Immunotherapy in Pancreatic Adenocarcinoma: Beyond "Copy/Paste"

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ABSTRACT

Immunotherapy has dramatically changed the cancer treatment landscape during the past decade, but very limited efficacy has been reported against pancreatic cancer. Several factors unique to pancreatic cancer may explain the resistance: the well-recognized suppressive elements in the tumor microenvironment, the functional and structural barrier imposed by the stroma components, T-cell exhaustion, the choice of perhaps the wrong immune targets, and microbial factors including gut dysbiosis and the unexpected presence of tumor microbes. Furthermore, we discuss various strategies to overcome these barriers.

Introduction

Immunotherapy has revolutionized cancer medicine, providing long-lasting disease control in malignancies that were previously uniformly fatal (1). One illustrative example is seen in metastatic melanoma, in which the median survival has doubled from 8–12 months to greater than 24 months with the advent of immunotherapy (2). In some cases—former President Jimmy Carter being one well-known example—immunotherapy can result in metastatic cancer cures. While the promise of immunotherapy has profoundly altered the management of many cancer types, this paradigm shift has not extended to pancreatic ductal adenocarcinoma (PDAC). Indeed, PDAC, with an incidence of 12.9 per 100,000 is much less common than cancers such as breast, lung, and prostate, and yet in 2019 was the third leading cause of cancer-related death (3), and it is projected to become the second leading cause of cancer-related death by 2030 (4).

Treatment options for PDAC remain quite limited. Surgical resection is the only curative option but possible in only 15% to 20% of cases due to diagnosis at advanced stages in most cases. Even following major surgery and adjuvant chemotherapy with 5-fluorouracil (5-FU), irinotecan, and oxaliplatin (FOLFIRINOX), disease recurrence is common; a recent study showed distant and/or locoregional recurrence over 50% at a median follow-up of 33.6 months (5). For those patients who are more commonly diagnosed with locally advanced or metastatic disease, medical therapies are limited to combination chemotherapy. For the most medically fit patients with good performance status, combination therapy with FOLFIRINOX has shown to improve progression-free survival (PFS) from 3.3 months with single-agent gemcitabine to 6.8 months with the combination. Still, median overall survival (OS) with this regimen remains poor at 11.1 months (6). For those patients who do respond to treatment, toxicities including myelosuppresion, peripheral neuropathy, diarrhea, and profound fatigue can frequently decrease the quality of life. The second regimen used is combination of gemcitabine and nab-paclitaxel which prolongs survival to 8.5 months compared with single-agent gemcitabine (7). While generally better tolerated, this regimen nonetheless comes with its own unique toxicities which include neuropathy and myelosuppression.

It is clear that more effective therapies are desperately needed for PDAC. Despite herculean research efforts from the scientific community and pharmaceutical industry to combat this disease, even incremental improvements in terms of toxicity profile and OS remain elusive. However, there are several promising avenues of research which have shown hope in making inroads in the fight against PDAC, including the use of immunotherapy. As Robert Vonderheide, professor and immunologist expert in pancreatic cancer at University of Pennsylvania (Philadelphia, PA), once stated at a national meeting, "copy–paste of immunotherapy from other cancers to pancreatic cancer will not work". To date, clinical trials in the realm of immunotherapy in PDAC have been disappointing. In a review article, Henriksen and colleagues pointed to over 20 published trials using checkpoint inhibitors in PDAC (8). A trial of CTLA4 monotherapy showed no responders. Two trials of PD-1 or PD-L1 inhibitor monotherapy showed no objective response for anti-PD-L1 monotherapy, and combined PD-L1 inhibition with CTLA4 inhibition showed a low disease control rate of only 9%. Trials of combination chemotherapy and immunotherapy have had equally lackluster results. For example, two clinical trials which evaluated combined CTLA4 inhibition and gemcitabine therapy showed similar efficacy rates to gemcitabine monotherapy. Another trial evaluated nivolumab with combination gemcitabine/nab-paclitaxel for patients with advanced PDAC, and showed a PFS of 5.5 months, identical to PFS seen in the original study from Von Hoff that led to approval of combination gemcitabine/nab-paclitaxel in 2013. The lack of ongoing trials of checkpoint inhibitor monotherapy point to absence of clinical response or OS demonstrated to date with use of these agents in PDAC.

Here we explore the potential explanations for PDAC tumor resistance mechanisms to immunotherapy and compile strategies to overcome resistance to immunotherapy in PDAC, an approach that still holds promise for the control of this disease.

PDAC Tumor Microenvironment is Highly Immunosuppressive

The PDAC tumor microenvironment (TME) has come into focus as a crucial adaptive mechanism by which cancer cells evade immune

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surveillance, enabling progressive invasion and distant metastatic spread (9). The PDAC TME is mostly comprised of pancreatic stellate cells (PSC), extracellular matrix (ECM), and immunosuppressive cellular populations (10). The TME can be heavily influenced by host factors including obesity, a known risk factor for development of PDAC (11). Gomez-Chou and colleagues have recently demonstrated that higher levels of lipocalin-2 (LCN2), an adipokine elevated in the serum and in adipose tissue of obese individuals, can adversely modulate the TME through a variety of mechanisms including activation of PSCs with subsequent stromal remodeling, and upregulation of tumor-associated macrophages (TAM), which are implicated in ductal metaplasia leading to PDAC development (12).

