the Helios system for processing with SPSS v 27 (IBM Armonk, NY) for statistical analysis. When appropriate, chi-square, student t-tests and analysis of variance (ANOVA) with posthoc Tukey method were performed for comparative analyses. Repeated measures of ANOVA with Bonferroni correction were utilized to detect differences in the trend of populations over time.

Statistical analyses

OS was defined as the time from the start of treatment for their PDA to the date of death, due to any reason. PFS was defined as the time from the start of initial IRE treatment to the date of first observed disease progression. All patients who received operative resection with IRE margin accentuation were excluded from PFS and OS analyses. The rates of OS and PFS were estimated by Kaplan-Meier method. Multivariable Cox survival regression was performed to determine independent predictors of PFS and OS after backward selection (criterion $P<0.05$) to include all variables of interest. All statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA), and *P* values less than 0.05 were considered significant. When appropriate, student t-tests or analysis of variance (ANOVA) with posthoc Tukey method were performed for comparative analyses.

Results

Patient demographics

Patients underwent surgical treatment for pancreatic adenocarcinoma between 12/1/2018-12/1/2019 and were followed clinically until study conclusion on 12/1/2020. Four patients (13%) underwent pancreaticoduodenectomy, while 26 patients (87%) underwent IRE alone

Table 1. Baseline characteristics

	IRE alone	IRE + concurrent chemotherapy	Overall	P	Resection
Mean Age (SD)	62.0 (9.5)	60.0 (9.6)	62.4 (9.1)	0.50	69.5 (6.36)
Recurrence (%)	3 (50%)	3 (60%)	6 (61.5%)	0.52	2 (100%)
XRT (%)	3 (50%)	2 (40%)	5 (39.5%)	0.52	0 (0%)
Preop CA 19-9 (SD)	107.0 (120.4)	36.1 (39.0)	78.6 (95.6)	0.50	45.5 (39.4)
Lymphocytes (SD)	52.1 (8.5)	53.7 (15.3)	52.9 (11.7)	0.85	
CD3 T cells (SD)	37.9 (9.0)	44.2 (13.4)	41.0 (11.3)	0.41	
CD4 T cells (SD)	27.0 (9.9)	30.0 (7.7)	28.5 (8.5)	0.61	
CD8 T cells (SD)	8.8 (3.1)	11.2 (4.0)	10.0 (3.6)	0.31	
CD4/CD8 ratio (SD)	3.4 (1.6)	2.7 (0.4)	3.1 (1.2)	0.42	

(In-Situ). Baseline characteristics are reported in **Table 1**. The mean age was 62.4 yrs (SD +/- 9.1) and did not differ between groups (P>0.05). Baseline CA19-9 was 69.2 (SD 89.3) and did not differ between treatment groups (P>0.05). Completion chemotherapy and chemoradiation therapy were utilized per protocol in 80% (n=24) of patients, with the other 6 receiving only completion chemotherapy. During the study period, 63% of patients had recurrence of disease (n=19/30) with a median disease-free interval of 21.7 months (range 10 to 44 months); 100% of patients who underwent resection had a recurrence (n=4/4) and 53% (n=16) of patients in the In-Situ IRE group.

Chemoradiotherapy was utilized in the adjuvant setting in 33% of patients (n=10/30): 10 in the IRE group and 0 in the resection group. Utilization of neoadjuvant or adjuvant chemoradiotherapy did not influence recurrence rates (P>.05); 50% of patients who did not receive radiation recurred and 50% who received radiation recurred. The average serum tumor marker CA 19-9 during the surveillance period was significantly lower among patients who did not recur (64.6 vs 720.0, P=0.03). No differences in CA19-9 existed at baseline and between treatment groups (P>0.05).

Mass cytometry

As reported above, mass cytometry was performed to characterize the immune cell profile of patients in the perioperative and surveillance periods (**Figure 1**).

The Cen-se' map revealed heterogeneous lymphocyte clusters, including the CD3 T-cell populations, CD4 T-cell populations, B-cell populations, monocyte populations, dendritic cell populations, etc. The CyTOF-generated data of cell subpopulations were identified as the percentage of intact live cells. Thus, we performed advanced analysis to create a heat map represented as pre-IRE, post-IRE (day 5 and day 90) in the patients who had no recurrence and the patient with recurrence. In the patient who had no recurrence, increases of both T-cell and B-cell lymphocyte subpopulations were found post-IRE in comparison with pre-IRE. However, in the patient with recurrence, decreased lymphocyte subpopulations of both T cells and B cells were found post-IRE, in comparison with pre-IRE (**Figure 2**).

