**Immunologic and Metabolic Features of Pancreatic Ductal Adenocarcinoma Define Prognostic Subtypes of Disease**

Jack Hutcheson, Uthra Balaji, Matthew R. Porembka, Megan B. Wachsmann, Peter A. McCue, Erik S. Knudsen, and Agnieszka K. Witkiewicz

**Abstract**

**Purpose:** Pancreatic ductal adenocarcinoma (PDA) is associated with an immunosuppressive microenvironment that supports the growth of the malignancy as well as immune system evasion. Here we examine markers of immunosuppression in PDA within the context of the glycolytic tumor microenvironment, their interrelationship with tumor biology and association with overall survival.

**Experimental Design:** We utilized tissue microarrays consisting of 223 PDA patients annotated for clinical stage, tumor size, lymph node involvement, and survival. Expression of CD163, FoxP3, PD-L1, and MCT4 was assessed by IHC and statistical associations were evaluated by univariate and multivariate analysis. Multimarker subtypes were defined by random forest analysis. Mechanistic interactions were evaluated using PDA cell lines and models for myeloid differentiation.

**Results:** PDA exhibits discrete expression of CD163, FoxP3, and PD-L1 with modest individual significance. However, combined low expression of these markers was associated with improved prognosis ($P = 0.02$). PDA tumor cells altered macrophage phenotype and function, which supported enhanced invasiveness in cell-based models. Lactate efflux mediated by MCT4 was associated with, and required for, the selective conversion of myeloid cells. Correspondingly, MCT4 expression correlated with immune markers in PDA cases, and increased the significance of prognostic subtypes ($P = 0.002$).

**Conclusions:** There exists a complex interplay between PDA tumor cells and the host immune system wherein immunosuppression is associated with negative outcome. MCT4 expression, representative of the glycolytic state of PDA, contributes to the phenotypic conversion of myeloid cells. Thus, metabolic status of PDA tumors is an important determinant of the immunosuppressive environment.

**Introduction**

Pancreatic ductal adenocarcinoma (PDA) is a highly aggressive malignancy and the fourth leading cause of cancer mortality in the United States. Most patients present with advanced disease and the prognosis of these patients is dismal, with 5-year overall survival less than 6% and a median survival of 4 to 6 months (1). Even when complete surgical resection is possible, recurrence is common and a majority of patients will die with distant metastasis within 3 years (2). As systemic treatments remain minimally effective, many studies have focused on the unique genetic characteristics of PDA to define new therapeutic targets (3). Meanwhile, recent studies have demonstrated unique aspects of metabolism and immune evasion in PDA that could offer rational targets for therapeutic intervention beyond genomic targets (1, 4–6).

PDA is characterized by the presence of desmoplastic stroma that account for as much as 90% of the total tumor volume (7, 8). Interactions between the tumor cells and the surrounding stroma create an inflammatory microenvironment that is conducive to tumor growth and progression. This chronic inflammation recruits predominantly T cells supported by additional leukocytes, including dendritic cells, macrophages, neutrophils, and B cells, to the primary tumor (7, 9). The clinical relevance of this ongoing inflammatory program is reflected in poor outcomes correlated with infiltrating inflammatory cells and the contributions of specific cell types are being increasingly elucidated (10, 11). For instance, neutrophil-lymphocyte ratio may represent a prognostic marker in PDA patients with a lower ratio representing improved prognosis and, despite the lack of a significant increase in the total cell number, B cells have now been implicated in PDA progression through their ability to impact myeloid cell function (12–14). Meanwhile, single-agent inhibitors of immune checkpoints have not demonstrated substantial efficacy in PDA (15). Thus, a better understanding of the mechanisms that regulate the PDA immunosuppressive environment could reveal specific disease subtypes with enhanced prognostic significance and allow for additional targeted treatment approaches.