The activities of TAMs include secretion of immunosuppressive cytokines and enzymes such as TGFβ and IL10, interference with the metabolism of effector T cells, recruitment of regulatory T cells (Treg), and suppression of cytotoxic $CD8^+$ T-cell activation (13). TAMs, in turn, upregulate PSCs, further contributing to the immunosuppressive microenvironment of PDAC (14).

Myeloid-derived suppressor cells (MDSC) are immune cells that play a critical role in pancreatic cancer by suppressing the function of effector T cells through numerous pathways, including through the use of reactive oxygen species (ROS), production of adenosine, and by inducing *de novo* Treg development through secretion of IFNγ and IL10 (15). Zhang and colleagues have shown that *in vivo* genetic depletion of myeloid cells in murine PDAC transgenic models resulted in tumor arrest and $CD8^+$ T-cell–dependent tumor regression (16).

Treg $CD4^+$ lymphocyte–infiltrating tumors are linked to worse outcomes in patients with PDAC (17). However, recently Zhang and colleagues have depleted Tregs in transgenic murine models of PDAC and unexpectedly, tumor progression was accelerated by reprogramming of the fibroblast population, highlighting the complex relationships between Tregs and fibroblasts in PDAC (18).

Another protumorigenic cell type that infiltrates the pancreas during tumorigenesis, particularly in the context of chronic pancreatitis, is Th17. These cells are the main source of IL17, a cytokine that binds IL17RA, which is overexpressed in pancreatic epithelium upon *Kras* oncogenic activation, resulting in increased immunosuppression through various mechanisms including induction of genes related to embryonic stemness and secretion of chemokines that attract MDSCs and neutrophils to the TME (19). GM-CSF is a cytokine that is also secreted in the context of oncogenic *Kras*-dependent pancreatic tumorigenesis, and also has the capacity of attracting suppressive myeloid cells that interfere with $CD8^+$ T-cell infiltration and tumor cell killing (20).

Neutrophils account for another immune-suppressive population present in the PDAC TME and their depletion in pancreatic cancer murine models have resulted in antitumorigenic effects (21). Mechanisms of immune suppression via neutrophils include the secretion of cytokines and chemokines, and suppression of cytotoxic $CD8^+$ T lymphocytes (22, 23). Production of neutrophil extracellular traps (NET) has been proposed as another mechanism of immunotherapy resistance induced by neutrophils. There are several known inducers of NETs, and recently IL17 has been described as an indirect NET inducer, as it needs to first interact with pancreatic tumor cells (24).

An additional crucial resistance mechanism of the PDAC TME is angiogenesis by way of VEGF, which leads to disorganized tumor vasculature, hypoxia, high interstitial pressures, and low pH in the TME, which serves to hinder the trafficking and effector function of immune cells. VEGF facilitates an immunosuppressive environment through multiple pathways in addition to angiogenesis, including the direct inhibition of cytotoxic T-cell trafficking and function, inhibition of antigen presentation, and recruitment of the abovementioned immunosuppressive cells including Tregs, MDSCs, and M2-like TAMs to the TME (25). The use of VEGFR inhibitors in combination with immunotherapy has been successful in several tumor types including hepatocellular carcinoma (HCC) and renal cell carcinoma (RCC), through normalization of the tumor vasculature, and is currently being evaluated in pancreatic cancer in combination with immunotherapy (26).

Combinatorial Strategies to Target the PDAC TME

Despite the complex interactions of these various cell signaling pathways in the PDAC TME, several trials are currently underway employing combination strategies to overcome the PDAC immunosuppressive TME. These completed and ongoing trials combine traditional chemotherapy or immunotherapy with drugs or antibodies that target the suppressive TME, rather than the cancer cells themselves (**Table 1**).

One such combinatorial strategy involves inhibition of colony-stimulating factor-1 receptor (CSF1R; which recruits TAMs to the TME) combined with immunotherapy. CSF1R inhibition has been shown to upregulate T-cell checkpoint molecules including PD-L1 and CTLA4 (27). Nywening and colleagues have shown that the use of small-molecule inhibitors against chemokine receptor type 2 (CXCR2) receptor expressed in the membrane of tumor-associated neutrophils and MDSCs can induce antitumor immunity against PDAC when combined with chemotherapy (28). CXCR2 and its ligands are responsible for recruiting neutrophils to the TME, which contribute to tumor immune evasion. Combined CCR2 and CXCR2 blockade, along with FOLFIRINOX chemotherapy, increase OS in a KPC mouse model. Loss of p53 can induce overexpression of CCL2, which further recruits suppressive myeloid cells into the TME, and promotes epithelial-to-mesenchymal transition (EMT), which has been associated with resistance to immunotherapy (29). The CCL2/CCR2 and chemokine receptor type 4 (CXCR4) receptor pathways that are involved in the recruitment of immunosuppressive monocytes to the TME and are currently under evaluation as drug targets in conjunction with chemotherapy and immunotherapy (10). Recent preclinical work has also demonstrated a decrease in total immune infiltration of suppressive myeloid cells through the inhibition of CCR1 (18). Genetic or pharmacologic deletion of neutrophils or NETs can reverse PDAC resistance to checkpoint blockade (24).

