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Review

From Friend to Enemy: Dissecting the Functional Alteration of Immunoregulatory Components during Pancreatic Tumorigenesis

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a 5-year survival rate of approximately 8%. More than 80% of patients are diagnosed at an unresectable stage due to metastases or local extension. Immune system reactivation in patients by immunotherapy may eliminate tumor cells and is a new strategy for cancer treatment. The anti-CTLA-4 antibody ipilimumab and anti-PD-1 antibodies pembrolizumab and nivolumab have been approved for cancer therapy in different countries. However, the results of immunotherapy on PDAC are unsatisfactory. The low response rate may be due to poor immunogenicity with low tumor mutational burden in pancreatic cancer cells and desmoplasia that prevents the accumulation of immune cells in tumors. The immunosuppressive tumor microenvironment in PDAC is important in tumor progression and treatment resistance. Switching from an immune tolerance to immune activation status is crucial to overcome the inability of self-defense in cancer. Therefore, thoroughly elucidation of the roles of various immune-related factors, tumor microenvironment, and tumor cells in the development of PDAC may provide appropriate direction to target inflammatory pathway activation as a new therapeutic strategy for preventing and treating this cancer.

Keywords: pancreatic cancer; immunotherapy; CTLA-4; PD-1; PD-L1

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) has very high mortality rate among cancers with less than 8% survival beyond 5 years [1]. As for the obscure clinical course of the disease, most patients have advanced stage and unresectable status at diagnosis. Metastatic PDAC has a dismal prognosis due to resistance to current therapy. Standard chemotherapy with gemcitabine shows modest effects with median overall survival (OS) ranging from 5 to 8 months [2,3]. Adjuvant chemotherapy with gemcitabine- or fluorouracil-based regimens improved the overall survival in resectable disease [4,5]. Combinations of chemotherapy have been reported with some benefit to survival in metastatic PDAC. For locally advanced and metastatic disease, polychemotherapy using FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan, oxaliplatin) improved the median survival to 11.1 months [6]. The phase III MPACT study showed that the median OS was significantly longer for nab-paclitaxel and gemcitabine compared to that for gemcitabine alone (8.7 vs. 6.6 months, hazard ratio (HR) = 0.72) [7].

However, the results are not satisfactory for clinical practice and the patients finally experience disease progression and recurrence.

Cancers develop multiple ways to escape immunosurveillance and process immunoediting [8]. The antitumor efficacy of the immune checkpoint blockade was displayed by blocking the downstream regulators of immunity including cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) or its ligand, programmed cell death ligand 1 (PD-L1). CTLA-4, also known as CD152, is a co-inhibitory molecule expressed on CD4+ CD25+ regulatory T cells, and binds with its ligands B7.1 and B7.2 expressed on antigen-presenting cells to halt immune response and reinforce signaling for cell cycle progression [9]. CTLA-4 blocking antibodies such as ipilimumab and tremelimumab arouse immune detection and stimulate T cell activation [10,11]. PD-L1, also known as CD274, expressed on antigen-presenting cells, binds to PD-1 (known as CD279) expressed on activated T cells, B cells, NK cells, and monocytes [12,13]. PD-L1 is generally considered to influence the immune response at later stages in peripheral tissues [14,15]. Targeting the PD-L1/PD-1 pathway enhances T cell proliferation and production of interferon-γ (IFN-γ), tumor necrosis factor-α, and IL-2, and is directly correlated with a regressive response in tumor size [16,17]. Anti-PD-1 antibodies such as pembrolizumab and nivolumab contribute remarkably to treating different cancers including metastatic melanoma, non-small cell lung carcinoma, renal cell carcinoma, urothelial carcinoma, Hodgkin's lymphoma, microsatellite instability-high (MSI-H) or mismatch repair-deficient solid tumors, and head and neck carcinoma [18–21]. Therefore, immune checkpoint blockade has revealed a promising benefit in treating a range of cancer types.

PDAC comprises a massive stroma and few tumor cells, which build a natural barrier and defense against immune cells and conventional cytotoxic agents [22]. The stroma containing extracellular matrix (ECM), fibroblasts, pancreatic stellate cells, immunoregulatory cells, and endothelial cells makes up 90% of the PDAC components. The remaining 10% components are cancer cells. Thus, the microenvironment mainly obstructs the therapeutic effects of drugs, in contrast to most other solid tumors. However, the microenvironment is dynamic and change continuously during pancreatic tumorigenesis from pre-neoplastic pancreatic intraepithelial neoplasm (PanIN) to invasive PDAC. In the meta-analysis, positive PD-L1 expression was highly correlated with poorer overall survival and pathologic differentiation in PDAC patients [23]. However, the relationship between PD-L1 and PDAC patients remains inconclusive. In this review, we focus on the functional transitions of associated immunoregulatory cells and their interplay during cancer development and treatment.

2. The Pancreatic Cancer Microenvironment

Abundant stroma forms a natural barrier leading to a hypoxic surrounding and accumulation of immunosuppressive cells and cytokines, which characterize pancreatic cancer with low immunogenicity and non-inflamed circumstances. Pancreatic cancer displays an immunosuppressive microenvironment that is immunologically ignorant and poorly infiltrated by T lymphocytes [24].

2.1. Pancreatic Stellate Cells-Stromal Fibroblast

During the development of chronic pancreatitis, activated cancer-associated fibroblasts (CAFs) derived from pancreatic stellate cells (PSCs) contribute to fibrosis and produce massive ECM during transformation to PDAC [25]. A prior study demonstrated that activated PSCs encompassed human PanIN, suggesting that PSCs participate in the development of early-stage PDAC [26].