Glycolysis plays an important role in inflammation and, specifically, lactate is a critical mediator of the macrophage inflammatory...
response (16–18). Alterations in glycolytic tumor metabolism also impact the contributions of other immune cells in PDA (19, 20). Since increased expression of the lactate exporter MCT4 (SLC16A3) also portends poor outcome in PDA (4), we hypothesized that the glycolytic imbalance in pancreatic cancer cells, represented by MCT4 expression, directly impacts the surrounding immune milieu and that these characteristics could be used to identify distinct disease subsets. To this end, in this study we examine the tumor microenvironment of PDA patients for indicators of an immunosuppressive phenotype and utilize these elements to define prognostic subtypes of disease. Furthermore, we demonstrate that the metabolic context of pancreatic cancer cells can influence the phenotype of associated immune cells and, by extension, expression of the lactate exporter MCT4 can further refine the disease-immune subtypes.

Materials and Methods

Tumor microarray construction and population study

Study cases were obtained from the surgical pathology files at Thomas Jefferson University with Institutional Review Board approval. The tissue microarray (TMA) contained tumor samples derived from 223 largely consecutive patients with PDA who had been treated at Thomas Jefferson University Hospitals between the years 2002 and 2010. Whole tissue section slides were constructed and TMAs were derived from them using a tissue arrayer (Veridim) as described previously (4). IHC was performed as described previously (21) on 4-μm TMA sections using a standard avidin–biotin immunoperoxidase method with antibodies specific for CD163 (1:100, clone 10D6, Leica), FOXP3 (1:50, clone 206D, Biolegend), PD-L1 (CD274, 1:200, clone E1L3N, Cell Signaling Technology), and MCT4 (1:250, developed and characterized by Dr. Nancy Philp (22)]. Staining was performed on a Ventana Benchmark automated stainer. TMA analysis

After staining, positive cells were counted and converted to a percentage of the total counted cells in a given field and expression of all examined markers was categorized as low or high based on either the median percentage of positive cells or 10% positivity (PD-L1), consistent with published reports (5, 23, 24). Correlation between markers was obtained using the spearman correlation method. Kaplan–Meier (KM) curves for both independent as well as combined markers were obtained using the survival package in R statistical software (25). Statistical significance and HR along with 95% confidence intervals for the KM curves was established using the log-rank P value obtained from Cox proportional hazard regression analysis. An unsupervised random forest (RF) clustering method was used for clustering analysis of immune markers (26). RF dissimilarity measure between markers was obtained and classified into clusters using the partitioning around medoids method; both implemented using RF and cluster packages in R. All heatmaps and three-dimensional scatterplots were obtained using heatmap.2 and rgl packages in R.

Immune infiltrate scoring

Lymphocytes and neutrophils were assessed in hematoxylin and cosin–stained whole tissue sections at 10× magnification in either the central area of the tumor (tumor cellular environment field) or at the tumor’s invasive margin (tumor stroma field). The presence of infiltrate was assessed using a four-degree scale in each area, where a score of 0 indicated no presence, 1 denoted a mild and patchy appearance of inflammatory infiltrate (rare), 2 signified a prominent inflammatory reaction (intermittent), and 3 represented dense infiltration (frequent).

Cells

Established pancreatic cancer cell lines (BXPC3, Capan-2, Hs766t, MIA PaCa-2, Panc-1, PL5, PL45) were cultured in DMEM +10% FBS. THP-1 cells were a generous gift from Dr. David Farrar and were maintained in RPMI1640 supplemented with 10% FBS, 1-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), nonessential amino acids, and β-mercaptoethanol (75 μmol/L). To visualize wound healing, PL45 cells were stably transfected with mCherry fluorescent protein. For MCT4 knockdown experiments, a mixture of Lipofectamine RNAiMAX transfection reagent (Life Technologies) and MCT4 siRNA (h2, sc-45892, Santa Cruz Biotechnology) was added to plates of 70% confluent pancreatic cancer cells as described by the manufacturer.

