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New avenues for the treatment of immunotherapy-resistant pancreatic cancer

Silva LGO *et al*. New avenues for immunotherapy-resistant PC

Abstract

Pancreatic cancer (PC) is characterized by its extremely aggressive nature and ranks 14th in the number of new cancer cases worldwide. However, due to its complexity, it ranks 7th in the list of the most lethal cancers worldwide. The pathogenesis of pancreatic cancer involves several complex processes, including familial genetic factors associated with risk factors such as obesity, diabetes mellitus, chronic pancreatitis, and smoking. Mutations in genes such as *KRAS, TP53*, and *SMAD4* are linked to the appearance of malignant cells that generate pancreatic lesions and, consequently, cancer. In this context, some therapies are used for pancreatic cancer, one of which is immunotherapy, which is extremely promising in various other types of cancer but has shown little response in the treatment of pancreatic cancer due to various resistance mechanisms that contribute to a drop in immunotherapy efficiency. It is therefore clear that the tumor microenvironment (TME) has a huge impact on the resistance process, since cellular and non-cellular elements create an immunosuppressive environment, characterized by a dense desmoplastic stroma with cancer-associated fibroblasts, pancreatic stellate cells, extracellular matrix, and immunosuppressive cells. Linked to this are genetic mutations in *TP53* and immunosuppressive factors that act on T cells, resulting in a shortage of CD8+ T cells and limited expression of activation markers such as interferon-gamma. In this way, finding new strategies that make it possible to manipulate resistance mechanisms is necessary. Thus, techniques such as the use of tumor microenvironment modulators that block receptors and stromal molecules that generate resistance, the use of genetic manipulation in specific regions, such as MicroRNAs, the modulation of extrinsic and intrinsic factors associated with T cells, and, above all, therapeutic models that combine these modulation techniques constitute the promising future of pancreatic cancer therapy. Thus, this study aims to elucidate the main mechanisms of resistance to immunotherapy in pancreatic cancer and new ways of manipulating this process, resulting in a more efficient therapy for cancer patients and, consequently, a reduction in the lethality of this aggressive cancer.

Key Words: pancreatic cancer; immunotherapy; resistance; tumor microenvironment; manipulation; combined immunotherapy

Core Tip: This study aims to analyze the main mechanisms of resistance to pancreatic cancer immunotherapy and the respective methods of manipulating these processes. Thus, this review provides a compilation of the main mechanisms of resistance to immunotherapy linked to the tumor microenvironment, genetic factors and those linked to T-cell immunosuppression. Finally, this study provides an insight into new avenues that can be followed to manipulate the factors linked to resistance, providing a more efficient treatment and a reduction in lethality.

INTRODUCTION

Pancreatic cancer (PC) is an extremely complex disease and represents a major challenge for oncology. Characterized by its highly aggressive nature, PC ranks 14th among cancers with the highest number of new cases worldwide, with around 495,773 cases reported in 2020 and an overall 5-year survival rate of 11%[1,2]. Moreover, PC also garners attention due to its high lethality and aggressiveness, accounting for 466,003 new deaths and securing the 7th position on the list of the most lethal types of cancer in 2020[1]. Additionally, the high level of complexity involved in managing PC stems from its late diagnosis and potent metastatic capability, which compromises treatment and prognosis[3].

The pathogenesis of PC involves a combination of factors related to life history and genetic alterations, ultimately leading to an individual's susceptibility[4]. The primary risk factors for developing pancreatic cancer include a family history of the disease, chronic pancreatitis, genetic disorders, smoking, and poor dietary habits[4-6]. Regarding

genetic alterations, cancer can stem from mutations in tumor suppressor genes and oncogenes, such as *KRAS, CDKN2A, TP53, SMAD4*, and *BRCA1/2*[7].

The treatment of PC relies on surgery, chemotherapy, radiotherapy, and immunotherapy[8]. However, this process is extremely complex due to the elevated rates of metastases that impede surgery in the majority of patients, the intricate nature of the surgical approach due to anatomical challenges, and the mechanisms of resistance to chemotherapy and immunotherapy present in the tumor microenvironment of pancreatic cancer[9,10].

It is important to note that immunotherapy has emerged as a crucial treatment in recent years for various types of cancer[11]. Nevertheless, resistance to these methods in PC underscores the necessity of comprehending these mechanisms to develop efficient strategies for addressing this new challenge. Therefore, this study aims to report the main mechanisms in PC that lead to resistance to immunotherapy and the new ways to overcome this obstacle.

THE ONSET OF PANCREATIC CANCER

The development of pancreatic cancer is notably linked to the extensive plasticity of acinar and ductal cells in pancreatic tissue[12]. In physiological situations, these cells already possess a wide capacity for cellular metaplasia (transdifferentiation) for regenerative purposes[12]. This potential for identity reprogramming extends beyond regenerative processes, becoming a favorable factor for pancreatic carcinogenesis[12]. Besides genetic familiar factors, non-hereditary risk events are associated with an increased risk of pancreatic cancer, including obesity[13], chronic pancreatitis[14], cigarette smoking[15], and diabetes mellitus, especially new-onset diabetes mellitus (NOD) after the 5th decade of life[16].

The onset of pancreatic cancer occurs through the malignant evolution of non-invasive precursor pancreatic lesions, which, according to a prospective epidemiological study, become significantly more common, larger, and more numerous with aging[17-19]. However, the presence of non-invasive precursor pancreatic lesions does not

necessarily imply future malignancy; the risk varies according to the level of identified dysplasia[20]. A 2015 international consensus recommends stratifying pancreatic lesions into two levels: "low-grade" for lesions with mild to moderate dysplasia and "high-grade," reserved for lesions with severe dysplasia ("carcinoma in situ" type), exhibiting significantly increased potential for progression to invasive carcinoma[21]. Morphologically, there are three primarily forms of noninvasive precursor lesions, including pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN)[17,21].