Multiple cellular pathways have been associated with regulating CAFs function. For example, hypoxia-inducible factor-1 alpha and galectin-1 induce the expression of sonic hedgehog (SHH) and Gli 1 to activate fibroblasts leading to impedance of blood perfusion and a growth advantage to tumor cells [27–32]. Another key player between CAFs and cancer cells is the fibroblast activation protein (FAP), which mediates cancer cell invasion and cell cycle activation by Rb protein inhibition [33]. These evidences suggest that abundant stroma creates an additive factor for tumor advanced progression. However, a recent study indicated that myofibroblast depletion led to the remodeling

of the tumor extracellular matrix without improving the efficacy of gemcitabine treatment. Notably, myofibroblast depletion resulted in tumors with a decreased effector and regulatory T cells (Teff/Treg) ratio and increased CTLA-4 expression. Myofibroblast depletion combined with anti-CTLA-4 antibodies significantly reduced the tumor burden in mice [34]. These evidences indicate that the cross-talk between cancer cells and stroma is sophisticated, but targeting the checkpoint blockade will likely offer a new strategy into combination therapies involving ECM remodeling and immune surveillance in advanced PDAC.

2.2. Regulatory T Cells (Tregs)

Tregs are characterized by expression of both CD4 and forkhead box P3 (FoxP3). Tregs are important to maintain immunological self-tolerance and have the ability to obstruct antitumor responses in several cancer types. High levels of Tregs are associated with immunosuppression and poor prognosis [35,36]. Infiltration of immunosuppressive cells such as Tregs and myeloid-derived suppressor cells (MDSCs) with procancerous signals is considered a factor contributing to the alteration of pro-inflammatory environment to tolerogenic immune surroundings [37]. Pancreatic cancer cells and PSCs produce Treg cell attractants CXCL10, CCL3, CCL4, CCL5, and vascular endothelial growth factor (VEGF) and interact with Treg cell-surface receptors CXCR3, CCR5, and neuropilin-1 to promote Treg cell migration and infiltration [38–42].

Activated Tregs create immunosuppressive circumstances via multiple mechanisms. For example, Tregs inhibit Teff activation and proliferation through IL-2 activation [43]. Tregs express inhibitory markers including CTLA-4 and inducible T cell costimulatory (ICOS) to accelerate apoptosis in Teff. Suppressive cytokines such as IL-10, IL-35, and transforming growth factor-β (TGF-β), are secreted by Tregs to diminish immune surveillance [44]. Importantly, pancreatic cancer mouse models have demonstrated that targeting CTLA4 in Tregs increases the infiltration of CD4+ T cells recruited to the tumor, suggesting that targeting Tregs is important for PDAC immunotherapy [45].

2.3. Myeloid Derived Suppressor Cells (MDSCs)

MDSCs are composed of heterogeneous immune cells with a phenotype of $CD33^{+}/HLA\text{-}DR^{-/low}$. MDSCs are associated with tumor progression, angiogenesis, senescence evasion, tumor metastasis, and chemoresistance. Their functions are repressive for T cell immunity and angiogenic to promote tumorigenesis [46,47]. Cytokines such as colony-stimulating factors are the main molecules stimulating generation of MDSCs from progenitor cells in bone marrow [48]. In mouse models, oncogenic Kras-induced GM-CSF production promotes MDSCs formation in the tumor microenvironment [49]. Besides, MDSCs are activated by several signals, including IL-4, IL-6, VEGF, and TGF-β [50–53].

It has been reported that mononuclear MDSCs have greater potential ability for suppressive control than polymorphonuclear MDSCs, and recruit FOXP3+ Treg cells by producing TGF-β, CCR5, and ARG-1 [54–56]. Hypoxic surroundings recruit MDSCs, activating the STAT3 pathway to secrete VEGF and basic fibroblast growth factor, which promote subsequent angiogenesis [57]. Depletion of MDSCs with Gr-1 antibody retarded the growth of pancreatic cancer cells and revealed a role for CD8 T cells in the disease [58].

2.4. Tumor-Associated Macrophages (TAMs)

Circulating monocytes are accumulated in the tumor microenvironment; they will proliferate and differentiate into different phenotypes of TAMs according to diverse stimulating signals. TAMs can be polarized into two different subtypes: (1) inflammatory M1-type macrophages, which are induced by IFN-γ, LPS, and IL-12, termed as “classical activation” and (2) precancerous M2-type macrophages, which are activated by IL-4 and IL-13, termed as “alternative activation” [59].

Besides cytokines, hypoxia and other signaling pathways such as IRF4, STAT6, MYC, PPARγ, and KLF4 have been reported to promote M2 polarization [60–64]. In general, M2 macrophages have an immunosuppressive phenotype and release cytokines, including IL-10, that induce a Th2

immune response [65]. In contrast, M1 macrophages are dominant and prone to tumor initiation and development in chronic inflammation [66]. After transformation to cancer, macrophages switch to the M2 phenotypes to invade and progress [67]. High infiltration of M2-type polarized TAMs presents poor prognosis in many cancers including pancreatic cancer. These evidences suggest that reversing the polarization of M2-type TAMs could provide a potential strategy in improving their immunosuppressive function in pancreatic cancer.

2.5. Dendritic Cells (DCs)

Antigen presentation via DCs is crucial to an effective antitumor T cell response. However, a paucity and disability of DCs is found in the pancreatic tumor microenvironment, with their location within the tumor being at the edges of cancer [68]. Similarly, the number of circulating DCs is relatively low in PDAC patients [69]. Studies indicate a positive correlation between high levels of blood DCs and better outcomes in patients with PDAC [70,71]. Notably, the co-inhibitory molecule indoleamine 2,3-dioxygenase (IDO), is increasingly expressed in the DCs of PDAC, which suppresses the T cell responses and promotes immune tolerance [72]. These data suggest that the tolerogenic functions of IDO can be targeted for treating pancreatic cancer, via modulation of DC function.