Macrophage differentiation and polarization

For macrophage differentiation assays, THP-1 cells were cultured for 72 hours in the presence of 50 ng/mL phorbol 12-myristate 13-acetate (PMA) or 50% conditioned media, as indicated. For polarization assays, following incubation in the presence of PMA, adherent THP-1 cells were washed and differentiation media was replaced with fresh supplemented RPMI with or without 50% conditioned media. Conditioned media was isolated from approximately 80% to 90% confluent established pancreatic cancer cell lines 72 hours postsplit, or posttransfection, centrifuged at 4,000 rpm for 10 minutes, and filtered with a 0.45-μm syringe filter to remove contaminating cells and debris before use. For protease K and lactic acid experiments, following conditioned media collection, samples were treated with protease K (50 ng/mL) for 30 minutes at 37°C followed by phenylmethylsulfonyl fluoride (PMSF, 5 mmol/L) for 1 hour at room temperature to inactivate protease K. Lactic acid (Sigma Aldrich) was added at 8 mmol/L. Lactate readings were acquired on an
Flow cytometry

Adherent THP-1 cells were scraped from culture dishes, collected by centrifugation, and resuspended in flow wash buffer (PBS + 1% FBS + 0.1% sodium azide). To stain for cell surface markers, cells were incubated in the presence of antibodies specific for galectin 3/Mac-2 (LGALS3, clone M3/38, Cedarlane Laboratories), CCR7/CD197 (clone 3D12), CD206 (MRCl, clone 19.2), and HLA-DR (clone L243; eBioscience). Cells were then washed and resuspended in 1% paraformaldehyde. Pancreatic cancer cells were collected by trypsinization and resuspended as described above.

For MCT4 staining, intracellular staining was performed as described previously (27) using anti-MCT4 antibody (clone H-90, Santa Cruz Biotechnology) conjugated with Alexa fluor 555 (Z/25305, Life Technologies). Following incubation, cells were washed with 0.1% saponin and then resuspended in flow wash buffer. All samples were collected on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (FlowJo).

Drug sensitivity

Cells from established pancreatic cancer cell lines were plated for 72 hours in the presence or absence of PMA-differentiated macrophages, pancreatic cancer cell-conditioned media–polarized macrophages, and gemcitabine (90 nmol/L). To the end of the time course, relative cell number was determined by CellTiter-Glo cell viability assay (Promega) and luminescence was read on a Synergy 2 multimode plate reader (Biotek Instruments).

Cancer cell invasiveness

THP-1 cells were plated in 12-well plates, differentiated for 72 hours with PMA, and then polarized with conditioned media overnight. The following day, fresh media was added and transwell membrane inserts with 0.4-um pores (Corning) were added onto each well. Pancreatic cancer cells (10,000 cells/membrane) were added to the top of each transwell membrane and cultured for 24 hours. The top of each insert was carefully washed with PBS and the remaining cells on the bottom of the membrane were fixed in 10% formalin and stained with hematoxylin. Membranes were cut from the transwell supports, placed on microscope slides, and imaged. The number of migrated cells was counted and the remaining cells on the bottom of the membrane were cut from the transwell supports, placed on microscope slides, and the remaining cells on the bottom of the membrane were imaged. The number of migrated cells was counted and the remaining cells on the bottom of the membrane were averaged number of cells was obtained from 10 high-powered fields. For wound-healing assays, equivalent numbers of either PMA-differentiated or conditioned media-polarized THP-1 cells were added to wells of culture dishes confluent with pancreatic cancer cells. Each well was scored with a pipet tip and imaged at the indicated time points at 40× total magnification on an EVOS Fl. Cell Imaging System (Life Technologies).

Results

Differential recruitment of immune cells to tumor and stromal compartments is associated with survival differences in PDA patients

Pancreatic ductal adenocarcinoma often arises in the background of chronic inflammation suggesting that perturbations in immune function can have significant impact related to tumor biology, disease prognosis, and therapeutic interventions. To elucidate the association of suppressive immune features in clinical cases a cohort of PDA cases (n = 223) was employed (Table 1). Within this cohort, inflammation, as defined by the presence of lymphocytes and neutrophils, was widely distributed throughout the cases (Supplementary Fig. S1A) and overall lymphocyte or neutrophil accumulation was not significantly associated with survival (Supplementary Fig. S1B).