Lymphoid markers allowed the determination of CD3+, natural-killer (NK), and B-cell subsets. Within the CD3+ population, CD4+ cells including T-regulatory cells, T-helper subsets 1, 2, and 17, and CD4+ memory subsets naïve, central memory, effector and terminal effector cell types were profiled. Additionally, CD3+ CD8+ memory subsets, gamma delta T cells, and mucosal-associated invariant T-cells were profiled as well as subsets of NK and B cells. Profiling was also performed of classical, transitional, and nonclassical monocytes, myeloid and plasmacytoid dendritic cells, and granulocyte populations of neutrophils, basophils, and eosinophils. This comprehensive panel was performed iteratively preoperatively and on successive postoperative days. This study included immune profiling of patients who underwent concurrent chemotherapy until and during operative IRE. We performed comparative analyses to determine whether systemic immunophenotype differed between resection alone and IRE group. Across every immune cell pro-

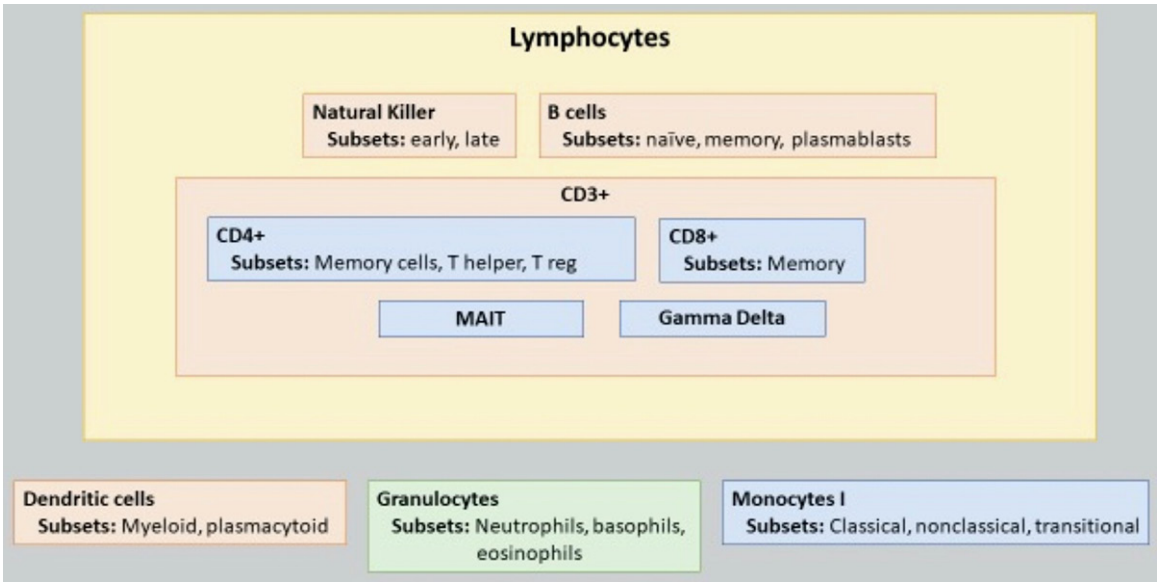

Figure 1. Mass cytometry characterization of immune cell phenotypes.

filed, no differences existed in the preoperative or early postoperative immunophenotype between groups (all P>0.05). Thus, these arms were combined for comparative analyses between the patient who recurred and those who did not (**Table 2**).

Lymphocyte populations

Repeated measures of ANOVA were performed to determined differences in total lymphocyte populations in patients that recurred following IRE and those who did not over the course of surveillance. There were no outliers, as assessed by boxplot. There was not homogeneity of variance (P=0.03) as assessed by Levene's Test or Mauchly's test of sphericity (P<0.005) therefore Greenhouse-Geisser correction was assumed for all subsequent repeated ANOVA. There was no statistically significant interaction between total lymphocytes and time (P=0.13). There was a statistically significant increase in total lymphocytes among patients who did not recur F (1, 9) =8.249, partial eta squared =0.478.

B-cell populations were evaluated at baseline in the preoperative setting and patients who did not recur had significantly higher total B-cell populations (4.2% vs 1.8%, P<0.007), as well as naïve B cells (3.4% vs 1.6%, P=0.014) and memory B cells (0.66% vs 0.17%, P=0.014). In the early postoperative period (postoperative days 1-14), patients who did not recur had significantly higher total B-cell populations compared to those who did recur (6.2% vs 1.8%, P<0.001). No differences existed in memory B-cell populations (0.5% vs 0.2%, P=0.17), but naïve B populations were significantly higher in those who did not recur (5.7% vs 1.3%, P<0.001). No differences existed in plasmablasts at baseline or in early perioperative settings. During the surveillance period from postoperative day 90 onward, there remained no significant difference in populations of memory B cells (0.7% vs 0.8%, P=0.8) and no differences were detectable in total B-cell populations, naïve B cells or plasmablasts (all P>0.05).