3. Immunotherapy in Pancreatic Cancer Treatment

The tumor microenvironment (TME) is involved in cancer biology in a more sophisticated manner in PDAC [73,74]. Evidences indicated that PDAC patients with both highest neoantigen number and the most abundant CD8+ T cell infiltrates have the longest survival time [75]. However, the activation signature of T cell is suppressed in genomic profiles of PDAC patients [76]. Immune cell and stromal signature integrating genomic and immunophenotypic classification of PDAC develops in recent studies [77,78]. The "immune rich" subtypes with rich in T and B cells, lower in FOXP3+ Tregs, and reduced CDKN2A and PIK3CA mutation rate, possess better outcome compared with "immune escape" and "immune exhausted" subtypes [78]. These signatures provide a new way to approach the immune response in PDAC patients with immunotherapy.

3.1. Checkpoint Inhibitors

Clinical trials of anti-PD-1/PD-L1 (BMS-936559, pembrolizumab, nivolumab, and atezolizumab) and anti-CTLA-4 (ipilimumab) monotherapies show no effects in unselected patients with advanced, pretreated, and progressive PDAC [18,19,79–81]. Only small groups of patients with mismatch repair (MMR) deficiency, which results in higher rates of somatic mutations and increased neoantigen production, showed higher clinical benefit with immune checkpoint inhibitors, despite the rare incidence rates in PDAC (less than 2%) [82–85]. Combination therapy is considered an alternative strategy to improve the clinical response via modulation of immune cell infiltration to the tumor region. These reports indicate that combination therapies display an advantage for the treatment of pancreatic cancer patients (Table 1). For example, acalabrutinib, a bruton's tyrosine kinase inhibitor, which were used in lymphoma suppressed MDSCs and TAMs and promoted therapeutic potency after combining with checkpoint inhibitors [86,87]. CXCR4 antagonist (BL-8040) is a robust mobilizer of immune cells to peripheral blood and is effective at inducing direct tumor cell death. Additional findings have suggested that CXCR4 antagonists may be effective at increasing the infiltration of antitumor T cells into the tumor region. Therefore, combining BL-8040 with a checkpoint blockade is predicted to increase the responsiveness of PDAC patients to immunotherapy [88]. The efficacy of checkpoint inhibitors can also be improved in combination with dendritic cell-based therapy in pancreatic cancer via blockade of PD-L1 on dendritic cells, which induces a Th-1 immune profile and reduces the release of Th-2 cytokines [89]. Gemcitabine, the standard chemotherapy for pancreatic cancer treatment, increases the Teff:Treg ratio and the activity of Teff. Nab-paclitaxel and gemcitabine may also cooperate well with Nivolumab in EMT remodeling [90–93]. Furthermore, low-dose cyclophosphamide improved the antitumor immune responses when used in early cancer vaccine trials [94]. Daily and metronomic

administration of cyclophosphamide effectively downregulated the number and function of Tregs in other cancers types [95,96]. In addition, an antivascular effect was also reported with low-dose cyclophosphamide in prostate cancer [97]. Although clinical trials of anti-CTLA-4 and anti-PD-1/PD-L1 therapies were well conducted, other checkpoint inhibitors including LAG-3, TIM-3, and A2AR have been applied in ongoing clinical trials [98].

3.2. Vaccinations

Immunotherapy with vaccination aims to arouse passive immune responses via administering tumor-specific antigens and expressed epitopes for CD8+ and CD4+ T cells. These include whole-cell vaccines, DC vaccines, DNA vaccines, and peptide vaccines, which are all listed in Table 2.

3.2.1. GVAX

GVAX is composed of tumor cells genetically modified to allogeneic cell lines that secrete the immune stimulatory cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), and are then irradiated to prevent further cell division. GVAX stimulates stem cells to produce granulocytes and monocytes and promotes cytolytic activity against tumor cells [99]. In a pilot study, neoadjuvant and adjuvant GVAX was administered with or without cyclophosphamide in resected PDAC. Post-GVAX triggers the PD-1–PD-L1 axis to helpfully provide better candidates than vaccine-naive patients for immune checkpoints and other immunomodulatory therapies [100]. Notably, vaccine cells and their combination with 5-FU-based chemoradiation improved the clinical outcomes in resected PDAC patients (24.8 months, 95% CI, 21.2–31.6) [101,102].

3.2.2. CRS-207

CRS-207 is composed of live-attenuated, double-deleted *Listeria monocytogenes* expressing human mesothelin. CRS-207 has the ability to trigger both innate and mesothelin-specific CD8+ T cell induction adaptive immune responses to enhance GVAX-mediated antitumor effects in metastatic PDAC, compared to GVAX alone [103,104]. However, a disappointing result was noted in the phase IIb study, which showed no overall survival advantage in combination with cyclophosphamide (CY)/GVAX + CRS-207 in patients with previously treated metastatic PDAC, compared to chemotherapy [105].

3.2.3. Algenpantucel-L

Algenpantucel-L is composed of two human allogenic irradiated cancer lines genetically modified to express murine alpha-1,3-galactosyl transferase (αGT), which leads to synthesis of α-galactosyl (αGal) residues on their cell surface. Vaccine tumor cells mediate hyperacute rejection and phagocytosis to reinforce the anticancer immune response via cells with αGal expression [106]. A phase II adjuvant study with algenpantucel-L addition to standard adjuvant chemoradiotherapy reported that 12-month disease-free survival was 62% and that 12-month overall survival was 86% [107]. However, phase III randomized trials showed no overall survival difference between chemoradiotherapy with or without algenpantucel-L (30.4 months in control vs. 27.3 months in the treatment group) [108].