Proper T-cell function is necessary for efficient recognition and subsequent elimination of tumor cells by the immune system. As such, suppression of T-cell function is associated with poor outcome in multiple malignancies, including pancreatic cancer (28). Under normal circumstances, FoxP3 regulatory T cells play a critical role in blunting the T-cell response to prevent autoimmunity; however, the role of these cells is subverted in the context of tumorigenesis and disease progression (29, 30). As PDA often contains abundant desmoplastic stroma, regulatory T cells were scored in the tumor cellular environment and tumor stroma (stroma). Approximately 50% of PDAs harbored FoxP3-positive infiltrate scored as high in at least one compartment (Fig. 1A). Higher levels of these cells only in the stroma were associated (HR = 1.395, P < 0.05) with worse survival and combined expression in both compartments was associated (HR = 2.038, P < 0.05) with decreased survival versus the absence of FoxP3 (Fig. 1B). These data suggest that the immune milieu of the surrounding stroma is an important determinant of disease outcome in addition to that of the tumor itself (Supplementary Table S1).

The presence of M2 (CD163+) macrophages was evaluated in both the tumor cellular environment and stroma (Fig. 1C). Expression of CD163 corresponded (HR = 2.058, P= 0.005) to overall survival when present in the tumor cellular environment, as opposed to stromal expression that only trended (HR = 1.481, P < 0.08) toward poor survival (Fig. 1D). Likewise, the combined absence of CD163 throughout both the tumor and stroma was suggestive (HR = 1.852, P < 0.08) of a favorable prognosis (Fig. 1D; Supplementary Table S2). Patients with tumors expressing low levels of both FoxP3 and CD163 had a significantly improved outcome [median survival (MS) = 45.6 months] when compared against patients with high levels of both markers (HR = 2.367, P > 0.017, MS = 21 months). Meanwhile, high CD163 expression in combination with low FoxP3 expression

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients 223 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>65 (27–89)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>121 (54)</td>
</tr>
<tr>
<td>Female</td>
<td>102 (46)</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
</tr>
<tr>
<td>0–2</td>
<td>42 (19)</td>
</tr>
<tr>
<td>2.1–4</td>
<td>123 (55)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>49 (22)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (4)</td>
</tr>
<tr>
<td>Node involvement</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>142 (64)</td>
</tr>
<tr>
<td>Negative</td>
<td>78 (35)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (1)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20 (9)</td>
</tr>
<tr>
<td>2</td>
<td>138 (62)</td>
</tr>
<tr>
<td>3</td>
<td>57 (26)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8 (3)</td>
</tr>
<tr>
<td>Vital status</td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>119 (53)</td>
</tr>
<tr>
<td>Dead</td>
<td>92 (42)</td>
</tr>
<tr>
<td>Unknown</td>
<td>12 (5)</td>
</tr>
</tbody>
</table>

Characteristics No. of patients 223 (%)
Immunologic and Metabolic Features can Define PDA Prognosis

Figure 1. Immunosuppressive cells are associated with poor outcome in PDA. A, representative images of high FoxP3 staining in the tumor cellular environment (TCE, >0%) and stroma (>9.5%) of patient sections. B, KM plots indicating survival probability in human patients with high or low accumulation of FoxP3+ cells in the tumor cellular environment and/or stroma. See Supplementary Tables S1 and S2. C, representative images of high CD163 staining in the tumor cellular environment (>30%) and stroma (>33%) of patient sections. D, KM plots indicating survival probability in human patients with high or low accumulation of CD163+ cells in the tumor cellular environment and/or stroma. See Supplementary Tables S3 and S4. E, KM plots indicating survival probability in human patients with high or low accumulation of CD163+ and FoxP3+ cells in the tumor stroma. See Supplementary Table S5.
was significantly associated (HR = 3.422, \( P < 0.005 \), MS = 13.8 months) with the shortest median survival (Fig. 1E, Supplementary Table S3).