Other current avenues for research include the use of radiotherapy and/or radiofrequency ablation in conjunction with immunotherapy to circumvent the naturally suppressive TME of PDAC (27). An "abscopal effect" has been posited as justification for use of radiotherapy in conjunction with a systemic immune-stimulatory agent in metastatic solid tumors. However, recent data indicate that in tumors with low immunogenicity such as PDAC, multiple levels of immune activation may be required to see a desired effect (30). One novel ablative strategy involves irreversible electroporation (IRE), a non-thermal ablative technique in which high-voltage electrical pulses are applied directly to the tumor. The phase II PANFIRE trial explored the use of IRE in patients with locally advanced or recurrent PDAC, in conjunction with cytotoxic chemotherapy. The target median OS exceeded, and a phase III trial in conjunction with immunotherapy is currently under way (31). Another combinatorial approach is the use of "super" IL15 in conjunction with immunotherapy, which acts as a long-acting cytokine to increase circulating natural killer (NK) cells, as well as activate $CD8^+$ T cells in the TME. The use of "super" IL15 has

Table 1. Summary of ongoing combinatorial clinical trials in PDAC.

Trial Name	Identifier	Sponsor	Treatment strategy	Resistance mechanism
Pilot study with cyclophosphamide, pembrolizumab, GVAX, and IMC-CS4 (LY3022855) in patients with borderline resectable adenocarcinoma of the pancreas	NCT03153410	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	Combination of vaccine, chemotherapy, checkpoint blockade, and CSF1-R inhibition	Suppressive TME/T-cell exclusion/exhaustion
A study to evaluate the safety and tolerability of SX-682 in combination with nivolumab as maintenance therapy in patients with metastatic panceatic ductal adenocarcinoma	NCT04477343	University of Rochester	Combination of checkpoint blockade and CXCR1/2 inhibition	Suppressive TME
Trial of neoadjuvant and adjuvant nivolumab and BMS-813160 with or without GVAX for locally advanced pancreatic ductal adenocarcinomas	NCT03767582	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	Combination of vaccine, checkpoint blockade, and CCR2/5 inhibition	Suppressive TME
Plerixafor and cemiplimab in metastatic pancreatic cancer	NCT04177810	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	Combination of bone marrow stimulant and checkpoint blockade	T-cell exclusion/exhaustion
Chemo4METPANC combination chemokine inhibitor, immunotherapy, and chemotherapy in patients with pancreatic adenocarcinoma	NCT04543071	Columbia University	Combination of chemotherapy, checkpoint blockade, and CXCR4 inhibitor	Suppressive TME/T-cell exclusion/exhaustion
LOAd703 oncolytic virus therapy for pancreatic cancer	NCT02705196	Lokon Pharmaceuticals	Combination of immunostimulatory oncolytic adenovirus, chemotherapy, and checkpoint blockade	T-cell exclusion/exhaustion
Study of CRS-207, nivolumab, and ipilimumab with or without GVAX pancreas vaccine (with cyclophsphamide; in patients with pancreatic cancer	NCT03190265	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	Combination vaccine and checkpoint blockade	T-cell exclusion/exhaustion
Paricalcitol and hydroxychloroquine in combination with gemcitabine and NabP for the treatment of advanced or metastatic pancreatic cancer	NCT04524702	Emory University	Combination of Vit D, chemotherapy, and an autophagy inhibitor	Fibrotic stroma
Study of pembrolizumab with or without defactinib following chemotherapy as a neoadjuvant and adjuvant treatment for resectable pancreatic ductal adenocarcinoma	NCT03727880	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	Combination of checkpoint blockade and FAK inhibition	Fibrotic stroma

Abbreviation: Vit D, Vitamin D.

been tested in patients with advanced solid tumor malignancies and found to be safe while inducing a strong immune response. Its efficacy in combination with checkpoint blockade is currently under investigation (32). Galunisertinib, a TGFβ receptor inhibitor that blocks this strongly immunosuppressive cytokine, is also currently under investigation in combination with both cytotoxic chemotherapy and immunotherapy (3). It should be noted that toxicity may be a limiting factor as researchers continue to explore combinations of chemotherapy and immunotherapy regimens in patients with PDAC (33, 34).

T-cell Exclusion or Inactivation

On the basis of the dense desmoplastic stroma and tumor infiltration with the immune-suppressive cells described above, recent models of the human PDAC tumor microenvironment point to immune exclusion as a key factor for resistance to therapies (35). Novel imaging techniques that allow for spatial analysis of multiple cellular types within PDAC tissue have found that high levels of total T-cell infiltration are associated with prolonged survival, desmoplastic elements do not impair cytotoxic T-cell infiltration, and close proximity of cytotoxic T cells to pancreatic cancer cells significantly correlates with prolonged patient survival (36). A second study on PDAC immune infiltrates spatial analysis also confirmed a significant association between high $CD8^+$ cell infiltration in the tumor as a whole and improved patient survival. Interestingly, the authors describe a favorable prognostic association with higher $CD8^+$ cell density in the tumor center but not in the tumor margin (37). These findings suggest that the combination of T-cell density data at the tumor center and the tumor margin could be applied as a prognostic tool in PDAC (37). Another recent study proposed that not only the degree of intratumoral T-cell infiltration, but the degree of "attack" as measured by the presence of intraepithelial lymphocytes indicate a more favorable prognosis. More specifically, $CD8^+$ intraepithelial attack was an independent favorable prognostic indicator for OS, while $CD8^+$-high intratumoral infiltration without $CD8^+$ T-cell intraepithelial attack was a poor prognostic factor (38).