Table 1. Summary of clinical trials on checkpoint inhibitor monotherapies or combination therapy in pancreatic cancer.

Molecules	Regimen	Phase	*n*	Patient Population	Results	ORR (Responder/*n*)	Survival	NCT Number
BMS-936559	Monotherapy	I	14	Advanced, pretreated	Negative	0%		NCT00729664
Ipilimumab	Monotherapy	II	27	Advanced, pretreated	Negative	0%		NCT00112580
	Combination with GVAX	Ib	30	Advanced, pretreated	Positive	0%	OS: 5.7 m 1 y OS: 27%	NCT00836407
Atezolizumab	Monotherapy	I	1	Advanced, pretreated	Negative	0%	PFS: 12.2 m	NCT02302807
Pembrolizumab	Monotherapy	I	1	Advanced, pretreated	Negative	0%		NCT02331251
	Monotherapy	II	4	Advanced, pretreated	Positive	50% (2/4)		NCT01876511
	Combination with acalabrutinib	II	28	Advanced, pretreated	Positive	7.1% (1/14)		NCT02362048
	Combination with BL-8040	II	37	Advanced, pretreated	Positive	3.4% (1/29)	OS: 3.4 m (all) 7.5 m (2 L)	NCT02826486
Nivolumab	Combination with nab-paclitaxel +/− gemcitabine	I	6	Advanced	Positive	18.2% (2/11) (−gem) 50% (3/6) (+gem)		NCT02309177
	Combination with cyclophosphamide/GVAX +/− CRS-207	II	90	Advanced	Positive		OS: 3.9 m (−CRS) 6.1 m (+CRS)	NCT01417000
	Combination with MoDC		7	Advanced, pretreated	Positive	28.6% (2/7)	OS: 8 m and 16 m (responder)	Investigator-initiated trail

The legends of abbreviations: *n*: patient numbers, ORR: overall response rate, OS: overall survival, PFS: progression-free survival, m: months, 2 L: second-line, gem: gemcitabine, and MoDC: monocyte derived dendritic cells.

Table 2. Summary of mechanisms and trials on vaccination treatments that modulate the tumor microenvironment.

Agents	Mechanisms on Immune System	Clinical Trials
GVAX	1. Increased tumor antigen recognition by the immune system through presentation by dendritic cells, including mesothelin 2. Induces intratumoral tertiary lymphoid structures and TILs 3. Upregulates PD-L1 membranous expression on cancer cells 4. Delayed-type hypersensitivity	1. Novel Allogeneic Granulocyte-macrophage colony-stimulating Factor-secreting Tumor Vaccine for Pancreatic Cancer: A Phase I Trial of Safety and Immune activation, NTC03122106. 2. Vaccine Therapy Combined With Adjuvant Chemoradiotherapy in Treating Patients With Resected Stage I or Stage II Adenocarcinoma (Cancer) of the Pancreas, NCT00084383. 3. Safety and Efficacy of Combination Listeria/GVAX Immunotherapy in Pancreatic Cancer, NCT01417000.
CRS-207	1. Stimulates potent innate and mesothelin-specific adaptive immunity and "boosts" the immune response initiated by GVAX 2. Induces T cells to leave the periphery and enter tissues	1. Safety and Efficacy of Combination Listeria/GVAX Immunotherapy in Pancreatic Cancer, NCT01417000. 2. Safety and Efficacy of Combination Listeria/GVAX Pancreas Vaccine in the Pancreatic Cancer Setting (ECLIPSE), NCT02004262.
Algenpantucel-L	1. Mediates hyperacute rejection 2. Anti-αGal antibodies bind to αGal epitopes causing complement- and antibody-dependent cell-mediated destruction of transplanted allografts	1. Immunotherapy Study for Surgically Resected Pancreatic Cancer, NCT01072981. 2. Vaccine Study for Surgically Resected Pancreatic Cancer, NCT00569387. 3. Vaccine Treatment for Surgically Resected Pancreatic Cancer, NCT00255827.
KRAS peptide (including GI4000 etc.)	1. Presented as a foreign antigen by MHC class I and II to CD4+ and CD8+ T cells, and induces cytotoxic effects 2. Using nonpathogenic yeast as a vehicle to carry antigens and present them to DCs 3. Lowers the expression of FoxP3 cells 4. Increases the ratio of CD4+CD25+ activated T cells to Tregs 5. Increase production of Th1-related cytokines and IL-6 6. Delayed-type hypersensitivity	1. Safety and Efficacy of the Therapeutic Vaccine GI-4000 in Combination With Gemcitabine Versus Placebo for the Treatment of Non-metastatic, Post-resection Pancreas Cancer, NCT00300950. 2. Vaccine Therapy in Treating Patients With Colon, Pancreatic, or Lung Cancer, NCT00019006. 3. Vaccine Therapy and Biological Therapy in Treating Patients With Advanced Cancer, NCT00019084. 4. Vaccine Therapy Plus Biological Therapy in Treating Adults With Metastatic Solid Tumors, NCT00019331. 5. Vaccine Therapy Plus QS21 in Treating Patients With Advanced Pancreatic or Colorectal Cancer, NCT00006387.
Telomerase peptide (GV1001)	1. Facilitates the transport of molecular cargo across the plasma membrane 2. Binds to MHC, activating hTERT-specific T cell responses 3. Integrates both T helper and CTL responses 4. Delayed-type hypersensitivity	1. Gemcitabine and Capecitabine With or Without Vaccine Therapy in Treating Patients With Locally Advanced or Metastatic Pancreatic Cancer, NCT00425360. 2. Immunochemoradiotherapy in Patients With Pancreatic Cancer, NCT01342224. 3. A Feasibility and Safety Study of Vaccination With Poly-ICLC and Peptide-pulsed Dendritic Cells in Patients With Metastatic, Locally Advanced, Unresectable, or Recurrent Pancreatic Adenocarcinoma, NCT01410968.
Antigastrin-17 vaccine (G17DT)	1. Gastrin is a driver of pancreatic cancer that stimulates growth through a markedly overexpressed CCK receptor 2. Reverse intense desmoplastic reaction of pancreatic cancer via modulating pancreatic stellate cells 3. Arrests the progression of PanINs	1. An Open Label Study to Evaluate G17DT Compared to Gemcitabine, NCT03200821. 2. An Open, Single-center Study to Determine the Antibody Response to Gastrimmune and Its Safety and Tolerability in Patients With Advanced Pancreatic Carcinoma, NCT02098291. 3. Safety and Efficacy of G17DT Immunogen Combined With Gemcitabine vs. Gemcitabine in the Treatment of Advanced Pancreatic Carcinoma, NCT00044031. 4. Sequential Trial of G17DT for the Treatment of Advanced Pancreatic Cancer, NCT02118077. 5. Single Centre Study to Determine the Antibody Response to G17DT in Patients With Advanced Pancreatic Cancer, NCT02098239
Anti-VEGFR vaccine (VEGFR2-169)	1. Reduces the number and function of Tregs 2. Inhibits the infiltration of other suppressive immune cells (MDSCs, macrophages) 3. Activation of CD4+ T cells 4. Increases the mature dendritic cell fraction 5. Changes the intratumoral cytokine levels, specifically those of IL-1β, IL-6, and CXCL1 6. Reduces the production and expression of interleukin-10 and TGF-β in TME	1. Gemcitabine With Antiangiogenic Peptide Vaccine Therapy in Patients With Pancreatic Cancer, NCT00622622 2. Antiangiogenic Peptide Vaccine Therapy With Gemcitabine in Treating Patient With Pancreatic Cancer (Phase1/2), NCT00655785