In the total, early, and late NK-cell populations, no differences existed at baseline with respect to recurrence status (all P>0.05). In the early postoperative period, Early NK cells were significantly higher among patients who did not recur (4.4% vs 2.5%, P=0.004). No differences in total NK or late NK subsets were identified (P>0.05). No differences in the surveillance period were identified in NK cells or subtypes with respect to recurrence status.

To improve detection of differences in the trends of these B- and NK-cell subset populations over time with respect to baseline and identify trends that may help in prediction of recurrence, repeated measures of ANOVA were undertaken comparing baseline, early postop-

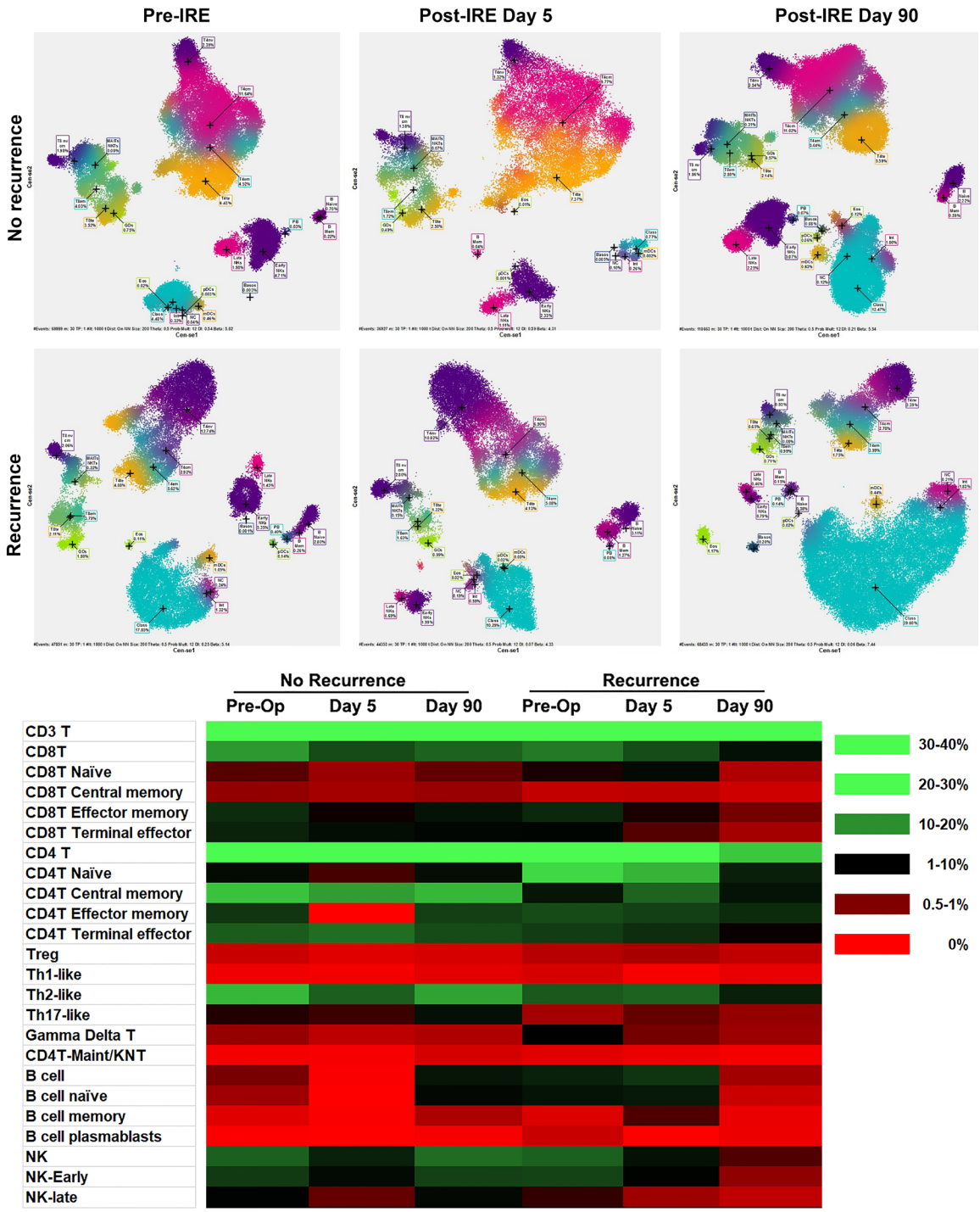

Figure 2. Representative Cen-se' maps of pre-IRE and post-IRE (day 5 and day 90) from a patient without recurrence and a patient with recurrence. In Cen-se' map, top-left clusters show CD3 T cells, top-right clusters show CD4 T cells. B-cell clusters are shown in the middle. Heat map was created by the cell subpopulations as the percentage of intact live cells.

erative (day 5) and surveillance (day 90) values. No differences in population trends were identified in B or NK subsets with respect to recurrence status (all P>0.05).