Pancreatic cancer cells influence macrophage differentiation in a cell line–dependent manner

To further characterize the relationship between pancreatic cancer cells and macrophages, THP-1 cells were stimulated with conditioned media from various pancreatic cancer cell lines (Fig. 2A). Although the conditioned media demonstrated marked increases in macrophage differentiation over media alone, the response varied greatly between the different cell lines in terms of morphology (Fig. 2B) as well as overall adherence (Fig. 2C). Phenotypically, these differences were characterized by the increased prevalence of a galectin-3 (Mac-2)–negative population in MIA PaCa-2 or PL45 conditioned media–treated THP-1 cells as compared with those cells treated with Capan-2 conditioned media (Fig. 2D). Furthermore, these galectin-3–negative cells expressed markers indicative of both M1 (CCR7, HLA-DR) and M2 (CD206) polarized macrophages (Fig. 2E and F).

Macrophages differentiated by pancreatic cancer cells enhance cancer cell invasiveness

Given that PDA cell–conditioned media differentially induced macrophage differentiation and activation, the impact these macrophages played on mechanisms of pancreatic cancer severity was gauged. Regardless of the initial stimuli, treated THP-1 cells had a discernable effect on gemcitabine response (Fig. 2G). However, the addition of the macrophages resulted in some cell death in the cocultures, as evidenced by decreased cellularity in THP-1–only groups. Conditioned media–differentiated macrophages promoted pancreatic cancer cell invasiveness, as measured by Boyden chamber (Fig. 2H) and wound-healing assays (Fig. 2I and J). Of note, expedited wound healing is a cell line–specific phenomenon, as evidenced by the differential impact PMA-differentiated macrophages have on Capan-2 cells as compared with the other cell types.

Combined expression of immunosuppressive markers defines a subset of PDA patients with improved prognosis

The presence of alternatively activated, M2 macrophages and regulatory T cells in the PDA tumors suggested local immune evasion, whereby tumor cells escape immune surveillance. Therefore, the expression of PD-L1, which is associated with suppression of local immune engagement, was evaluated in the PDA cohort. Tumor-specific expression of PD-L1 was observed in the majority of PDA cases (Fig. 3A) and was associated (HR = 1.619, \( P = 0.056 \)) with overall survival (Fig. 3B) suggesting that the presence of PD-L1 could be important for the maintenance of immune evasion.

Tumors that were dually absent of FoxP3 and PD-L1 (Fig. 3C), or CD163 and PD-L1 (Fig. 3D) demonstrated prolonged overall survival. In all patients, the median survival was 21 months, whereas these dual negative cases had a median survival of greater than 45 months (Supplementary Tables S5 and S6). Importantly, this was not a feature of all combinations of these markers, as analysis of CD163– or FoxP3–negative stroma combined with the absence of PD-L1 expression had no such survival advantage. These data suggested that interactions between the immune markers could be clinically significant and define a subset of PDA patients with favorable prognosis.

RF analysis was used to cluster cases based on the presence of CD163, PD-L1, and FoxP3 (Fig. 3E). Using this approach, four clusters of cases that had distinct principle component features were defined (Fig. 3F). In particular, cluster 3, consisting of approximately 25% of cases in the cohort, was largely devoid of CD163, FoxP3, and PD-L1, and had improved overall survival that was statistically significant versus clusters 1 and 2 and trended for improved survival versus cluster 4 (Supplementary Table S7). In comparison with all other clusters, the cases in cluster 3 were significantly associated with improved outcome exhibiting a median survival in excess of 50 months (Fig. 3G; Supplementary Table S8).

Pancreatic cancer cell–mediated changes in glycolytic metabolites influence macrophage differentiation independent of cytokines

Local factors alter the function of immune cells in the tumor microenvironment. Established PDA cell lines secrete varying amounts of cytokines that would impact immune function, including high expression of CSF-1 by MIA PaCa-2 cells (31). To determine whether the differences in macrophage activation were attributable to variations in cytokine secretion, THP-1 cells were cultured in protein-depleted conditioned media. Although protein depletion caused a significant reduction in macrophage adherence (Fig. 4A), the phenotypic differences remained (Fig. 4B). These data suggest that although secreted proteins (e.g., cytokines and chemokines) are important mediators of THP-1 adherence, additional factors are sufficient to skew macrophage polarization.