3.2.4. *Kras* Vaccines

It is well known that *Kras* point mutation triggers PanIN formation, which consequently progresses to PDAC development [109]. *Kras* mutation, mainly in codon 12, is commonly found in more than 90% of PDAC patients. Clarifying the detailed mechanisms may provide a useful strategy for immunotherapeutic treatments. It is known that mutant Kras peptides are processed and presented as foreign antigens by both MHC class I or II molecules [110,111], which become accessible to cytotoxic T cells. The products of mutant Kras antigens are expressed distinctively in tumor tissues compared to normal tissues. Vaccine peptides were custom synthesized to the corresponding mutation and injected to the patient to induce mutation-specific immune responses [112]. Besides, using nonpathogenic yeast as a vehicle to present mutated Kras peptide to DCs is another strategy for activating the immune system [113]. Unfortunately, the initial use of Kras vaccine in patients induced a transient T cell response but provided no clinical benefits [114]. However, combination with GM-CSF in adjuvant treatment induced a 58% response rate and longer median survival of responders than nonresponders (148 days vs. 61 days, respectively), and the survival of resectable patients was better than that of unresectable patients in a phase I/II trial [115]. Previous studies with long-term follow-up demonstrated 17 of 20 (85%) immune responders with a median survival of 28 months. The 10-year survival was 20% (4/20 evaluable) versus 0% in a comparable nonvaccinated cohort [116,117]. Another study of resectable pancreatic cancers with Kras mutation showed that concurrent use of Kras vaccine and GM-CSF resulted in 25% (9/24) immune responders. Median recurrence-free survival time was 8.6 months and median OS was 20.3 months [118].

3.2.5. Telomerase Vaccine

Telomerase is overexpressed in 85 to 90% of pancreas cancer cases, and immunogenic telomerase peptides have been defined as a target of anticancer drugs. Telomeres protect the end of the chromosome from DNA damage or from fusion with neighboring chromosomes. Telomerase is active in normal stem cells and in most cancer cells, but is nearly absent from most somatic cells. GV1001, a 16-aa hTERT peptide, belongs to cell-penetrating peptides facilitating hTERT-specific T cell response-mediated MHC activation. Notably, a telomerase peptide vaccine could interact with a variety of HLA-class II molecules including HLA-DR, -DP, and -DQ loci, thereby eliciting T helper (Th) responses commonly found in vaccinated patients [119]. Evidences indicate that higher immune responses are positively correlated with better survival [120]. In a phase I/II trial of unresectable PDAC, different dosages of GV1001 combined with GM-CSF were evaluated for immune response and survival in nonresectable PDAC patients. Median survival for the intermediate dose-group was significantly longer than that for the low- ($p = 0.006$) and high-dose groups ($p = 0.05$). Two phase III studies were conducted using a combination of chemotherapy agents, but showed no survival benefits in patients with metastatic PDAC.

3.2.6. Anti-Gastrin Vaccine

Gastrin is expressed in the developing fetal pancreas; however, gastrin expression disappears in the embryo and is re-expressed in PanINs [121]. A gastrin-stimulating growth-mediated autocrine mechanism is commonly observed in pancreatic cancer [122]. Cholecystokinin (CCK) and gastrin are known to function in directly activating PSCs to further induce pancreatic fibrogenesis [123]. Antigastrin-17 vaccine (G17DT) is well known as a gastrin immunogen with a diphtheria toxoid (DT) carrier protein and plays an important role in regulating the tumor microenvironment. Two randomized clinical trials showed improved survival in responders compared to the placebo [124,125]. However, G17DT combined administration with gemcitabine showed inferior survival compared to gemcitabine plus placebo [126].