CD3+ cells were evaluated in all patients and there was no differences in CD3+ cells existed at baseline within the early postoperative period or in the surveillance period (all P>0.05). The

Table 2. Baseline preoperative characteristics by recurrence status in patients treated by electroporation

Cell Population	No Recurrence (SD)	Recurrence (SD)	P
Lymphocytes	55.9 (13.2)	49.9 (10.5)	0.44
CD3 T cells	42.6 (15.1)	39.5 (7.0)	0.69
CD4 T cells	27.3 (10.3)	29.6 (7.3)	0.70
CD8 T cells	11.3 (4.7)	8.8 (1.6)	0.30
CD4/CD8 ratio	2.7 (1.1)	3.5 (1.2)	0.30
GD T cells	3.0 (2.5)	0.8 (0.6)	0.10
Mucosal Associated Invariant T cells	1.0 (0.7)	0.3 (0.3)	0.08
B Cells	4.2 (1.0)	1.8 (1.1)	0.007*
NK Cells	9.2 (5.9)	8.5 (3.8)	0.84
Monocytes	17.3 (4.5)	14.4 (5.8)	0.40
Granulocytes	4.3 (4.3)	9.6 (8.6)	0.26

Note: *, Statistical significance.

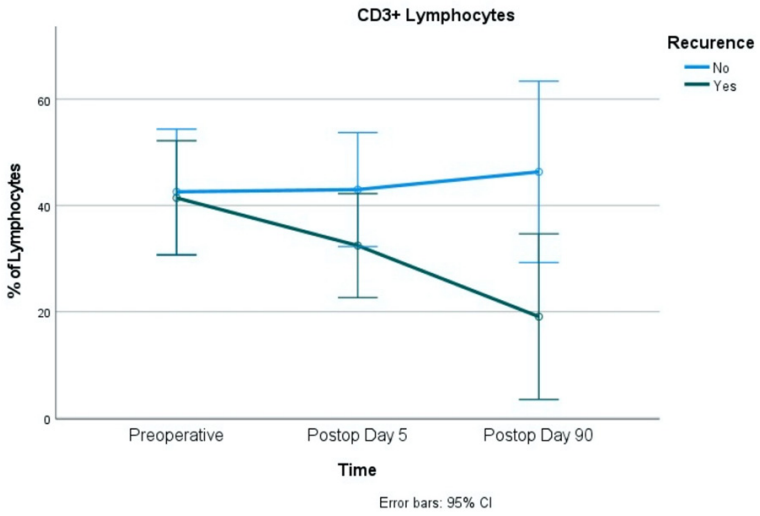

Figure 3. CD3+ lymphocyte populations evaluated from preoperative to postoperative day 90, demonstrating a statistically significant increase in CD3+ among patients who did not recur later in their disease follow up.

trend in total CD3+ cells was significant, however (**Figure 3**). There was a statistically significant increase in CD3+ among patients who did not recur F (1, 9) =6.055, partial eta squared =0.402 (P=.036).

CD8 subtypes were evaluated in all patients and mass cytometry provided characterization of CD8 subtypes including CD8 naïve T cells, CD8+ central memory cells, CD8+ effector memory cells, and CD8+ terminal effector cells. No differences were detected in baseline values of CD8+ T cells or its subsets (all P>0.05). In the early postoperative period (postoperative days 1-14), no differences were noted in total CD8 lymphocyte populations between patients who recurred versus those who were disease free at study conclusion (P=0.24). Among CD8 subtypes, no differences were noted among CD8 naïve cells, CD8 effector memory cells, or CD8 terminal effector cells (all P>0.05). Mean CD8 central memory was significantly higher among those without recurrence (2.6% vs 0.8%, P=0.09).

During the surveillance period from postoperative day 90 on, again no differences were noted in total CD8 populations with respect to recurrence status (P=0.55). Among CD8 subtypes, no differences were noted in populations of terminal effector cells (P=0.16), effector memory cells (P=0.16), or naïve cells (P=0.13). CD8 central memory cells remained significantly higher among those without recurrence (3.3% vs 1.1%, P=0.047). Repeated measures of ANOVA were undertaken to determine significance in the change in populations over time. No significant changes in cell populations of CD8+ subsets were identified in the preoperative, early postoperative or surveillance periods (all P>0.05). Collectively, CD8+ central memory populations were significantly higher following IRE in patients who did not recur without change in the trend of these populations over time compared to patients who ultimately recurred.