Given these findings of a microenvironment characterized by effector T-cell exclusion, current research has focused on enhancing both the quantity and the quality of the immune infiltrate in the pancreatic tumor milieu. One promising avenue of research has explored the inhibition of CXCR4, an alpha chemokine receptor that inhibits T-cell chemotaxis. Using time-lapse confocal microscopy on fresh PDAC tumors treated in organotypic slice culture, researchers demonstrated enhanced tumor-cell apoptosis through the combined use of PD-1 and CXCR4 blockade, as compared with PD-1 monotherapy or control (35). These results showed a striking shift in the distribution of $CD8^+$ T cells from the fibroblastic stroma into the immediate juxtatumoral stroma using combined PD-1 and CXCR4 blockade, indicating that the sequestration of already clonally expanded tumor-reactive T cells, rather than lack of immunogenicity, is responsible for the lack of efficacy of immunotherapy in PDAC, and that this sequestration can be reversed through targeted therapy (35). However, the transient nature of these T-cell responses may be a limiting factor, and likely more robust T-cell priming is vital to not only expanding preexisting antitumor T-cell clones, but recruiting new T-cell clones to the TME (35). One proposed strategy involves activation of CD40 found on both dendritic cells and B cells, which has been shown in mouse models to reverse T-cell exhaustion through the upregulation of cytokines, antigen-presenting molecules, costimulatory molecules, and adhesion molecules (39). Chemotherapy with gemcitabine and nab-paclitaxel, followed by CD40 activation led to the establishment of effective, T-cell–dependent immunity and memory in mouse models compared with CD40 alone, presumably due to the "spill" of antigens induced by chemotherapy (40). The addition of PD-1 or CTLA4 inhibition has been shown to further extend the activity and durability of response to chemotherapy plus CD40 activation (41). Clinical trials with various CD40 antibodies in combination with chemotherapy and/or immunotherapy are now underway across multiple tumor types, including PDAC (**Table 1**; ref. 39).

Another approach to improve T-cell activation is the use of vaccines. The laboratory of Elizabeth Jaffee at Johns Hopkins University (Baltimore, MD) has pioneered the development of vaccines for pancreatic cancer for more than 20 years (42–44). The first vaccine developed was granulocyte-macrophage colony-stimulating factor gene transfected tumor cells vaccine (GVAX), which is capable of recruiting antigen-presenting cells (APC), mostly dendritic cells (DC), to the vaccination site which results in priming of $CD8^+$ T cells by a mechanism of cross-priming (42, 43). The second vaccine, CRS-207, is made of deactivated Listeria-expressing mesothelin, a tumor-associated antigen (TAA) highly expressed in PDAC, with the goal of also priming T cells. Several murine studies have shown efficacy of combining vaccines with checkpoint inhibitors (45), and previous and current trials are testing combinatorial strategy (46). A trial with GVAX/cyclophosphamide followed by CRS-207 with or without nivolumab was completed, and though it did not meet the primary endpoint of improvement in OS, there were beneficial effects seen within the TME including an increase of $CD8^+$ T cells and decrease in $CD68^+$ myeloid cells (47).

In the future, the development of vaccines for PDAC will likely continue and will likely include combination with chemotherapy and other checkpoint blockade agents or TME-targeting agents.

Highly Fibrotic TME

The fibrotic and desmoplastic stroma in PDAC has been postulated as a structural and functional barrier for therapeutics delivery and efficacy (48). PSCs form the main component of tumor stroma and are inactive in healthy tissue, but secrete extracellular matrix proteins including collagen and fibronectin in the presence of tissue injury or tumorigenesis, decreasing access of immune cells to the tumor (49). In mice coinjected with pancreatic cancer cells plus PSCs, the suppressive immune-cell population of regulatory T cells, M2-type macrophages, and MDSCs was significantly increased, while the immune-cell populations of $CD8^+$ T cells, $CD4^+$ T cells, and M1 macrophages in the tumor tissues were significantly decreased compared with mice solely injected with pancreatic cancer cells (49). The PDAC stromal reaction is mostly driven by the conversion of quiescent to activated PSCs. One strategy to combat the fibrotic PDAC stroma is by targeting of the vitamin D receptor (VDR), which is expressed in the PDAC stroma. Therapy with calcipotriol, which binds VDR, decreases inflammation and fibrosis in a pancreatitis murine model (50). On the basis of this finding, combination of Vitamin D with chemotherapy has been proposed, and clinical trials are underway.

Hedgehog (Hh) signaling, which is absent in the normal adult pancreas, is known to be upregulated in pancreatic cancer stem cells (CSC), facilitating tumorigenesis through paracrine signaling to stromal fibroblasts, which in turn stimulates growth of the fibrotic and immune-suppressive tumor stroma (51). The targeting of Sonic hedgehog (Shh), a soluble ligand overexpressed by PDAC cells, presented a reasonable new avenue of therapeutic research after preclinical studies revealed that Shh inhibition resulted in major improvement in outcomes by facilitating entry of chemotherapy into pancreatic tumors (52). On the basis of these remarkable results, high expectations were built over the clinical trial using standard chemotherapy (gemcitabine/nab-paclitaxel) plus vismodegib (an Hh inhibitor) in patients with metastatic PDAC. Unfortunately, results from a phase II single-arm therapy reported no clinical benefit in this patient population (53, 54).