3.2.7. Anti-VEGFR Vaccine

Vascular endothelial growth factor receptor 2 (VEGFR2) is well characterized as a crucial regulator in driving tumor angiogenesis. VEGFR2 is a target for Treg recognition; therefore, VEGFR2+ Tregs demonstrate highly immunosuppressive activity in the tumor microenvironment [127]. In addition, VEGF inhibits the maturation of DCs and increases immune tolerance [128–130]. A phase I study combining VEGFR2-169 with gemcitabine in advanced PDAC demonstrated a 67% disease control rate and 8.7 months of median overall survival time [131].

4. Other Immunotherapies and Targeted Therapies Modulating the Tumor Microenvironment

Stromal heterogeneity of numerous immune cells and components is involved in future therapeutic targets. Several therapies against stroma were investigated in clinical trials and are listed in Table 3. All-trans retinoic acid (ATRA) (NCT03307148) regulates the immune response via eradicating monocytic MDSCs, diminishing the suppressive capacity of granulocytic MDSCs in sarcomas [132], enhancing CD8+ T cell infiltration around cancer cells, and activating PSCs mediated by CXCL12 from PSCs in PDAC [133,134]. Paricalcitol (NCT02030860), a less calcemic analog of $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$, upregulates the expressions of p21 and p27 in vitro and in vivo leading to G0/G1 arrest in PDAC [135]. Paricalcitol also blocks high levels of vitamin D receptors on stellate cells and inactivates stroma production in PDAC [136]. Defactinib (NCT02546531), a second-generation inhibitor of focal adhesion kinase (FAK) and proline-rich tyrosine kinase-2, inhibits tumor cell survival and promotes anoikis. FAK signaling mediates the physical attachment of cells to ECM and promotes formation of a fibrotic and proinflammatory tumor microenvironment. Inhibition of FAK signaling leads to significant reduction in pancreatic tumor growth in animal models. FAK inhibitors displayed markedly reduced tumor fibrosis, decreased immunosuppressive MDSCs, and restored unresponsive Kras (G12D)/Trp53 null/Pdx1-cre (KPC) in mouse models of PDAC sensitive to PD-1 blockade [137,138]. Pexidartinib (NCT02777710), an inhibitor of M-CSF-receptor (M-CSFR) and c-kit tyrosine kinase, decreases $CD206^+$ $F4/80^+$ TAM (M2-like TAMs) number and blood vessel density, but improves the CD8+ T cell/Treg-ratio in malignant mesothelioma [139]. CXCR4 is a receptor specific for CXCL12 (also called stromal-derived-factor-1), a potent molecule with chemotactic activity for lymphocytes. Multiple targeted therapies including olaptesed and plerixafor (NCT03168139, NCT03277209, NCT02179970), target CXCR4/CXCL12 interaction, leading to modulation of the immune microenvironment. Olaptesed increases lymphocyte infiltration into solid tumor–stroma spheroids of different cancer cell lines, thereby synergizing with the anti-PD-1 checkpoint blockade [140]. Plerixafor may hinder the survival, growth, and migration of CXCR4-expressing cancer cells and inhibit endothelial progenitor cells to reduce tumor vasculogenesis in ovarian and breast cancers [141,142]. In Ewing Tumors, plerixafor diminishes PDGFB expression and results in compromised tumor vasculature and apoptosis in vivo [143]. Hypoxia and anti-angiogenesis also obstruct the proliferation of pancreatic cancer cells, and similar agents including evofosfamide (NCT03098160), apatinib (NCT02726854), and ziv-aflibercept (NCT02159989) reduce the oxygen and nutrient supply to the tumor. In several cancers including PDAC, Lenalidomide (NCT01547260) exerts diverse antitumor effects through anti-angiogenesis and recruitment of tumor antigen-specific T cells, thus enhancing natural killer (NK) cell cytotoxicity [144–146]. In addition, lenalidomide promotes T cells and induces proliferation, cytokine production, and cytotoxic activity, with decreased TNF-α and IL-12 production [145,147]. Trabedersen (NCT00844064), a TGF-β antagonist, functions to overcome TGF-β2-mediated immunosuppression in pancreatic cancer cells. Trabedersen induces LAK (lymphokine activated killer) cell-mediated cytotoxicity in patients with pancreatic cancer, malignant melanoma, and colorectal carcinoma [148,149]. TGF-β inhibitors enhance immunosurveillance and reinforce immune recognition in the tumor microenvironment.

Table 3. Summary of mechanisms in targeting the tumor microenvironment (TME).