Total CD4 cell populations or CD+ subsets in the preoperative setting did not differ with respect to ultimate recurrence (all P>0.05). Total CD4+ populations in the early postoperative period did not differ between patients who recurred or did not recur (P=0.141). Among CD4+ subtypes, no differences were noted among CD4 naïve, CD4 effector memory, or CD4 terminal effector cells (all P>0.05). Patients who did not have a recurrence had significantly

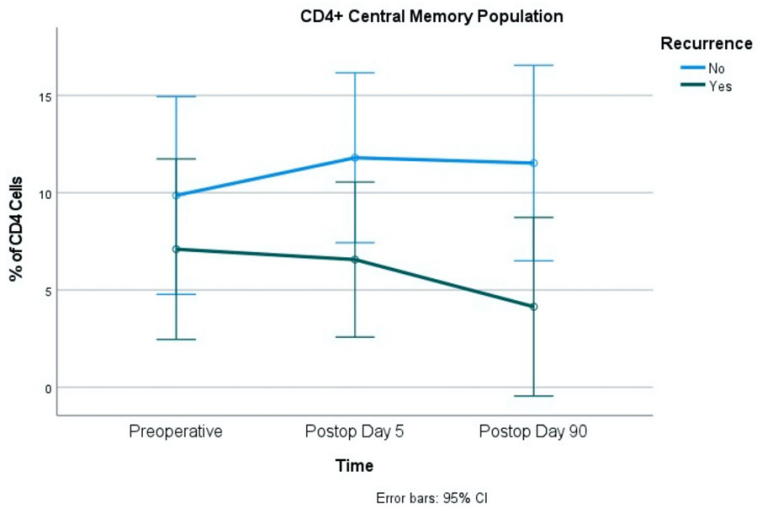

Figure 4. CD4+ central memory cell populations over time. Collectively, baseline levels of CD4 cells were similar, but patients who did not recur had higher levels of CD4 central memory populations in the early postoperative period and maintained higher levels of effector memory cells in later surveillance periods.

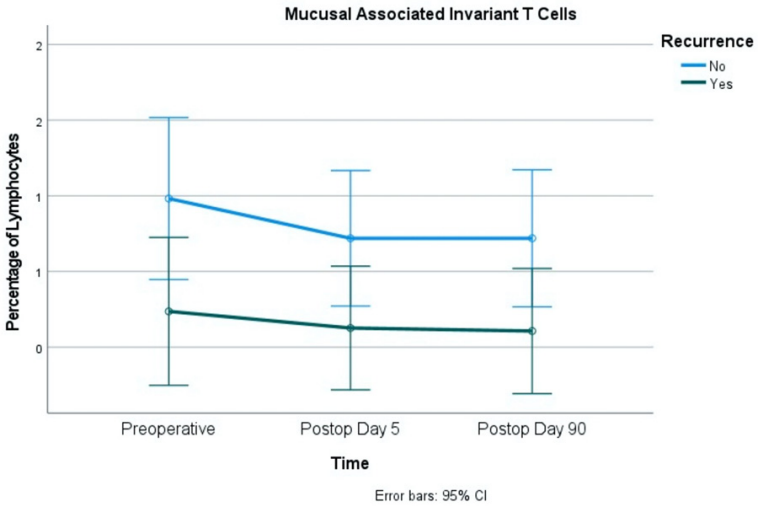

Figure 5. Mucosal associated invariant T cell populations over time. Statistically significant higher levels of MAIT were seen among patients at 90 days who did not recur later in their disease surveillance.

higher levels of CD4 central memory populations in the early postoperative period (11.3% vs 6.0%, P=0.02) than those who did recur.

During the surveillance period, no differences were noted among total CD4 populations with respect to recurrence status, or among CD4 naïve cells, or CD4 terminal effector cells (all P>0.05). CD4 effector memory cells were significantly higher among those who did not recur (10.3% vs 6.4%, P=0.04). CD4 central memory was not significantly higher (11.2% vs 5.6%, P=0.053). However, during repeated measures of ANOVA, there were statistically significant higher levels of CD4+ central memory populations among patients who did not recur F (1, 9) =5.222, partial eta squared =0.367 (P=.048). Collectively, baseline levels of CD4 cells were similar, but patients who did not recur had higher levels of CD4 central memory populations in the early postoperative period, higher levels of effector memory cells in later surveillance periods, while the trend of CD4 central memory populations in those who recurred decreased significantly over time (**Figure 4**).