Glycolysis has also been identified as a regulator of the inflammatory response (32). The PDA microenvironment is glycolytic in comparison with surrounding tissue and has been associated with poor outcome in PDA (4). As such, we investigated how the glycolytic environment impacts immune cell activation in PDA. Similar to our previous report (4), various established PDA cell lines differentially express MCT4 (Fig. 4C), which serves to export...
increased levels of lactate produced via glycolysis to the extracellular environment. Accordingly, the cell lines with increased MCT4 expression consume more glucose and secrete more lactate than those with lower MCT4 expression (Fig. 4D). Meanwhile, the addition of exogenous lactic acid to conditioned media negated the phenotypic differences induced by the different PDA cell lines (Fig. 4E). To determine whether MCT4 expression on PDA cells influenced immune response, MCT4 was knocked down in PDA cell lines (Fig. 4F). Conditioned media from these cells was added to undifferentiated THP-1 cells and in all cases, MCT4 knockdown resulted in decreased THP-1 cell differentiation, as measured by macrophage adherence, when compared with control cells (Fig. 4H). Although the addition of lactic acid did not increase THP-1 adherence in these cells following MCT4 knockdown (Fig. 4H), this treatment was sufficient to rescue CCR7 and CD206 expression (Fig. 4I). These data are consistent with our earlier findings (Fig. 4A and B) in suggesting differential roles for glycolytic metabolites in adherence and polarization.

The inclusion of MCT4 expression enhances the prognostic value of immunosuppressive biomarkers

Given the role of MCT4 in regulating immune function, additional prognostic power could be added by incorporating immunologic markers with MCT4 expression. Thus, we investigated the relationship of MCT4 expression in PDA with the immune-related markers. These data showed that MCT4 status in both the tumor cellular environment and stroma were positively correlated with CD163 expression in the tumor cellular environment (Fig. 5A).
All other markers exhibited a tenuous or nonsignificant association with MCT4 in the tumor cellular environment, but interestingly stromal MCT4 correlated with veritably all immunologic features (Fig. 5A). These findings suggest that although there is a relationship between the glycolytic state of the tumor and the immunologic features of disease, the dual absence of both is associated with increased overall survival. RF clustering revealed the presence of discrete clusters that were separated on the basis of distinct principle component features (Fig. 5B). Cluster 4 lacked almost all immune markers and the expression of MCT4 in the stroma. In KM analysis, this cluster harbored particularly favorable overall survival (Fig. 5C; Supplementary Table S9). Importantly, this cluster was significant (HR = 3.852, P < 0.005, MS > 53 months) versus all other cases (MS = 20.6 months) and remained significant in multivariate analysis including grade and lymph node status (Fig. 5D; Supplementary Tables S10 and S11).
Discussion

On the basis of underlying tumor metabolism and mutation status, PDA actively promotes an inflammatory and immuno-suppressive microenvironment by recruiting macrophages and other immune cells. The composition of the cellular immune microenvironment in PDA is associated with variable patient outcome. We examined the tumor microenvironment of patients with PDA and identified tumor characteristics that were independently associated with prognosis. In addition, PDA can influence macrophages recruited to the tumor through alterations in the metabolic environment. MCT4 expression, a surrogate marker for the glycolytic tumor microenvironment, influenced macrophage differentiation in vitro and provided additional prognostic discrimination in patients.