In light of the clinical findings, a PDAC mouse model was generated with genetic deletion of *Shh*, which resulted in Shh-deficient tumors with significantly reduced stroma. However, these genetically altered mice lacking *Shh* expression developed PDAC tumors faster and had a significantly decreased survival compared with control mice, indicating that *Shh*—and by extension the fibrotic PDAC tumor stroma itself—may in fact restrain tumor progression and aggressiveness (55). Interestingly, these stroma-depleted tumors were found to be more sensitive to VEGFR inhibition, very likely due to increased blood vessel density (55). A second study also used transgenic mouse models to selectively deplete aSMA myofibroblasts in a PDAC mouse model, with a resultant decrease in tumor stroma but an increase in poorly differentiated tumors and significantly diminished survival were found compared with control PDAC mice (56). Myofibroblast-depleted tumors had an increase in the percentage of Tregs, with an overall decrease in the ratio of effector T cells to Tregs. Myofibroblast depletion also resulted in increased CTLA4 expression and anti-CTLA4 antibodies significantly increased mice survival (56). Analysis of PDAC specimens revealed that low aSMA was associated with worse survival (56). Recently, the groups that led these clinical trials reported no differences in number of cancer stem cells or stromal density betwen tumors from visomodegib-treated patients compared with those untreated, questioning the activity of the drug in influencing these components (53, 54).

Focal adhesion kinase (FAK) and specifically FAK1 is a nonreceptor tyrosine kinase which is implicated in activating proinflammatory cytokines and upregulating pathologic fibrosis. A 2016 study published in Nature Medicine showed that FAK1 is upregulated in PDAC, and tumors with high FAK1 expression had higher levels of total stromal collagen and collagen I deposition. The same group demonstrated a

synergistic effect in a KPC mouse model between a FAK inhibitor, gemcitabine chemotherapy, and anti-PD-1 immunotherapy as measured by reduced tumor burden and improved OS. Mice treated with this regimen had significantly increased number of $CD8^+$ tumor-infiltrating lymphocytes (TIL) compared with mice treated with gemcitabine and anti-PD-1 immunotherapy alone (57). Several FAK inhibitors are currently under investigation in clinical trials.

Another stromal-related reason for the lack of penetration of drugs is attributed to the extremely high interstitial fluid pressures (IFP) seen in the PDAC microenvironment. A study using KPC mice with autochthonous PDAC tumors demonstrated IFPs in excess of 10 times that of mice with normal pancreata. The elevated IPF in KPC mice was directly associated to hyaluronic acid (HA) deposition in the pancreatic tumor ECM. Administration of pegylated recombinant human hyaluronidase (PEGPH20), an engineered form of the enzyme hyaluronidase with longer half-life, resulted in rapid appearance of patent blood vessels in the TME and normalization of IFP. The combination of PEGPH20 with gemcitabine in spontaneous murine models of PDAC resulted in prolonged OS compared with gemcitabine alone (58). Given this promising preclinical data, a phase Ib/II trial of PEGPH20 in combination with gemcitabine/nab-paclitaxel compared with nab-paclitaxel alone was performed, but it did not show improvements in PFS (5.7 months vs. 5.2 months, $P = 0.11$; ref. 59). However, a subgroup analysis based on HA content showed a significant improvement in overall response rate (52% vs. 24%, $P = 0.038$) in patients with tumors of high HA compared with low HA (59). In parallel, a Phase IB/II randomized study of FOLFIRINOX plus human hyaluronidase PEGPH20 compared with FOLFIRINOX alone was performed in patients with metastatic pancreatic cancer with good performance status [Eastern Cooperative Oncology Group (ECOG) 0–1]. Surprisingly, the trial was closed to accrual after interim analysis showed not only increased toxicity in the form of thrombotic events and GI bleeding in the hyaluronidase arm, but a dramatically worse median OS (7.7 months) in the combination group compared with the FOLFIRINOX arm (14.4 months). The authors postulate that the poor outcomes seen in the hyaluronidase arm could be due to dose delays and/or reductions from increased adverse events, drug–drug interactions with components of FOLFIRINOX, or more complex effects on the TME which have yet to be elucidated (60). On the basis of these results, a phase III trial based of combination of PEGPH20 and gemcitabine/nab-paclitaxel compared with nab-paclitaxel alone was planned for patients with high HA expression in metastatic PDAC. The results of this study, recently disclosed, showed that the treatment arm of PEGPH20 in combination with gemcitabine and nab-paclitaxel failed to demonstrate an improvement in median OS compared with gemcitabine and nab-paclitaxel alone (11.2 months compared with 11.5 months, HR = 1.00, $P = 0.97$; ref. 61). These results ended the development of PEGPH20 for metastatic PDAC.

These studies illustrate the complex nature of the fibrotic TME, and hint that the fibrotic stroma may play a role in suppressing tumorigenesis and disease progression while also dampening the host immune response. The combination of stromal-directed therapy with immunotherapy could represent a new avenue for research in PDAC.

Choice of Immune Targets

Knowledge of the mechanisms and receptors which become unregulated during T-cell exhaustion in pancreatic cancer would be important for development of effective therapy (62). Because checkpoint inhibitors targeting PD-1/PD-L1 and CTLA4 are effective in a large portion of patients with melanoma, the same compounds represent the first checkpoint blockade agents tested against PDAC, with disappointing results as previously mentioned. In murine models of PDAC, it has been described that CTLA-4 inhibition was not sufficient to increase infiltration of $CD8^+$ T cells in the pancreatic TME (63).

A recent study compared the density of immune cells expressing PD-1/PD-L1, and found them significantly higher in melanoma compared with PDAC (64). The same study reported over-expression of V-domain Ig suppressor of T-cell activation (VISTA), an inhibitory checkpoint molecule, in PDAC in comparison with melanoma, and postulated that VISTA represents a more dominant inhibitory pathway and may represent a more efficacious target for immunotherapy in PDAC (64).