Cell characterization was also possible in additional CD3+ subsets. Gamma delta T cells (GDT) are highest in abundance in gut mucosa and have been postulated to have roles in both adaptive immunity and innate immune responses. Mucosal-associated invariant T cells (MAIT) demonstrate innate cytolytic function but may play a role in immunotherapy through ligand modulation [29]. T-regulatory and T-helper subsets have been demonstrated to play a role in immune modulation.

In this study, no differences were found in IRE-treated patients with respect to recurrence status in the preoperative, early postoperative, or late surveillance cell populations of GDT, MAIT, immunosuppressive T regulatory CD4+ cells, or T helper subsets Th-1, 2 or 17 (all P>0.05). There were statistically significant higher levels of MAIT among patients over time (**Figure 5**) who did not recur on repeated measures of ANOVA (F (1, 9) =8.116, partial eta squared

=0.474 (P=.019)). Collectively, no significant differences in population levels were found regarding recurrence status, but MAIT levels were sustained at higher amounts in patients who did not recur.

Monocytes may play a prominent role in tumor progression through immunosuppressive functions but also can differentiate into tumor-induced macrophages [30]. Dendritic cells have emerged as a major target of therapeutic strategies through inducing anti-tumor immunity but can have substantial immune suppression in the local tumor milieu [31]. We sought to determine differences in these populations and within their subsets. No differences were identified in baseline, early postoperative, and surveillance populations with respect to recurrence status in total monocytes or monocyte subsets, plasmacytoid dendritic cells or myeloid dendritic cells (all P>0.05). No differences in the repeated measures of ANOVA populations over time were detected among monocyte populations (all P>0.05). There were statistically significant higher levels of plasmacytoid dendritic cells among patients who did not recur F (1, 9) =7.075, partial eta squared =0.440 (P=.026).

Granulocyte subpopulations included neutrophils, basophils and eosinophils. No differences with respect to recurrence status were detected among granulocytes or subpopulations at baseline (all P>0.05). No differences were detected in the cell populations of granulocytes, basophils, neutrophils, or eosinophils in the early postoperative period with respect to recurrence status (all P>0.05). In the surveillance period, there were significantly lower levels of neutrophils in those who did not recur (2.9% vs 15.6%, P=0.038). The repeated measures of ANOVA did not detect differences in the change in proportion of these cell populations over time (all P>0.05).

No differences were noted in utilization of chemoradiation for recurrence status (P=0.59) or in adjuvant chemotherapy (P=1.0). However, patients who received chemoradiation did have increased populations of CD4 central memory cells (13.8% vs 6.0%, P=0.009) in the surveillance period of the study. This finding was not correlated with recurrence when controlled for chemoradiation. No differences were noted in CD8 subsets or the remainder of CD4 subsets. Chemotherapy utilization had no impact on cell populations of CD4 or CD8 subsets with respect to recurrence (all P>0.05).

Discussion

We present our data utilizing mass cytometry to characterize the immune profile following local therapy for PDAC. Recent data has accumulated in murine models that combination IRE + anti-PD1 therapy led to increased intratumoral CD8+ T cells with higher CD8-to-Treg ratios [10]. As repeated measures of intratumoral immune profiles are simply not clinically feasible, we have investigated systemic immune profile changes following IRE [11, 12]. Following IRE, systemic reduction in circulating CD4 Treg between postoperative days 1-5 have been previously reported [12]. On that foundation, concurrent PD1 blockade with IRE was investigated demonstrating increased in CD4(+) effector memory populations by postoperative day 90 [11]. This study expands the immune profile surveillance through a large cohort of cell populations including T cells, B cells, dendritic cells, monocytes and many other subsets during the early postoperative and follow up phases of care.

In the present study, we have shown that patients without evidence of recurrence have a robust early immune response to IRE with the establishment of significantly higher levels of CD4 and CD8 central memory populations as well as enhanced early NK response. These central memory levels are maintained throughout the surveillance period. Central memory, especially of CD8 origin, likely confers greater antitumor immunity compared to other memory subsets [32]. The migration of CD4 central memory subsets to peritoneal solid organs may lead to an activated anti-tumor phenotype [33]. These systemic changes are apparent early in the postoperative course and can be monitored in the clinical setting.

The hallmark of PDAC on histologic review is characterized by dense desmoplasia. This fibrosis provides a mechanical barrier and hinders immune infiltration and cytotoxic therapy exposure [5, 6]. Systemic stromal depletion strategies have been disappointing in clinical trials, however, and are limited by systemic toxicity [34]. A multimodal approach with thorough understanding of the tumor microenvironment,

stroma, and systemic immunophenotype will likely be critical to deliver effective local and systemic therapy. Irreversible electroporation, with its non-thermal apoptotic mechanism of cellular death, has been shown to overcome stromal immunosuppression [10]. The local architecture with tumor-restraining collagen is preserved and immunosuppressive T regulatory cells are not able to infiltrate the ablated tissue.