Agents	Mechanisms	References
ATRA	1. Eradicates monocytic MDSCs and diminishes the suppressive capacity of granulocytic MDSCs 2. Enhances the CD8+ T cell infiltrate around cancer cells 3. Activate PSCs mediated by CXCL12 from PSCs	[132–134]
Paricalcitol	1. Inhibits CDK2, CDK4, Cyclin D1, Cyclin E, and Cyclin A 2. Upregulates the expressions of p21 and p27 in vitro and in vivo 3. Blocks high levels of vitamin D receptors on stellate cells and inactivates stromal production	[135,136]
Defactinib	1. Inhibits physical attachment of cells to the ECM 2. Inhibit tumor fibrosis and proinflammatory tumor microenvironment 3. Decreases immunosuppressive MDSCs 4. Render the previously unresponsive KPC mouse models sensitive to PD-1 blockade	[137,138]
Pexidartinib	1. Decreases $CD206^+$ $F4/80^+$ TAM (M2-like TAMs) numbers and blood vessel density 2. Improves the CD8+ T cell/Treg-ratio	[139]
Olaptesed	1. Targets CXCL12 2. Increases lymphocyte infiltration into solid tumor–stroma spheroids, thereby synergizing with the anti-PD-1 checkpoint blockade 3. Lowers the monocyte-to-lymphocyte ratio	[140]
Plerixafor	1. Targets CXCR4 2. Hinders the survival, growth, and migration of CXCR4-expressing cancer cells 3. Inhibits endothelial progenitor cells to reduce tumor vasculogenesis 4. Diminishes PDGFB expression resulting in compromised tumor vasculature and apoptosis in vivo	[141–143]
Lenalidomide	1. Anti-angiogenesis 2. Expands tumor antigen-specific T cells and enhances natural killer (NK) cell cytotoxicity 3. Promotes T cells inducing proliferation, cytokine production, and cytotoxic activity 4. Decreases TNF-α and interleukin-12 production	[144,145,147]
Trabedersen	1. Reverses TGF-β2-mediated immunosuppression of pancreatic cancer cells 2. Increases LAK (lymphokine activated killer) cell-mediated cytotoxicity to pancreatic cancer cells 3. Prevents angiogenesis	[148,149]

5. Perspectives and Conclusions

Though the results of checkpoint inhibitors do not meet the expectations, PD-1/PD-L1 pathway is still considered vital to regulate antitumor activity in pancreatic cancer. The residual components including PSCs, Tregs, MDSCs, TAMs, and DCs in the tumor microenvironment play a decisive role in reversing immune dysfunction and modulating the effect of checkpoint inhibitors. Early phase clinical trials in PDAC showed that monotherapy with blockade of the PD-1/PD-L1 pathway did not attain satisfactory responses. However, the safety profiles and toxicity with anti-PD-1 monoclonal antibodies are acceptable, leading to increased interest in targeting PD-1/PD-L1 in PDAC. After combining PD-1/PD-L1 antibodies with agents altering TME, such as chemotherapies, targeted therapies, and vaccination, higher response rates are demonstrated in advanced and pretreated PDAC patients. Unlike other types of neoplasms, passive immunity is more crucial and acts as an "enhancer" for immunologically ignorant pancreatic cancer. Vaccination may turn the tumor microenvironment from a "cold" tumor to "hot" tumor and arouse immunosurveillance and immunoediting, after which the anti-PD-1 antibody boosts tumor cell destruction. Diverse combinations of vaccine therapies are undergoing clinical trials and are listed in Table 4. Multimodalities of treatment strategies will be needed for overcoming the fibrotic stroma, suppressive immune cells, and malignant cancer cells. Early phase clinical trials conducted with oncolytic viruses, TGFβ inhibitors, and SMO inhibitors in advanced PDAC patients have shown preliminary promising results [149–154]. Novel strategies targeting the immune check points and stroma-associated therapies have also revealed impressive results in preclinical studies. For example, blockade of the TGFβ pathway combined with

nivolumab treatment provides immune restoration to enhance immune responses and promote tumor regression [155]. As TGFβ inhibitors are currently used in recurrent/refractory high-grade glioma [156], triple combinations with checkpoint inhibitors and vaccination may be a potential and novel therapeutic strategy in PDAC patients. On the other hand, these combinations may conquer the resistance mechanisms to the PD-1/PD-L1 pathway blockade (Figure 1).