PanIN are noncystic proliferative lesions located in pancreatic ducts up to 5mm, characterized by the replacement of normal cuboid/columnar epithelial tissue by flat/papillary epithelial cells with varying levels of cytological and architectural atypia[22]. Low-grade PanINs (traditionally described as PanIN 1A, PanIN 1B, and PanIN 2) exhibit mild-to-moderate cytological atypia[23]. In contrast, high-grade PanINs (or PanIN 3) are predominantly papillary, featuring loss of polarity, irregular stratification, severe cytological atypia, and eventual intraluminal necrosis[24].

In addition, some alterations in oncogenes or tumor suppressor genes underlying pancreatic cancer are identified early in PanIN. The endogenous expression of the *KRASG12D* mutation, associated with telomere shortening, represents the first events in low-grade PanINs, driving all stages of PanIN progression to pancreatic adenocarcinoma (PDAC)[25–27]. The frequency of the *KRASG12D* mutation in PanINs increases according to the degree of dysplasia, becoming more frequent in high-grade PanINs, and being present in >90% of PDAC cases[24,28,29].

Conversely, mutations in tumor suppressors *TP53*, which plays a role in cellular damage repair and apoptosis, and *SMAD4*, whose protein is a transcription factor for growth inhibition and apoptosis-related genes, are widely described in established PDAC[30–33]. However, these mutations appear almost exclusively in advanced neoplastic stages (high-grade PanINs)[34], being rarely found in isolated PanIN lesions, i.e., without invasive pancreatic cancer[35–37].

Intraductal papillary mucinous neoplasm are macroscopic mucin-producing cystic neoplasms that communicate with the pancreatic ductal system[38]. Similar to PanINs, IPMNs are predominantly composed of columnar cells with a papillary configuration, exhibiting varying degrees of cytological atypia[38]. However, they substantially differ in diameter, generally being larger than 1.0 cm in IPMNs[38]. IPMNs can be classified based on the duct involved: main-duct or branch-duct type; or the predominant cell type: pancreatobiliary, intestinal, or gastric types[24]. Main-duct IPMNs are most frequently associated with high-grade dysplasia, generally consisting of pancreatobiliary- and intestinal-cell types, which are associated, respectively, with an increased risk of tubular adenocarcinomas and colloid carcinomas, according to recent meta-analysis[39–41].

An IPMN lesion may be accompanied by an invasive carcinoma in two ways[42]. The first scenario involves the IPMN serving as a direct precursor to the existing carcinoma, commonly main duct IPMNs accompanied by colloid carcinomas[42]. The second possibility is the coexistence of an IPMN alongside an independently established carcinoma, with branch-duct IPMNs being more likely in this context[42]. Main risk factors for IPMN progression to PDAC include main pancreatic duct dilation, a size of >= 3 cm, and the presence of associated solid components[43,44].

Like PanINs, *KRAS* mutations are consistently reported in IPMNs, tipically manifesting in early stages of dysplasia (low-grade)[45]. Mutations in *TP53* and *SMAD4* tend to develop in more advanced stages of neoplasia[46,47]. *SMAD4* mutation is strongly associated with neoplastic capacity[47]. *GNAS* mutations are frequent and highly specific to IPMNs, potentially aiding in their differentiaton from other cystic lesions[48].

Also in this context, mucinous cystic neoplasm is the least common precursor of pancreatic cancer, and are almost exclusive to women aged 40-50 years[24]. Similarly to IPMNs, MCNs are composed of mucin-producing columnar epithelial cells, but differ in the presence of a subepithelial ovarian-type stroma, a pathognomonic finding of MCN[48,49]. Generally located in the body and tail of the pancreas (with less than 10% in the pancreatic head), MCNs are rarely multifocal[25,50]. In comparison to IPMNs, MCNs have a lower risk of evolving into invasive carcinoma[51]. Predictive factors for

malignancy include cyst diameter and the presence of mural nodules[51]. While MCNs typically exhibit slow growth, high exposure to sex hormones during pregnancy can trigger rapid enlargement[52]. Histological types of invasive carcinoma frequently associated with MCNs include tubular adenocarcinomas, mucinous non-cystic (colloid) carcinomas, undifferentiated carcinomas, undifferentiated carcinomas with osteoclast-like giant cells, adenosquamous carcinomas, and sarcomas[25]. While there is a lack of studies focusing on the genetic bases of MCNs, mutations in *KRAS, TP53, SMAD4*, and *CDKN2A/p16* have been verified[53].

Finally, it is important to note that the tumor microenvironment (TME) plays a fundamental role in the establishment and persistence of pancreatic neoplasia. Histologically, the tumor microenvironment of PDAC is characterized by a dense stroma composed of cellular and acellular components, initiating development from the early stages of neoplastic precursors[54]. The cellular component of the stroma forms a network that includes: myeloid cells (macrophages, neutrophils, regulatory cells, cytotoxic cells), cancer-associated fibroblasts, neurons, and endothelial cells[54]. Interaction between these agents co-stimulates the production of molecules such as growth factors, matricellular proteins, tissue inhibitors of metalloproteinases, and cytokines[54]. Such structural changes are intimately related to tumor maintenance and progression, altering vascular density and tissue perfusion[29].

SURGERY, CHEMOTHERAPY AND RADIOTHERAPY FOR PANCREATIC CANCER

The management of PC is contingent upon the disease stage. Consequently, the application of surgical interventions is reserved for individuals presenting with resectable tumors devoid of distant metastases, possibly in conjunction with adjuvant chemotherapy[17]. Within this framework, surgery is undertaken with the objective of achieving complete tumor resection, thereby fostering a more favorable prognosis for the patient. However, pancreatic tumor excisions constitute anatomically intricate procedures, frequently culminating in incomplete resection[55]. Moreover, the

pancreatoduodenectomy, a commonly employed procedure, is associated with a morbidity rate of up to 45%[55]. This is compounded by the circumstance that a considerable proportion of pancreatic cancer diagnoses occur at an advanced stage, characterized by metastasis, rendering surgery unviable. This elucidates the intricacies associated with performing surgical interventions on pancreatic tumors[56].