Further study is warranted. Actionable efforts to to augment development of central memory through early immunotherapy or additional local or systemic options is needed. As this study shows, surveillance of immune response can be achieved through peripheral blood draw. Limitations of this study do exist. This is a small cohort of heavily pre-treated patients with PDAC. A larger prospective cohort is needed to validate these findings.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Robert C G Martin, Division of Surgical Oncology, University of Louisville, 315 E. Broadway-M10-Rm. #311, Louisville, KY 40202, USA. Tel: 502-629-3355; E-mail: Robert.Martin@louisville.edu

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

[2] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2020. CA Cancer J Clin 2020; 70: 7-30.

[3] Ducreux M, Cuhna AS, Caramella C, Hollebecque A, Burtin P, Goere D, Seufferlein T, Haustermans K, Van Laethem JL, Conroy T, Arnold D and Committee EG. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2015; 26 Suppl 5: v56-68.

[4] Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA, Kiezun A, Hammerman PS, McKenna A, Drier Y, Zou L, Ramos AH, Pugh TJ, Stransky N, Helman E, Kim J, Sougnez C, Ambrogio L, Nickerson E, Shefler E, Cortes ML, Auclair D, Saksena G, Voet D, Noble M, DiCara D, Lin P, Lichtenstein L, Heiman DI, Fennell T, Imielinski M, Hernandez B, Hodis E, Baca S, Dulak AM, Lohr J, Landau DA, Wu CJ, Melendez-Zajgla J, Hidalgo-Miranda A, Koren A, McCarroll SA, Mora J, Crompton B, Onofrio R, Parkin M, Winckler W, Ardlie K, Gabriel SB, Roberts CWM, Biegel JA, Stegmaier K, Bass AJ, Garraway LA, Meyerson M, Golub TR, Gordenin DA, Sunyaev S, Lander ES and Getz G. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 2013; 499: 214-218.

[5] von Bernstorff W, Voss M, Freichel S, Schmid A, Vogel I, Johnk C, Henne-Bruns D, Kremer B and Kalthoff H. Systemic and local immunosuppression in pancreatic cancer patients. Clin Cancer Res 2001; 7: 925s-932s.

[6] Skelton RA, Javed A, Zheng L and He J. Overcoming the resistance of pancreatic cancer to immune checkpoint inhibitors. J Surg Oncol 2017; 116: 55-62.

[7] Marabelle A, Le DT, Ascierto PA, Di Giacomo AM, De Jesus-Acosta A, Delord JP, Geva R, Gottfried M, Penel N, Hansen AR, Piha-Paul SA, Doi T, Gao B, Chung HC, Lopez-Martin J, Bang YJ, Frommer RS, Shah M, Ghori R, Joe AK, Pruitt SK and Diaz LA Jr. Efficacy of pembrolizumab in patients with noncolorectal high microsatellite instability/mismatch repair-deficient cancer: results from the phase II KEYNOTE-158 study. J Clin Oncol 2020; 38: 1-10.

[8] Martin RC 2nd, Kwon D, Chalikonda S, Sellers M, Kotz E, Scoggins C, McMasters KM and Watkins K. Treatment of 200 locally advanced (stage III) pancreatic adenocarcinoma patients with irreversible electroporation: safety and efficacy. Ann Surg 2015; 262: 486-494; discussion 492-484.

[9] Li X, Xu K, Li W, Qiu X, Ma B, Fan Q and Li Z. Immunologic response to tumor ablation with irreversible electroporation. PLoS One 2012; 7: e48749.

[10] Zhao J, Wen X, Tian L, Li T, Xu C, Wen X, Melancon MP, Gupta S, Shen B, Peng W and Li C. Irreversible electroporation reverses resistance to immune checkpoint blockade in pancreatic cancer. Nat Commun 2019; 10: 899.

[11] O'Neill C, Hayat T, Hamm J, Healey M, Zheng Q, Li Y and Martin RCG 2nd. A phase 1b trial of concurrent immunotherapy and irreversible electroporation in the treatment of locally advanced pancreatic adenocarcinoma. Surgery 2020.

[12] Pandit H, Hong YK, Li Y, Rostas J, Pulliam Z, Li SP and Martin RCG. evaluating the regulatory immunomodulation effect of irreversible electroporation (IRE) in pancreatic adenocarcinoma. Ann Surg Oncol 2019; 26: 800-806.