LAG-3 represents another inhibitory molecule present on PDAC TILs which works by binding MHCII molecules on tumor cells. LAG-3 has been found upregulated on infiltrating lymphocytes in PDAC (65). Galectins, in particular Gal1, Gal3, and Gal9, have been found to be upregulated in the human PDAC TME (66). Galectin signaling has been linked to tumor-cell proliferation and T-lymphocyte apoptosis. Gal9 binds to the TIM-3, an inhibitory receptor expressed in T cells and NK cells (67). Inhibition of Gal9 renders PDAC sensitive to immunotherapy (68). Another inhibitory receptor is TIGIT, expressed on NK cells and T cells, which is capable of directly inhibiting T-cell proliferation and cytokine secretion (69, 70). Balli and colleagues found that cytolytic-high tumors had higher expression of several immune checkpoints, except for PD-L1 which was uniformly low. They also proposed categorizing PDAC based on coexpression of CTLA4, TIGIT, TIM-3, and VISTA for clinical targeting purposes (71). Combinatorial therapies targeting these various novel receptors, depending on an individual patient's expression, require further exploration, as they represent a potential promising approach for PDAC treatment.

Neoantigens in PDAC

The low surface presentation of neoantigens in PDAC has been postulated as one of the reasons that explain why PDAC is considered an "immunologically cold" tumor, unresponsive to most immunotherapies (72). Neoantigen abundance has been linked to mutational burden and lymphocyte infiltration, with melanomas and RCC having the highest number of mutations, and with pancreatic cancer having the lowest. Mutant-specific neoantigens are also associated with TILs in both tumor types as well (73). The extreme example of correlation between mutations, neoantigen abundance, and lymphocyte infiltration is represented by tumors with mismatch repair impairment (74). Unfortunately, this tumor subtype represents less than 1% of all cases of PDAC (75).

A study that analyzed publicly available data from The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) revealed that while PDAC tumors lack the neoantigen diversity of melanomas, most tumors have mutations that result in candidate neoantigens, many of which would be expected to have efficient presentation by HLA class I (76). The same study also showed that despite the lower number of neoantigens, T-cell infiltration in PDAC was not very different from melanoma, although the cytotoxic capacity of those T cells was markedly lower in PDAC (76).

Consistent with these findings, Balli and colleagues found that rather than being associated with mutational burden or neoepitope abundance, immune activation in PDAC was inversely associated with expression of genes related to pancreatic differentiation status, suggesting that intrinsic oncogenic processes drive immune reactivity in PDAC (71).

A study which sequenced and analyzed TCRs from both PDAC short-term and long-term survivors revealed that the quality of neoantigens, rather than the quantity, was predictive of long-term survivorship (77). The study also suggested that neoantigens which mimic microbial epitopes have higher likelihood of TCR recognition. A recent study postulated that melanoma cancer cells can present peptides derived from intracellular bacteria on their HLA-I and HLA-II molecules to elicit immune reactivity. More functional work is required to further validate if these mechanisms may influence *in vivo* immune activation and responses to therapy (78).

The Microbiome in PDAC

The role of the gut microbiome on tumor responses to chemotherapy and immunotherapy has been extensively studied in several tumor types (79–82). Preclinical studies have shown that bacterial ablation by antibiotics or generation of germ-free mouse models blunt responses to immunotherapy and chemotherapy (83, 84). However, pancreatic cancer does not seem to follow the same pattern. As observed in several studies, PDAC-associated microbes have been associated with immunosuppression, and their ablation is linked to improved responses to therapies. In genetically engineered mouse models (GEMM) of PDAC, studies have shown differential evolution of the gut microbiome throughout the pancreatic tumorigenesis process compared with control mice (85–87). Rederivation of GEMM in germ-free conditions resulted in delayed tumorigenesis, which was rescued by oral reconstitution with fecal content from cancer mice. Furthermore, bacterial ablation in pancreatic cancer mouse models led to a reduction in immune-suppressive M2-like tumor-associated macrophages, an increased intratumoral CD8:CD4 T-cell ratio, as well as an increase in PD-1 expression (85). Combination therapy of anti-PD-1 antibodies and antibiotics resulted in synergistic antitumoral effect when compared with anti-PD-1 therapy alone (85). A second study showed the effect of antibiotics in slowing growth in an implantable subcutaneous model of pancreatic cancer, as well as a reduction in metastases (88).

In summary, data in PDAC suggests that antibiotics can potentially improve therapy responses. However, careful consideration must be given to this strategy, considering the toxicities associated with long-term use of broad-spectrum antibiotics including the acquisition of antibiotic multi-resistance.

Human studies have reported that patients with pancreatic cancer also present a different gut microbiome with lower alpha-diversity (85, 89). Geller and colleagues have described that pancreatic tumors harbor their own microbiome, with Gammaproteobacteria being the most abundant bacteria present in PDAC specimens (90). This bacteria class has been linked with resistance to the chemotherapy drug gemcitabine through the expression of the bacterial enzyme cytidine deaminase, which metabolizes gemcitabine to its inactive form (90). Riquelme and colleagues have analyzed the microbial content of tumors from pancreatic cancer short-term survivors (STS) and long-term survivors (LTS) from two geographically distant cohorts and found higher tumor microbial diversity LTS tumors. Furthermore, a tumor microbial signature was detected in PDAC LTS which directly associated with higher infiltration of cytotoxic T cells (91).