Chemotherapeutic interventions for pancreatic cancer encompass three distinct regimens: neoadjuvant, adjuvant, or first-line strategies[55]. The neoadjuvant approach is employed preemptively, preceding surgical resection, with the aim of diminishing tumor size. Conversely, the adjuvant regimen is administered post-surgical resection, while patients with metastatic pancreatic cancer receive first-line chemotherapy[55,57]. Noteworthy chemotherapy protocols for PC include gemcitabine, nab-paclitaxel, and FOLFIRINOX[57]. However, the anticipated efficacy of chemotherapy in treating pancreatic cancer has not been fully realized, as the intricate oncological landscape of PC is characterized by pronounced chemoresistance[58]. Within this context, gemcitabine emerges as the chemotherapy agent exhibiting the highest degree of chemoresistance to date. This phenomenon can be attributed to various factors inherent in PC, such as components of the tumor microenvironment, the release of inflammatory enzymes, altered signaling pathways involving cells like fibroblasts and pancreatic stellate cells, and genetic alterations, including MicroRNA[58].

Radiotherapy has been incorporated into neoadjuvant, adjuvant, and first-line treatment regimens for patients with metastatic and advanced PC[59]. While chemoradiotherapy in neoadjuvant and adjuvant settings has demonstrated a marginal increase in patient survival, the majority of diagnoses occur at an advanced disease stage. Consequently, the use of chemotherapy and radiotherapy as first-line treatments becomes imperative[59,60]. Nevertheless, the application of radiotherapy in the treatment of patients with metastatic PC yields conflicting data and falls short of anticipated effectiveness. This underscores the necessity for novel clinical studies dedicated to scrutinizing the role and efficacy of radiotherapy in addressing the complexities of this disease[60].

IMMUNOTHERAPY IN PANCREATIC CANCER

Immunotherapy stands as a groundbreaking frontier in the realm of cancer treatment. The concept of leveraging the body's own immune system to target cancerous cells has brought about a profound shift in the overall survival rates for several types of cancer[61-63]. Moreover, it distinguishes itself by presenting fewer side effects in comparison to conventional approaches, such as chemotherapy[64].

In the context of Pancreatic Cancer, however, the use of immunotherapy, particularly immune checkpoint inhibitors (ICIs), as a standalone treatment in unselected patients has not demonstrated the same level of success observed in other tumor types[65]. From this perspective, anti-CTLA-4 drugs are already a reality in immunotherapy treatment, and drugs of this class, such as Ipilimumab, have received approval in both the United States and Europe[66]. However, both Ipilimumab and Tremelimumab (anti-CTLA-4) proved unsuccessful in clinical trials focused on treating pancreatic cancer[67,68]. It is also important to mention the anti-PD-1/PD-L1 drugs, with Nivolumab and Pembrolizumab being the primary representatives; however, these drugs have not demonstrated significant success in studies targeting PC, largely due to the complexity of this cancer model[66,69,70].

In another scenario, the investigation of vaccines in pancreatic cancer therapy has also become a subject of study in the eager pursuit of an efficient treatment to combat resistance to this complex cancer[71]. Thus, various vaccine models already exist in the scientific world and are currently undergoing testing for PC, with the primary ones being GVAX (cell-based) and vaccination with *Listeria monocytogenes*[71]. However, these methods have also demonstrated limitations in the ongoing analyses[71].

Finally, it is important to highlight Adoptive Cell Transfer (ACT) as an immunotherapy model that has also been employed in treating PC[72]. The method is based on CAR T cells produced from T cells extracted from an individual and genetically altered to enhance their efficiency against cancer when reintroduced into the patient[73]. Despite this, clinical studies targeting various aspects of adoptive cell therapy for pancreatic

cancer, such as mesothelin and EGFR, have demonstrated limited responses and minimal impact on patient survival[74,75].

Therefore, it is clear that understanding the mechanisms in the pancreatic cancer TME that induce resistance to immunotherapy treatment is crucial for developing new techniques to overcome the complexity imposed by this oncological model.

MECHANISMS OF RESISTANCE TO IMMUNOTHERAPY IN PANCREATIC CANCER

The role of the tumor microenvironment in immunosuppression and resistance

The tumor microenvironment of pancreatic cancer is characterized by a complex network of cellular and non-cellular elements that create a highly immunosuppressive environment. It is composed of a dense desmoplastic stroma, comprising cancer-associated fibroblasts (CAFs), pancreatic stellate cells (PSCs), extracellular matrix (ECM) and immune suppressive cells[76].

Initially, the notable capacity of CAFs to generate and remodel the ECM plays a pivotal role in immunotherapy resistance in pancreatic cancers. These cells comprise distinct subtypes, each exerting specific influences on the tumor microenvironment. Firstly, α-smooth muscle actin (α-SMA) + myofibroblasts (myCAFs), which are TGF signaling-dependent, contribute to the synthesis of extracellular matrix components[77,78]. On the other hand, inflammatory CAFs (iCAFs), exhibit elevated expression of IL-6, which is related to cancer progression[78,79], while MHC class II+ CAFs (apCAFs) are able to present antigens but lack costimulatory molecules, potentially leading to deactivation of CD4+ T cells and further immune suppression in the TME [**Figure 1**][80,81].

In the context of immunotherapy resistance, myCAFs emerge as a key player in this process, as they lead to the development of a dense, fibrotic stroma around the tumor, which provides a physical barrier that impedes the infiltration of immune cells and, consequently, impairs the effectiveness of immunotherapeutic agents[77,82]. Also, evidence suggests that secreted phosphoprotein 1 (SPP1) derived from CAFs in hepatocellular carcinomas apparently increases resistance to tyrosine kinase inhibitors

(TKIs), through the induction of epithelial-to-mesenchymal transition[83,84]. However, as the direct correlation between SPP1 and TKI resistance remains unexplored in pancreatic cancer, further research is required to clarify this issue.