The immune system is designed to recognize and remove pathologic insults, including malignancy, with the survival of cancer cells suggesting a host immune deficiency or an active evasion by cancer cells. To this end, the makeup of the immune milieu in the PDA tumor microenvironment and the effects these cells have on pathogenesis and prognosis has garnered increased attention. Since cytotoxic (CD8⁺) T cells are abundant in PDA (14), the lack of tumor cell death would seem to be the result of an immuno-suppressive microenvironment instead of an intrinsic defect of adaptive immunity. This is further supported by immunotherapy models where targeting the T-cell checkpoint PD-L1 improved the efficacy of systemic therapy (23). However, the use of immune checkpoint blockade therapeutics as single-agent treatments has resulted in only minimal success (15, 33, 34), suggesting that some additional aspects of PDA or the tumor microenvironment are promoting immuno-suppression.

The combined blockade of both myeloid differentiation signaling (CSF1R) and T-cell checkpoint signaling (PD-L1) has been shown to be more effective than either approach individually (35). Although these data suggest that myeloid cell–mediated promotion of immuno-suppression directly contributes to the general lack of efficacy of single-agent immunotherapeutics in PDA, the mechanisms that promote this phenomenon remain unclear. Thus, although the emergence of this suppressive immune environment supporting PDA development provides new possibilities to distinguish disease subtypes and highlights new opportunities for therapeutic interventions, additional insights are necessary to guide these beneficial outcomes.

PDA is associated with a significant desmoplastic stroma that harbors infiltrating immune cells. Previously, increased tumor infiltration of FoxP3-, CD163-, or PD-L1-expressing cells or M2 macrophages has been associated with tumor progression or poor outcome (5, 24, 36, 37). We expand on this finding by demonstrating the importance of the tumor stroma to the development of this suppressive phenotype. PDA stroma has been shown to

Figure 5. Suppressive immune features and MCT4 expression define prognostic disease subtypes in PDA. A, Pearson correlation heat maps of immune markers and MCT4 expression. B, heat maps of unsupervised RF clustering of immune marker and MCT4 expression. C, KM plots indicating survival probability in human patients based on the previously defined immune marker and MCT4 expression clusters. See Supplementary Table S12. D, KM plots indicating survival probability in human patients from the cluster of lowest complex expression (cluster 4) with those from clusters with high expression of at least one marker (clusters 1, 2, 3, 5). See Supplementary Tables S13 and S14.
support the recruitment and accumulation of immune cells, demonstrating a phenotype high in proinflammatory cytokine and chemokine expression (7). RF clustering of our suppressive immune phenotypes reflects the association between suppressive immune cells in the stroma and inferior outcomes (clusters 1 and 2). Although the exact mechanism is not clear, the stroma in PDA promotes primary tumor growth by creating an immunosuppressive microenvironment. Further examination of the relationship between the primary tumor and the stroma is important to better understand the biology of this aggressive cancer and to help devise novel therapies.

It has recently been demonstrated that macrophages are critical for PDA tumor growth and that KRAS status plays an important role in macrophage recruitment and activation (38–40). To explore this further, here we utilized a system by which cells from the monocytic THP-1 line were treated with conditioned media isolated from established PDA cell lines in vitro. Our data demonstrate that these PDA cell lines differentially influence macrophage development. For example, the phenotype induced by conditioned media isolated from MIA PaCa-2 and PL45 cells (CCR7⁺CD206⁺) strongly resembles that of myeloid-derived suppressor cells (MDSC), which would further enhance the immunosuppressive environment (41). These cells also express the increased HLA-DR expression as compared with cells differentiated by Capan-2 conditioned media. It has previously been shown that the expression of HLA-DR on MDSCs is associated with their ability to induce CD4⁺ T-cell tolerance, further reducing the capability for an effective antitumor immune response (42). However, HLA-DR expression in these cells also provides a mechanism by which specifically designed CD4⁺ T cells can convert these MDSCs, decreasing their suppressive efficacy (42). This suggests that PDA influences the immune microenvironment by modulating macrophage populations.