Humanized microbial mouse (HMM) models have been generated through transfer of human fecal microbial transplants (FMT) into mice transplanted with orthotropic pancreatic tumors. These models showed a protective effect in tumor growth when using FMT from LTS donors with no evidence of disease and healthy control donors (to a smaller extent), compared with tumors from mice who received FMT from patients with pancreatic cancer which had the largest tumors. These finding suggest that the gut microbial dysbiosis present in PDAC supports tumor growth, and its disruption is fundamental for antitumoral efficacy (91). The protective effect of FMT was lost when mice had no $CD8^+$ T cells, providing preliminary evidence that modification of the gut/tumor microbiome is able to induce an antitumor response and activation of the immune system in tumor-bearing mice (92). A clinical trial that utilizes FMT in patients with resected PDAC is currently being launched at MD Anderson Cancer Center. Very recently, two publications have reported approximately 30% responses when FMT from responders to immunotherapy was combined with reinduction with checkpoint blockade for patients with melanoma who were considered refractory to immunotherapy (93, 94).

Chimeric Antigen Receptor T-cell Therapy: Limitations and Promise

Treatment with T cells transduced with a chimeric antigen receptor (CAR-T) is a novel treatment approach (95) that has shown remarkable efficacy in some blood cancers but has had limited responses in most solid tumors including PDAC (95–98). Early trials using CAR designs that lacked a costimulatory domain and provided signaling only through CD3 were universally unsuccessful due to poor persistence of the CAR-T and early development of hypofunction. The introduction of the costimulatory domain, along with the practice of lymphodepletion preceding treatment led to the first signs of high efficacy in B-cell–derived cancers we see today (99, 100). Unfortunately, these innovations have not been sufficient to drive to clinical success in PDAC.

One challenge in solid tumors is finding a safe antigen to target. However, there are targets that are enriched on the surface of solid tumor cells compared with normal epithelial tissue, suggesting that there are CAR-T doses at which efficacy can be achieved without causing lethal destruction of epithelial tissue (101–103). Target engagement between CAR-T and PDAC cells occurs slowly, creating an additional challenge, as T cells, once activated and exposed to consistent antigen, begin a process of terminal differentiation towards an exhausted and hypo-functional state (29).

PDAC has relatively few endogenous cancer reactive T cells (102). CAR-T provides additional reactive T cells that can then be enhanced with other immunotherapies (104). CAR-T has the potential to be more potent than endogenous T cells as they can be easily engineered *ex vivo*. As an example, enforced expression of cDNA encoding the chemokine receptor CCR2, found in the myeloid lineage cells that infiltrate PDAC in great numbers, led to an increase in trafficking by CAR-T to tumors (105). Other examples include the use of switch receptors to change signaling effects of suppressive compounds (TGFBR and FAS switch receptors) from suppressive to stimulatory (106). To address CAR-T exhaustion, regions of the IL2 receptor were included into the CAR construct, resulting in IL2/JAK/STAT signaling upon target engagement, which delays exhaustion (107). Research points to the transcription factors AP1, TCF7, and c-Jun signaling as being key to maintaining a memory-like phenotype and the capacity for long-term function. Enforced expression of those transcription factors alongside CAR can prevent an exhaustion phenotype (108, 109).

CAR-T can also be engineered to secrete cytokines such as IL12 or IL18, or other immune stimulants such as flagellin, and that such secretion enhances CAR-T potency and endogenous T-cell recruitment (110, 111). CAR-T can be engineered for PDAC therapy to secrete cytokines that bias them and other endogenous T cells toward a

memory phenotype, such as IL7 or chemokines to promote recruitment of endogenous immune cells, such as CCL19 (112, 113). Perhaps most exciting are examples demonstrating use of CAR-T to secrete scFv that block PD-1 itself, which could enable intratumoral immune activation without the toxicities of systemic delivery (114). A recent study describes synergy of joint administration of CAR-T following intratumoral injection with an oncolytic virus engineered to secrete the proinflammatory TNFα and the T-cell growth and survival factor IL2 (115). Another rational combination includes CAR-T with FAKi and checkpoint inhibition to clear stroma and enable better CAR-T trafficking (57, 116). Combining CAR-T with chemotherapeutics or radiation that enhance immunogenic cell death also seems promising (77, 117). Finally, treatment with targeted inhibitors that slow PDAC growth while sparing T cells could give CAR-T a "fighting chance" against large, established, aggressively proliferating PDAC tumors.

DNA damage repair deficiency not as promising in PDAC

Cells are capable of inducing five types of DNA damage pathways with the goal of maintaining the integrity of their genome. DNA damage repair (DDR) pathways include mismatch repair (MMR), homologous recombination, nonhomologous end joining (NHEJ), base excision repair (BER), and nucleotide excision repair (NER; ref. 118). A deficiency in a DDR pathway conduces to genomic abnormalities that can result in tumorigenesis promotion (119) but also in therapeutic opportunities.

DDR defects are also relevant in inducing sensitivity and efficacy to immunotherapy. Microsatellite instability/mismatch repair deficiency (MSH/dMMR) is an FDA-approved biomarker for immune checkpoint blockade, independent of tumor types (120). MMR deficient tumors are presumably more susceptible to immune therapy due to high expression of PD-L1 and high numbers of infiltrating lymphocytes due to a more immunogenic phenotype compared to microsatellite stable tumors. Despite this, a recent study of pembrolizumab in patients with noncolorectal solid tumors that were MSI-high (MSI-H) showed a lower response in patients with PDAC. In the KEYNOTE-158 study, the overall response rate (ORR) was 34.3% in a population of patients with a variety of MSI-H solid tumors including endometrial, gastric, cholangiocarcinoma, and small intestine; however, ORR was 18.2% in the subset of patients with PDAC (121). This is likely due to the uniquely immune-suppressive TME of PDAC, as discussed below.