[13] Callery MP, Chang KJ, Fishman EK, Talamonti MS, William Traverso L and Linehan DC. Pretreatment assessment of resectable and borderline resectable pancreatic cancer: expert consensus statement. Ann Surg Oncol 2009; 16: 1727-1733.
[14] Varadhachary GR, Tamm EP, Abbruzzese JL, Xiong HQ, Crane CH, Wang H, Lee JE, Pisters PW, Evans DB and Wolff RA. Borderline resectable pancreatic cancer: definitions, management, and role of preoperative therapy. Ann Surg Oncol 2006; 13: 1035-1046.
[15] Holland MM, Bhutiani N, Kruse EJ, Weiss MJ, Christein JD, White RR, Huang KW and Martin RC 2nd. A prospective, multi-institution assessment of irreversible electroporation for treatment of locally advanced pancreatic adenocarcinoma: initial outcomes from the AHPBA pancreatic registry. HPB (Oxford) 2019.
[16] Martin RC. Irreversible electroporation of locally advanced pancreatic head adenocarcinoma. J Gastrointest Surg 2013; 17: 1850-1856.
[17] Martin RC 2nd. Use of irreversible electroporation in unresectable pancreatic cancer. Hepatobiliary Surg Nutr 2015; 4: 211-215.
[18] Martin RC 2nd. Irreversible electroporation of locally advanced pancreatic neck/body adenocarcinoma. J Gastrointest Oncol 2015; 6: 329-335.
[19] Martin RC 2nd, Kwon D, Chalikonda S, Sellers M, Kotz E, Scoggins C, McMasters KM and Watkins K. Treatment of 200 locally advanced (Stage III) pancreatic adenocarcinoma patients with irreversible electroporation: safety and efficacy. Ann Surg 2015; 262: 486-494.
[20] Martin RC, Philips P, Ellis S, Hayes D and Bagla S. Irreversible electroporation of unresectable soft tissue tumors with vascular invasion: effective palliation. BMC Cancer 2014; 14: 540.
[21] Martin RC. Irreversible electroporation of locally advanced pancreatic head adenocarcinoma. J Gastrointest Surg 2013; 17: 1850-1856.
[22] Dunki-Jacobs EM, Philips P and Martin II RC. Evaluation of resistance as a measure of successful tumor ablation during irreversible electroporation of the pancreas. J Am Coll Surg 2014; 218: 179-187.
[23] Martin RC. Irreversible electroporation of locally advanced pancreatic neck/body adenocarcinoma. J Gastrointest Oncol 2015; 6: 329.
[24] Kwon D, McFarland K, Velanovich V and Martin RC 2nd. Borderline and locally advanced pancreatic adenocarcinoma margin accentuation with intraoperative irreversible electroporation. Surgery 2014; 156: 910-920.
[25] Holland MM, Bhutiani N, Kruse EJ, Weiss MJ, Christein JD, White RR, Huang KW and Martin RCG 2nd. A prospective, multi-institution assessment of irreversible electroporation for treatment of locally advanced pancreatic adenocarcinoma: initial outcomes from the AHPBA pancreatic registry. HPB (Oxford) 2019; 21: 1024-1031.
[26] Akinwande O, Ahmad SS, Van Meter T, Schulz B and Martin RC. CT findings of patients treated with irreversible electroporation for locally advanced pancreatic cancer. J Oncol 2015; 2015: 680319.
[27] Schulz B, Ou J, Van Meter T and Martin RC. Early nontumorous CT findings after irreversible electroporation of locally advanced pancreatic cancer. Abdom Radiol (NY) 2016; 41: 2142-2149.
[28] Martin RC 2nd, Durham AN, Besselink MG, Iannitti D, Weiss MJ, Wolfgang CL and Huang KW. Irreversible electroporation in locally advanced pancreatic cancer: A call for standardization of energy delivery. J Surg Oncol 2016; 114: 865-871.
[29] Lukasik Z, Elewaut D and Venken K. MAIT Cells Come to the Rescue in Cancer Immunotherapy? Cancers (Basel) 2020; 12.
[30] Kiss M, Caro AA, Raes G and Laoui D. Systemic Reprogramming of Monocytes in Cancer. Front Oncol 2020; 10: 1399.
[31] Wylie B, Macri C, Mintern JD and Waithman J. Dendritic cells and cancer: from biology to therapeutic intervention. Cancers (Basel) 2019; 11.
[32] Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA and Restifo NP. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A 2005; 102: 9571-9576.
[33] MacLeod MK, Clambey ET, Kappler JW and Marrack P. CD4 memory T cells: what are they and what can they do? Semin Immunol 2009; 21: 53-61.
[34] Doherty GJ, Tempero M and Corrie PG. HALO-109-301: a Phase III trial of PEGPH20 (with gemcitabine and nab-paclitaxel) in hyaluronic acid-high stage IV pancreatic cancer. Future Oncol 2018; 14: 13-22.