Additionally, the combination of a dense stroma and limited vascularization induces severe hypoxia within the tumor microenvironment, triggering the stabilization of hypoxia-inducible factors 1 (HIF1) and 2 (HIF2)[82]. CAF-specific deletion of HIF2 is associated to increased survival in pancreatic cancer, by reducing the intratumoral recruitment of M2 macrophages; and therapeutic HIF2 inhibition leads to increased response to immune checkpoint blockade[85]. These findings highlight the critical role of hypoxia in shaping the pancreatic tumor microenvironment and influencing immunotherapy resistance.

Finally, pancreatic stellate cells also seem to play a role in desmoplasia, as they become activated in response to signals from pancreatic cancer cells and contribute to the formation of the fibrotic stroma[82]. PSCs are associated with the secretion of immunosuppressive molecules, including CXCL2, IL-6 and galectin-1, sustaining immunosuppression within the TME[86]. These dynamic interactions between PSCs and pancreatic cancer cells sustain underlying mechanisms that promote immunotherapy resistance [Figure 1].

Therefore, therapeutic agents that target the aforementioned components could be a potential next step in overcoming immunotherapy resistance in pancreatic cancers.

Genetic/epigenetic factors

Evidence also suggests that genetic/epigenetic factors may play a role in immunotherapy resistance in pancreatic cancer, even though current literature provides minimal information on the subject. Some studies indicate that mutations in the *p53* gene are associated with alterations in the innate immune response, which may underlie tumorigenesis and promote immunotherapeutic resistance in pancreatic adenocarcinomas[87]. In this regard, the *Trp53*^{R172H} mutation in pancreatic cancer cells

has been identified as a promoter of neutrophil accumulation, potentially contributing to resistance against immunotherapy[88].

T cell-associated immunotherapy resistance: intrinsic and extrinsic mechanisms

Pancreatic ductal adenocarcinoma is typically associated with a low mutation burden, resulting in a paucity of neoantigens and a scarcity of tumor-infiltrating effector T cells[89,90]. This gives rise to an "immunologically cold" tumor microenvironment, which is characterized by a dearth of tumor antigen-specific CD8+ T cells and a limited expression of activation markers such as interferon-gamma (IFN-γ) and Granzyme B[91-95]. In turn, CD4+ helper T cells are more abundant within the TME compared to CD8+ T cells, displaying diverse immunological effects, including both anti- and pro-tumor activities across various phenotypes such as effector CD4+ T helper (Th) 1, Th2, Th17, FoxP3+Tregs, and γδ T cells[96]. Nevertheless, the evaluation of antigen-specific CD4+ T cells remains elusive in both animal and human PDAC models[97]. Moreover, These observations imply a deficit or impediment in adaptive T cell immunity, recognized as the primary factor contributing to the concerning resistance against immune checkpoint blockade therapies[81].

Accordingly, PDAC appears to utilize two primary strategies to circumvent anti-tumor immune responses: (1) intrinsic T-cell receptor (TCR)-mediated exhaustion, and (2) extrinsic TME-driven immunosuppression[81,98]. Indeed, CD8+ T cells targeting tumor-specific antigens can trigger cell death. Nonetheless, in cases where the tumor persists and these cells face continual antigen exposure, sustained TCR activation leads to their differentiation into exhausted T (Tex) cells[99-101]. Tex cells express cell surface inhibitory receptors (IRs) such as programmed cell death-1 (PD-1), Mucin-3/T-cell immunoglobulin (TIM-3), and T-cell activation gene (LAG-3)[94,102-105]. Upon interaction with their specific ligands expressed on cells within the tumor microenvironment, Tex cells undergo a progressive decline in effector function, differentiation state, and proliferative capacity[81]. Notably, these cells not only experience a reduction in functionality, such as decreased tumor necrosis factor (TNF)-α and IFN-γ expression,

but also demonstrate a gradual increase in interleukin-10 (IL-10) expression within the TME[106]. These alterations thus contribute to the establishment of a local immunosuppressive milieu **[Figure 2]**.

On the other hand, extrinsic factors encompass elements in the TME that hinder T cell function[107]. The oncogenic activation of *KRAS* in pancreatic cells initiates pancreatic intraepithelial neoplasia at the outset of cancer development, which triggers an immunosuppressive milieu orchestrated by tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T (Treg) and B (Breg) cells, compounded by inhibitory cytokines and metabolic limitations[108–110].

In this scenario, macrophages represent the predominant leukocyte population identified within PDAC[111]. Studies have unveiled the origin of TAMs in PDAC from two primary sources: (1) bone marrow-derived inflammatory monocytes, and (2) embryonic-derived tissue-resident macrophages[112,113]. These cells typically exhibit an immunosuppressive phenotype, which is characterized by the expression of immune checkpoints, inhibitory ligands, and the secretion of immunoregulatory cytokines such as IL-10[114–116].

Concurrently, the recruitment of bone marrow-derived myeloid cells toward PDAC involves a complex process orchestrated by granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), IL-3, vascular endothelial growth factor (VEGF), and the orchestrated interplay between the C-X-C chemokine ligand 12 (CXCL12)/C-X-C chemokine receptor 4 (CXCR4) or C-C chemokine ligand 2 (CCL2)/C-C chemokine receptor 2 (CCR2) signaling cascades[111,117,118]. Ultimately, tumor cell production of GM-CSF and G-CSF and tumor cell production of IL-1β fosters the proliferation of immature myeloid cells and drives their acquisition of a suppressive phenotype (MDSCs)[119–121].

Additionally, stromal-associated fibroblasts are known to produce C-X-C chemokine ligand 13 (CXCL13), which serves as a recruitment signal for IL-35-producing regulatory B cells within the TME[122]. This phenomenon further exacerbates PDAC

immune evasion by harnessing IL-35-mediated inhibition, effectively suppressing T cell proliferation[123].

Collectively, TAMs, MDSCs, and Bregs exhibit a robust capacity to suppress the proliferation in both CD4+ and CD8+ T cells[124]. They also generate elevated levels of immunosuppressive cytokines, such as IL-10, IL-27, and transforming growth factor β (TGF-β)[106,122,124]. This coordinated activity facilitates the recruitment of regulatory Foxp3+CD4+ Tregs, which may also impede the antitumor immunity of CD8+ T cells at local intratumoral sites[125,126]. In summary, these factors collectively reduce T cell infiltration, impair their function, or promote their exhaustion within the TME, contributing to resistance against immunotherapy interventions **[Figure 2]**.