There is a paucity of data regarding the use of alternative biomarkers in PDAC such as tumor mutational burden (TMB). One study found a low rate of 0.5% of high TMB (defined as ≥ 20 mutations/megabase) in a targeted genomic analysis of over 3,500 PDAC samples (122). The KEYNOTE-028 trial examined use of TMB as a predictive biomarker in patients receiving single-agent pembrolizumab across 20 different PD-L1–positive solid tumors. However, with an ORR of 0% (out of 24 patients included in the trial), patients with PDAC were excluded from biomarker analysis (123).

An example of another therapeutic opportunity based on a DDR pathway defect is represented by platinum agents and poly(ADP ribose) polymerase (PARP) inhibitors as therapy for HR aberrant tumors caused by mutations such as BRCA1/2 (124, 125). Recently, the POLO study showed that the PARP inhibitor olaparib is an efficient maintenance therapy for patients with metastatic PDAC and BRCA1/2 germline mutations, after platinum therapy (126). Zhang and colleagues showed that DNA repair inhibition by pharmacologic blockade or siRNA silencing of ataxia telangiectasia mutated (ATM) results in induction of type I IFN-mediated innate immune response in pancreatic cancer that is increased by

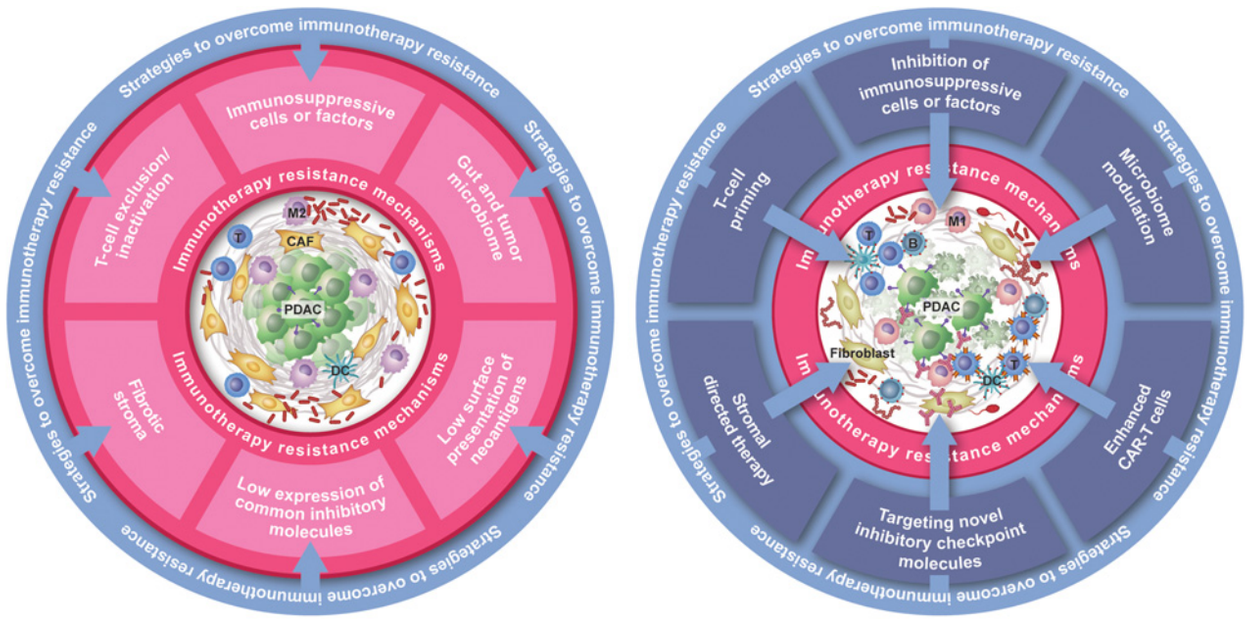

Figure 1.

Approaches to overcoming resistance mechanisms to immunotherapy in PDAC. Illustration by Jordan Pietz (Medical Illustrator at the Creative Communications Department, MD Anderson Cancer Center). © 2021 The Board of Regents of the University of Texas System. Copyright used with the permission of The Board of Regents of the University of Texas System through The University of Texas MD Anderson Cancer Center.

radiation, and results in increased sensitivity to PD-L1 blockade (127). Ongoing trials are testing combination of therapies specific for DDR-deficient tumors (ex: PARP inhibitors) with immune checkpoint blockade (**Table 1**; ref. 128).

Conclusions

In the past decade, we have seen how immunotherapies have proven successful in many cancers. Unfortunately, this success has not been replicated in pancreatic cancer, which remains resistant to this breakthrough in oncologic care. The challenges cited in this review that are particularly potent in PDAC include poor T-cell trafficking to tumors, stromal barriers, abundance of immune suppressive cells and secreted factors, and T-cell exclusion and exhaustion. These various factors are summarized in **Fig. 1**.

We expect that combinatorial therapy based on checkpoint inhibitors or CAR-T cells given in addition to compounds directed against the TME immunosuppressive components, as well as microbial modulation, may represent a potential avenue to efficacy in PDAC. Possibly, heterogeneity in individual patient TME characteristics, microbial composition, and immune checkpoint expression may require "personalized" immunotherapeutic combinatorial approaches. We look forward to results from these ongoing combinatorial trials.

Authors' Disclosures

P.K. Mazur is a scientific cofounder, consultant, and stockholder of Amplified Medicines, Inc. and Ikena Oncology, Inc. No disclosures were reported by the other authors.

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