Tumor-associated macrophages have been shown to promote tumor growth through various mechanisms including TLR4/IL10–mediated epithelial-mesenchymal transition, promigratory factor secretion, and enhanced drug resistance (37, 43–46). Here, we show that macrophages increase invasiveness in established PDA cell lines, as demonstrated by increased recruitment of PDA cells (Fig. 2H) and expedited wound-healing response (Fig. 2I and J) in the presence of macrophages differentiated by PDA-conditioned media. MIA PaCa-2 and PL45 cells were able to overcome the effect of PMA-differentiated macrophages on wound healing whereas Capan-2 cells were not. Thus, our data suggest a PDA cell–specific mechanism that facilitates tumor growth and prevents antitumor response, in line with previous reports (38, 39, 47). Taken together, these data highlight the complex regulation of the immune environment in PDA.

Although it has been shown that tumor-derived cytokines such as GM-CSF can directly influence the immune milieu in and around the tumor (38, 39), our data indicate that a cytokine-independent mechanism also contributes to skew immune cell differentiation (Fig. 4B). PDA can directly influence macrophages, myeloid cells, and the tumor microenvironment by altering the local metabolic conditions. It has become increasingly evident that glycolysis plays an important role in regulating immune response, particularly where increased glycolysis produces glycolytic by-products like lactic acid (32). In macrophages, high levels of intracellular lactate result in decreased responsiveness to LPS, and thus macrophage MCT4 expression is associated with more effective inflammatory response (16). Conversely, tumor-derived lactic acid is capable of skewing macrophages toward the anti-inflammatory, tumor-promoting M2 phenotype (48). Here we show decreased macrophage differentiation in THP-1 cells treated with conditioned media from cell lines where MCT4 has been knocked down. These data provided sufficient rationale for examining how PDA expression of MCT4 is associated with immune markers of disease.

Extensive profiling of established PDA cell lines has revealed distinct stratification of these lines on the basis of their metabolic characteristics. These findings demonstrate that various cell lines are fundamentally predisposed toward different metabolic programs, including a subset of cells with an elevated reliance on glycolysis (49). Along these lines, we have recently shown that enhanced glycolysis in PDA, represented by the expression of MCT4, is associated with prognosis. Our findings demonstrated that glycolysis was largely suppressed in PDA cell lines with higher endogenous levels of MCT4 (e.g., PL45 and MIA PaCa-2) whereas cell lines with lower endogenous MCT4 expression were generally unaffected (4). Here we have demonstrated that MCT4 knockdown is sufficient to attenuate lactate efflux and reduce glucose uptake (Fig. 4G), consistent with our previous findings, which also indicated that MCT4 is necessary to maintain proper lactate production (4). Strikingly, patients that had low expression of MCT4 in conjunction with diminished markers of suppressive immune cells demonstrated significantly improved survival as compared with other clusters. These data suggest that a combination of immune and metabolic features in PDA define subsets of disease that correspond to prognosis. Taken together, these data suggest that the individual metabolic state of PDA malignancies may portend the efficacy of potential immunotherapies and that, ultimately, a combined approach comprised of immunotherapy and a targeted metabolic therapy may increase treatment efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Hutcheson, M.R. Porembka, P.A. McCue, E.S. Knudsen, A.K. Witkiewicz

Development of methodology: J. Hutcheson, A.K. Witkiewicz

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Hutcheson, M.B. Wachsmann, P.A. McCue, A.K. Witkiewicz

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Hutcheson, M.R. Porembka, M.B. Wachsmann, E.S. Knudsen, A.K. Witkiewicz

Writing, review, and/or revision of the manuscript: J. Hutcheson, U. Balaji, M.R. Porembka, M.B. Wachsmann, E.S. Knudsen, A.K. Witkiewicz

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.S. Knudsen

Study supervision: A.K. Witkiewicz

Grant Support

This work was supported by grants from NIH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 4, 2015; revised January 7, 2016; accepted January 25, 2016; published OnlineFirst February 8, 2016.
References


Clinical Cancer Research

Immunologic and Metabolic Features of Pancreatic Ductal Adenocarcinoma Define Prognostic Subtypes of Disease


Updated version
Access the most recent version of this article at:

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/02/06/1078-0432.CCR-15-1883.DC1

Cited articles
This article cites 45 articles, 14 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/22/14/3606.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/22/14/3606.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.