NEW TECHNIQUES TO OVERCOME RESISTANCE TO IMMUNOTHERAPY IN PANCREATIC CANCER

Manipulation of the tumor microenvironment

Over the past decade, there has been significant interest in the pancreatic tumor microenvironment, particularly in its capacity to influence therapy response. The focus has shifted towards recognizing the tumor microenvironment as a key factor and obstacle affecting the effectiveness of immunotherapy in pancreatic ductal adenocarcinoma[127].

Numerous promising therapies targeting various mechanisms are currently undergoing preclinical and clinical development. These approaches encompass novel strategies to enhance T-cell responses, modify myeloid and stromal compartments, and attract new immune cells to the tumor microenvironment of PDAC[128].

From this perspective, targeting the tumor stroma holds potential advantages in the treatment of pancreatic cancer. The matricellular protein Secreted Protein Acidic and Rich in Cysteine (SPARC), produced by CAFs, has the ability to bind albumin[127]. This led to the hypothesis that SPARC could enhance the accumulation of nab-paclitaxel within the pancreatic cancer microenvironment, thereby augmenting its anti-tumor efficacy[129]. In a phase III study combining albumin-bound paclitaxel (nab-paclitaxel)

with gemcitabine, the results indicated an increased intracellular concentration of gemcitabine, possibly attributed to the disruption of the tumor stroma and the reduction of cancer-associated fibroblasts[129].

Despite the promising outcomes mentioned earlier, efforts to target the tumor stroma have yielded contradictory consequences. Matrix metalloproteinases, a family of proteolytic enzymes essential for maintaining tissue homeostasis, play a crucial role in cancer invasion when expressed abnormally[130]. However, attempts to target matrix metalloproteinases using marimastat and tanomastat did not show any discernible benefits when combined with gemcitabine[131,132].

Furthermore, follow-up studies have indicated that depleting the stroma may, in fact, promote tumor growth, highlighting the intricate and multifaceted role that stroma plays in tumor biology. This underscores the complexity of the interactions within the tumor microenvironment and suggests that a nuanced approach is needed when considering stroma-targeted therapies in cancer treatment[133].

In preclinical mouse models of pancreatic cancer, the depletion of stroma by inhibiting the Hedgehog cellular signaling pathway has been demonstrated to enhance the delivery of gemcitabine to tumors[134]. This intervention resulted in improved survival and reduced metastasis by increasing the intracellular concentration of gemcitabine. The Hedgehog pathway's involvement in the formation of desmoplasia highlights its role in impairing drug delivery in the context of pancreatic cancer[134]. Despite the conflicting results of tumor response to stroma-depleting therapies, the tumor microenvironment plays a significant role in tumor biology and in modulating the immune recognition of pancreatic cancer.

Another therapeutic avenue under investigation involves targeting hyaluronic acid, which is abundant in pancreatic cancers and contributes to angiogenesis and chemoresistance[135]. In a phase II study involving untreated, metastatic pancreatic cancer patients, the targeting of hyaluronic acid using PEGPH20 (a pegylated formulation of recombinant hyaluronidase) in combination with nab-

paclitaxel/gemcitabine resulted in a significant improvement in progression-free survival (PFS) and overall survival (OS)[136].

Still in this scenario of trying to manipulate the microenvironment, it is known that the TME in pancreatic cancer represents a formidable therapeutic challenge when using traditional immunotherapies. Nevertheless, there is a shift towards utilizing combination approaches to reprogram the tumor microenvironment, aiming to unlock the potential benefits of immunotherapy. Early results from these endeavors are showing promise in the pursuit of more effective treatments for pancreatic cancer.

As aforementioned, targeting immunosuppressive cells within the TME enhances the likelihood of efficacy in immunotherapy treatments. One primary target is colony-stimulating factor 1 receptor (CSF1-R), located on tumor-associated macrophages. The binding of colony-stimulating factor 1 (CSF1) to CSF1-R facilitates TAM proliferation and extended survival, promoting tumor growth, resistance to treatments, and metastasis[137]. Inhibition of CSF1-R results in fewer TAMs, leading to a heightened immune response, increased tumor regression, and improved survival[138].

Looking at another relevant pathway for manipulation, it is notable that pancreatic ductal adenocarcinoma tumors show infiltration of M2 macrophages, which have an immunosuppressive function. This phenotype is characterized by the expression of CD206, CSF-1R, and IL-10, along with reduced expression of major histocompatibility complex class II (MHC class II)[81]. The CSF-1 pathway plays a crucial role in the differentiation and survival of M2 macrophages. Inhibiting the CSF-1 pathway has been demonstrated to redirect TAMs toward the M1 phenotype, leading to distinct remodeling of the TME[139–141].

From a molecular perspective, it is important to analyze that C-X-C chemokine ligand 12, a chemokine produced by cancer-associated fibroblasts, is frequently expressed at elevated levels in the PDAC tumor microenvironment. This creates a network of dense stroma, which, in turn, hinders the migration of immune cells and the recognition of cancer cell antigens. The elevated levels of CXCL12 in the PDAC TME play a role in creating an immunosuppressive environment, thereby diminishing the effectiveness of

immune responses directed at cancer cells[142]. In preclinical studies, interrupting the interaction between CXCL12 and its receptor, CXCR4, enhanced the impact of immune checkpoint inhibitors in models of PDAC[142,143].

Also in this scenario, pancreatic tumor cells, fibroblasts, and other stromal cells release TGFβ, a cytokine that contributes to the creation of an immunosuppressive structure in the TME[144]. Using the small molecule inhibitor galunisertib to target TGFβ, combined with gemcitabine as the initial treatment for PDAC, resulted in only a marginal improvement in median overall survival (mOS) compared to gemcitabine alone and did not achieve statistical significance[145]. Following this, galunisertib was evaluated in conjunction with durvalumab in a cohort of 32 patients with advanced PDAC and demonstrated restricted effectiveness, yielding only one partial response[146]. Novel approaches, such as exploring a bifunctional fusion comprising a monoclonal antibody targeting TGFβ along with other immune checkpoint inhibitors, are currently being investigated[147].