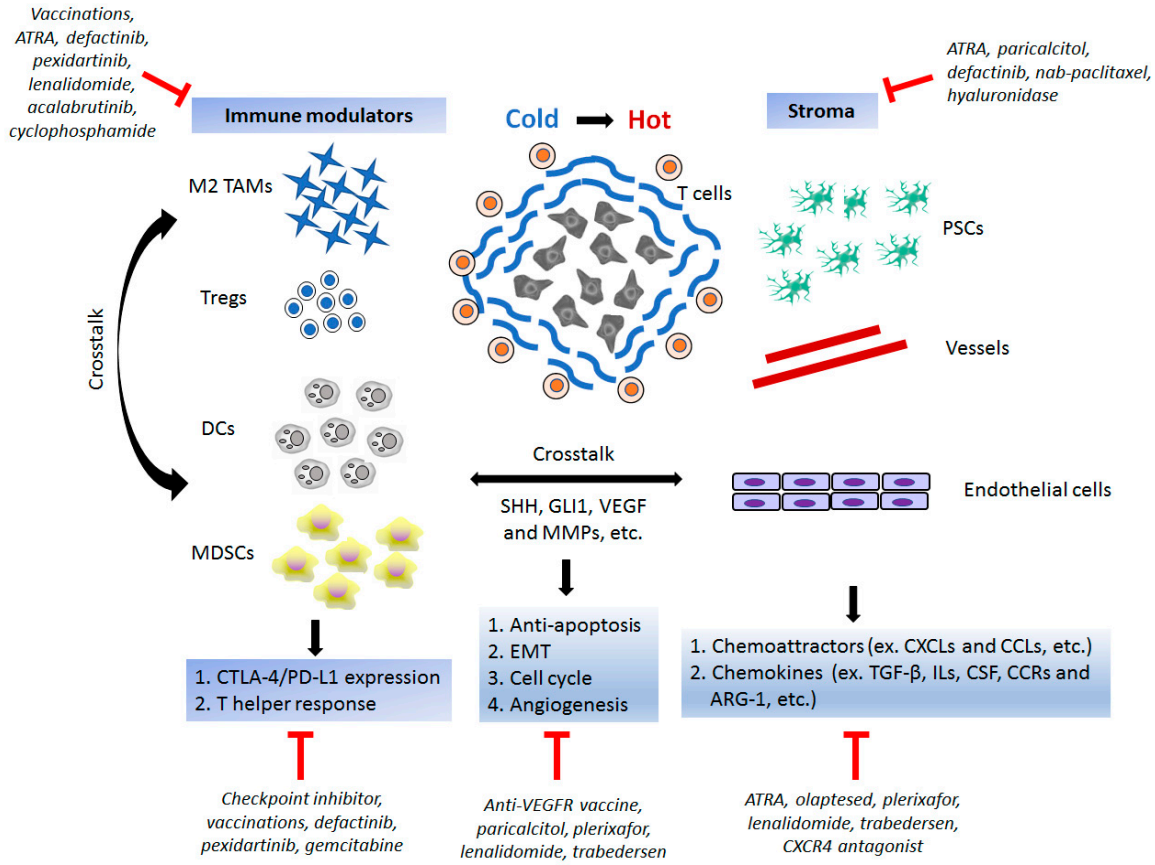

Figure 1. The response to immunotherapy in pancreatic ductal adenocarcinoma (PDAC) relies on destroying cancer cells as well as on breaking the stromal barrier and flaring up immune function. Immunotherapy targeting the PD-1/PD-L1 pathway and TME has disrupted the traditional method of cancer treatment and countered the immune-related adverse events in patients. In addition to direct cytotoxicity on cancer cells, it is important to dissect the immunosuppressive microenvironment with various cytokines and immune cells. Furthermore, recognition of mechanisms modulating the PD-1/PD-L1 pathway and TME will identify more potential therapeutic targets in the future.

Table 4. Summary of ongoing clinical trials on vaccination.

Agents	Ongoing Clinical Trials
GVAX	1. Neoadjuvant/Adjuvant GVAX Pancreas Vaccine (With CY) With or Without Nivolumab Trial for Surgically Resectable Pancreatic Cancer, NCT02451982, status: recruiting 2. Study With CY, Pembrolizumab, GVAX, and SBRT in Patients With Locally Advanced Pancreatic Cancer, NCT02648282, status: recruiting 3. Study of CRS-207, Nivolumab, and Ipilimumab With or Without GVAX Pancreas Vaccine (With Cy) in Patients With Pancreatic Cancer, NCT03190265, status: recruiting 4. Phase 2 GVAX Pancreas Vaccine (With CY) in Combination With Nivolumab and SBRT for Patients With Borderline Resectable Pancreatic Cancer, NCT03161379, status: recruiting 5. Pancreatic Tumor Cell Vaccine (GVAX), Low Dose Cyclophosphamide, Fractionated Stereotactic Body Radiation Therapy (SBRT), and FOLFIRINOX Chemotherapy in Patients With Resected Adenocarcinoma of the Pancreas, NCT01595321, status: active, not recruiting 6. Pilot Study With CY, Pembrolizumab, GVAX, and IMC-CS4 (LY3022855) in Patients With Borderline Resectable Adenocarcinoma of the Pancreas, NCT03153410, status: recruiting 7. Vaccine Therapy With or Without Cyclophosphamide in Treating Patients Undergoing Chemotherapy and Radiation Therapy for Stage I or Stage II Pancreatic Cancer That Can Be Removed by Surgery, NCT00727441, status: active, not recruiting
CRS-207	1. Study of CRS-207, Nivolumab, and Ipilimumab With or Without GVAX Pancreas Vaccine (With Cy) in Patients With Pancreatic Cancer, NCT03190265, status: recruiting 2. Epacadostat, Pembrolizumab, and CRS-207, With or Without CY/GVAX Pancreas in Patients With Metastatic Pancreas Cancer, NCT03006302, status: recruiting 3. GVAX Pancreas Vaccine (With CY) and CRS-207 With or Without Nivolumab, NCT02243371, status: active, not recruiting 4. Study of Safety and Tolerability of Intravenous CRS-207 in Adults With Selected Advanced Solid Tumors Who Have Failed or Who Are Not Candidates for Standard Treatment, NCT00585845, status: terminated
Algenpantucel-L	1. Immunotherapy and SBRT Study in Borderline Resectable Pancreatic Cancer, NCT02405585, status: terminated 2. Low Dose Vaccine Study for Surgically Resected Pancreatic Cancer, NCT00614601, status: terminated 3. Long Term Follow-Up Study for Subjects Previously Treated With Algenpantucel-L (HyperAcute-Pancreas) Immunotherapy, NCT03165188, status: recruiting
KRAS peptide	1. QUILT-3.070: Pancreatic Cancer Vaccine: Subjects With Pancreatic Cancer Who Have Progressed on or After Standard-of-care Therapy, NCT03387098, status: recruiting 2. QUILT-3.060: NANT Pancreatic Cancer Vaccine: Molecularly Informed Integrated Immunotherapy in Subjects With Pancreatic Cancer Who Have Progressed on or After Standard-of-care Therapy, NCT03329248, status: active, not recruiting 3. QUILT-3.080: NANT Pancreatic Cancer Vaccine, NCT03586869, status: recruiting 4. QUILT-3.039: NANT Pancreatic Cancer Vaccine: Combination Immunotherapy in Subjects With Pancreatic Cancer Who Have Progressed on or After Standard-of-care Therapy, NCT03136406, status: active, not recruiting 5. QUILT-3.088: NANT Pancreatic Cancer Vaccine, NCT03563144, status: not yet recruiting 6. Vaccine Therapy in Treating Patients With Pancreatic Cancer That Has Been Removed by Surgery, NCT00389610, status: active, not recruiting
Telomerase peptide (GV1001)	1. hTERT Immunotherapy Alone or in Combination With IL-12 DNA Followed by Electroporation in Adults With Solid Tumors at High Risk of Relapse, NCT02960594, status: active, not recruiting

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