Therefore, the combination of treatment strategies aimed at stimulating the immune response and overcoming barriers in the tumor microenvironment represent a promising avenue for improving the treatment of patients with pancreatic cancer.

Manipulation of genetic/epigenetic factors

The genetic mutation background of PC is well known, being found in *CDKN2A, MLH1, BRCA2, ATM, KRAS* and *BRCA1*. The most prevalent change of an oncogene in pancreatic cancer cells is the mutation of *KRAS*. Other than that, some tumor suppressor pathways are genetically inactivated, such as *INK4a/ARF (p16), TP53, DPC4/Smad4*[148–153].

Thus, the key principles of gene therapy are to induce immune effects that combat tumors with different signaling pathways, delivering genetic material to cells, focusing on the resolution of a disorder[154]. An effective gene therapy regimen is dependent upon the following factors: efficient delivery of the gene, therapy specifically targeted at the tumor, and careful selection of optimal targets[155].

Against this backdrop, there are many possibilities that surround gene-therapy on pancreatic cancer cells. Gene editing and gene transfer can be utilized as a therapeutic intervention, employing an array of vectors and molecular tools, including interference RNA and genome editing techniques, which have shown promise in bridging preclinical cancer research and clinical trials[156,157].

In addition, various strategies have been applied to eliminate tumor cells based on known genetic alterations. Gene transfer strategies with *TP53* have been utilized to treat multiple cancers[158]. However, attempts to restore *TP53* expression during tumor growth have yielded disappointing results, indicating the limited efficacy of gene transfer *in vitro*[158].

Still in the scenario of genetic manipulation, suicide gene therapy is a major topic of discussion, based on the transfer of a suicide gene with a strong neighboring antitumor effect that can compensate the weakness of gene expression within the tumor[155]. The classic suicide gene strategy is the herpes simplex virus thymidine kinase gene (HSV-TK gene)[159]. This therapy is capable of causing toxicity and cell death, through metabolites and inhibition of DNA synthesis[159,160], It also elicits a robust immune response targeting tumor cells by releasing tumor antigens, resulting in a reaction against additional tumor cells by the body[159–161]. HSV-TK delivery *via* adenovirus and retrovirus have shown great anti-tumor efficiency in pancreatic cells both *in vitro* and *in vivo*[160,162].

Other examples of suicide/prodrug gene system that has been also tested, with success, in PC models is the cytochrome P450/isofosfamide system, was developed through *in vitro* and *in vivo* proof of concept to conduct phase I and II trials in patients with pancreatic cancer, the treatment has shown significant success in improving survival rates[158,163–165].

Other than that, MicroRNA (miRNA) is another potent point for therapeutic approach. Several studies have shown that miRNAs play important roles in the development of pancreatic tumors and also in the process of resistance to various therapies, including immunotherapy[166–168]. Loss of miRNA expression may result in significant

dysfunctionality and promote carcinogenesis, owing to their crucial role in modulating apoptosis, cell cycle, and differentiation[158].

Thus, the methods for modulating intracellular miRNA levels primarily consist of miRNA replacement therapy and anti-miRNA oligonucleotides. In miRNA replacement therapy, oligonucleotide mimics are used to increase miRNA levels, while in anti-miRNA oligonucleotides, miRNA silencing is induced[169,170]. However, some barriers still exist when thinking in miRNA therapy such as low *in vivo* stability, improper biodistribution, insufficient cell specificity, disruption and saturation of endogenous RNA machinery, as some examples[171].

In this scenario, several miRNAs that are upregulated or downregulated in pancreatic cancer have demonstrated their contribution to tumor cell growth by targeting specific molecules[172-174]. An elevation in circulating miRNAs, such as *miR-21, miR-25, miR-155* and *miR-196*, demonstrated a strong correlation with chemotherapy resistance among pancreatic cancer patients[175,176]. In this sense, an experimental study showed that targeting the oncogenic *miRNA21* could suppress tumor growth in pancreatic cancer *in vitro* and *in vivo*[177]. However, no miRNA therapeutics have been tested clinically for pancreatic cancer treatment[178].

From another perspective of genetic manipulation, oncolytic virotherapy is one of the most promising anti-cancer therapies using agents with high antitumor potency and strong oncolytic effect[154,179]. Natural pathogens have either been selected or designed to specifically infect and destroy cancer cells, being engineered in a way that enables the production of cytokines, antigens, or suicide genes[158]. Oncolytic adenoviruses have been considered highly eligible vehicles for delivery of therapeutic genes to treat cancer due to their tumor-restricted replication capabilities[158,180], and because of them being non-pathogenic and with a high selectivity and cytotoxicity to cancer cells[181].

With this in mind, it is important to point out that a known viral vector is the herpes simplex virus type 1 (HSV-1) designed with an efficient blockade of experimental tumor growth, used alone or in combination with gemcitabine[182]. A study with a HSV that showed an promising anti-cancer activity was Myb34.5, which has been assessed

preclinically in pancreatic cancer models, being used inducing apoptosis and inhibition of pancreatic tumor growth[183]. Other studies are being conducted, dealing with possible viruses that can be used on a therapeutic concept, such as the oncolytic parvovirus, H-1PV, in some clinical trials[184,185].

Numerous genetic alterations that directly contribute to pancreatic tumorigenesis have been identified or are being actively studied; because of it, novel therapies for pancreatic cancer patients by targeting specific genes are a promising future, associated with a lot of new trials searching novel possibilities[154,156,158]. This approach to personalized medicine can be utilized for patients with pancreatic cancer, providing appropriate treatment that is tailored to their individual needs.

Manipulation of intrinsic and extrinsic mechanisms associated with T cells

The mechanisms of resistance to immunotherapy present in PC are rooted in an intense interaction between molecules and cells of the TME and cells of the immune system, with this impact being particularly focused on the activity of T cells, leading to a reduction in the tumor activity of these cells[186]. Thus, novel approaches to treating immunotherapy-resistant PC involve manipulating the activity of T cells and immune system cells that directly interact with these anti-tumor cells[187].

One of the primary immunotherapy strategies used in oncology is to block the immune checkpoint, but resistance to existing methods has prompted the need to employ immunomodulators, such as PD1-IL2v[188,189]. PD1-IL2v is a bispecific antibody molecule that binds to PD-1 on CD8 T cells and incorporates a modified IL-2 molecule in its structure[189]. This modification stimulates the cytotoxic activity of CD8 T cells without binding to CD25 present on Treg cells, consequently avoiding the activation of these regulatory cells[189]. Thus, the study conducted by Tichet *et al* in mice was based on the combination of anti-PD-L1 with the immunomodulator PD1-IL2v and achieved promising results, including tumor regression, improved efficiency of anti-tumor T cells, increased infiltration of CD8+ T cells into the TME, modulation of tumor-

associated macrophages, and demonstrated a positive response for cancers resistant to immunotherapy[189].

From another perspective, Siglec-15 was detailed by Wang J *et al* in 2019, demonstrating that it can be expressed in cancer cells and tumor-associated macrophages, with increased expression in macrophages leading to the inhibition of anti-tumor T cell proliferation[190]. Consequently, Siglec-15 has become a new target for therapy based on blocking the immune checkpoint, despite the absence of published studies to comprehend all its functions in PC[190,191]. Sun *et al* describe Siglec-15 as a potential therapeutic target for cancer based on ongoing clinical studies[191]. Therefore, as analyzed by Chen X *et al*, Siglec-15-based therapy may offer a promising solution for pancreatic cancer patients resistant to anti-PD-1 treatment[192]. It can be utilized alone or in conjunction with other immune checkpoint blockade techniques, resulting in increased infiltration of CD8+ T cells into the tumor microenvironment[192].

Another growing immunotherapy technique in cancer treatment is Adoptive Cell Therapy, based on the *ex vivo* manipulation of T cells to expand their anti-tumor activity[193]. After manipulation, these cells are reinserted into the individual to exert more potent anti-tumor effects[193]. Despite the various factors contributing to resistance to immunotherapy in pancreatic cancer, impacting the efficiency of ACT for this type of cancer, the primary obstacle remains the immunosuppressive and challenging-to-access TME, hindering the infiltration of immune cells[72]. In this regard, Rataj F *et al* demonstrated a promising approach to overcoming resistance by developing a fusion protein with the extracellular domain of the PD-1 receptor fused to the intracellular T-cell activation domain of CD28[194]. This fusion protein was implanted into CD4+ T cells using the ACT technique. When these modified cells were introduced into the TME, the PD-1 domain interacted with its ligand PD-L1[194]. However, instead of inducing immunosuppression, the activated CD28 protein coupled to PD-1 resulted in increased antitumor activity of CD4+ T cells through enhanced cytokine secretion and stimulation of the cytotoxic activity of CD8+ T cells[194].

Still from the perspective of manipulating the extrinsic and intrinsic mechanisms associated with T cells, even more recent studies have concentrated on the use of nanomedicines to overcome resistance to immunotherapy[195]. In this context, Jung JY *et al* conducted a study in humanized mice employing an siRNA nanoparticle targeting PD-L1 as the therapeutic approach[196]. The study yielded promising results, leading to an increase in CD8+ T cells in the TME as a result of the blockade of PD-L1 induction caused by the nanoparticle absorbed by cells in the tumor microenvironment[196].

When analyzing molecular pathways, it is known that the use of cytokines as immunomodulators is an immunotherapeutic strategy employed in cancer treatment, but it has not yielded promising responses when used alone in PC, primarily due to immune resistance mechanisms[197]. Many therapies utilizing cytokines with immunosuppressive activity are employed as adjuvants to other immunotherapy models[197]. Nonetheless, more targeted studies investigating the action of specific cytokines may lead to new strategies for addressing immunotherapy-resistant pancreatic cancer.

With this in mind, Huang J *et al* conducted a study with the aim of inhibiting the immunosuppressive activity resulting from the action of IFN-γ, a pivotal cytokine in the process of resistance to immunotherapy[198]. According to this analysis, the action of gamma interferon leads to the production of proteins such as indoleamine 2,3-dioxygenase 1 (IDO1) and CD274, which possess immunosuppressive properties and are included in the category of therapies based on inhibiting the immune checkpoint[198]. The study utilized dinaciclib to block the expression of these proteins induced by IFN-γ in murine models and achieved promising results in curtailing the immunosuppressive activity of IFN-γ, reducing cancer immune invasion, and blocking the expression of immune checkpoint[198].

In a similar vein, Tsukamoto M *et al* demonstrated in a study involving 235 patients that TNF-α overexpression is directly associated with increased PD-L1 expression in TME cells, leading to immunosuppression through the neutralization of cytotoxic T cells[199]. Consequently, anti-TNF-α may also exhibit promising efficacy in addressing resistance

to existing immunotherapy in PC by impacting the PD-L1 receptor, which is immunosuppressive and also a target of immune checkpoint blockade[199]. Nevertheless, additional clinical trials aimed at analyzing the impact of anti-TNF-α on PD-L1 expression are still necessary.

Combined immunotherapy

The mechanisms of resistance to immunotherapy in the treatment of pancreatic cancer have brought a new challenge for medicine: to identify therapeutic combinations that help overcome resistance, thus increasing the efficiency of immunotherapy and improving the patient's prognosis[200].

From this perspective, Mehla K *et al* utilized a murine monoclonal antibody (mAb-AR20.5), which modulates the TME by binding to Mucin 1 (MUC1), in conjunction with PolyICLC (a vaccine adjuvant) and anti-PD-L1 in their murine models for the treatment of pancreatic cancer[201]. The combined therapy induced superior anti-tumor activity, leading to the rejection of tumor cells expressing MUC1 and heightened cytotoxic activity of CD8+ T cells[201]. This resulted in immune modulation and promising tumor control for pancreatic cancer through the use of an TME modulator, an immune checkpoint blocker and a vaccine adjuvant[201].

In the same context, it is pertinent to examine the promising aspect of using TME modulators alongside immune checkpoint inhibitors, as demonstrated by Rana M *et al*[202]. They conducted a preclinical study with murine models, employing an inhibitor of the TGF-β receptor, responsible for TME progression, and an immune checkpoint blocker (anti-PD-L1/anti-CTLA-4)[202]. The study resulted in the inhibition of tumor growth, enhanced CD8 T cell infiltration, and increased the population of M1 macrophages in the TME[202]. Additionally, Cappellesso F *et al* adopted a similar approach by analyzing the single-cell RNA of individuals with pancreatic cancer and identifying solute carrier family 4 member 4 (SLC4A4), primarily responsible for maintaining the acidity of the TME and, consequently, tumor progression[203]. This led to the association of an SLC4A4 inhibitor with ICI, such as anti-PD-1/anti-CTLA-4, in

studies with mice, resulting in improved survival and overcoming resistance mechanisms that impact treatment alone[203]. Finally, Datta J *et al* employed MEK and STAT3 inhibitors, crucial components of existing resistance mechanisms in the TME, in conjunction with anti-PD-1 in mice[204]. This approach yielded promising responses in terms of enhanced survival and increased anti-tumor response, driven by the greater recruitment of cytotoxic T cells in the TME[204]. These preclinical studies underscore the significant clinical potential of combining TME modelers with immune checkpoint blockers, opening up new possibilities for innovative therapies in the treatment of resistant pancreatic cancer.

In the context of resistance mediated by immunosuppressive molecules, Zhang Y *et al* demonstrated the involvement of IL-17 in the process of triggering the inactivation of CD8 T cells and shaping the TME[205]. Subsequent to this analysis, a study in murine models was established utilizing a triple combination of anti-IL17/IL17R/PD-1 antibodies[205]. This approach resulted in a reduction in tumor size based on increased sensitization of the TME to the action of ICI, a fact corroborated when replacing anti-PD-1 with anti-CTLA-4 in the combination[205]. Similarly, Nelson A *et al* devised a triple combination involving natural killer T cells (NKT), a recombinant oncolytic virus designed to express the cytokine IL-15 (VSV-IL-15), and anti-PD-1[206]. The study's triple therapy tested in mice exhibited promising results, including prolonged tumor regression and complete elimination of the tumor in 20% of the mice[206].

Another compelling approach was demonstrated by Saung MT *et al* using a cancer vaccine (GVAX) in conjunction with anti-PD-1, anti-CSF-1R, and the chemotherapy drug gemcitabine in murine models[207]. The combination yielded enhanced survival of the mice, along with more efficient infiltration of anti-tumor cells and a reduction in myeloid cells[207]. **[Table 1]** summarizes all the pre-clinical studies reported and their respective findings.

The realm of combined immunotherapy to overcome resistant pancreatic cancer has expanded in recent years, and clinical trials are already underway to tackle this significant challenge. Thus, Bockorny B *et al* conducted a phase IIa study to assess the

efficacy of the combination of a CXCR4 blocker, BL-8040 (motixafortide), and pembrolizumab (anti-PD-1) for the treatment of 37 patients[208]. They achieved a disease control rate of 34.5% and an increase in overall survival of 7.5 months, attributed to greater infiltration of anti-tumor CD8 T cells and a reduction in immunosuppressive cells such as myeloid-derived suppressor cells and T regs[208]. Similarly, Overman M *et al* used a Bruton's tyrosine kinase (BTK) inhibitor combined with anti-PD-1 in a randomized phase II clinical trial with 40 patients[209]. Although the combination was tolerated and showed limited clinical activity, with a disease control rate of only 21.1%, blood analysis revealed a reduction in myeloid-derived suppressor cells[209]. Consequently, these clinical trials reveal a relevant potential, paving the way for new studies aimed at manipulating the TME in combination with ICI and other treatment models, seeking improved results for the treatment of patients with pancreatic cancer.

Finally, it is important to analyze the clinical studies carried out with a combination of different pancreatic cancer vaccine models. From this perspective, Le DT *et al* employed a combination of the GVAX vaccine and CRS-207 (live attenuated mesothelin-expressing *Listeria monocytogenes*) in a clinical trial with 90 patients[210]. They observed prolonged survival in these patients treated with this vaccine combination, with little toxicity in the therapeutic process[210]. Similarly, Nair N *et al* used the same immunotherapy combination in a phase IIa clinical trial with 38 patients[211]. They found that patients with higher CD8+ T expression achieved a longer overall survival under this therapeutic regimen[211]. **[Table 2]** summarizes all the clinical studies reported and their respective findings.

CONCLUSION

In summary, pancreatic cancer has evolved into a complex challenge for the medical community, given the intricate resistance to immunotherapy treatments and other applied therapies. Due to its distinctive tumor environment coupled with underlying genetic and immunosuppressive factors, various mechanisms of opposition to immunomodulatory methods manifest, with the primary one associated with immune

checkpoint inhibitors. Given this perspective, it is imperative to progress to unexplored stages, elucidating and presenting solutions that enable science to overcome the challenges posed by this demanding oncological model. In this context, emerging approaches aimed at modulating the tumor microenvironment to reduce immunosuppression are proving promising, as are innovative techniques for modulating the immune system. The goal is to enhance the efficacy and infiltration of anti-tumor cells by manipulating the intrinsic and extrinsic systems within the immune system. Based on these considerations, a combination of these techniques is feasible to achieve more auspicious prognoses and results, as evidenced in clinical studies exploring the efficacy of a combination of a CXCR4 blocker and pembrolizumab, yielding promising results, and the combination of two vaccine models, such as GVAX and CRS-207. In this way, a new path is emerging that presents itself as a promising prospect for overcoming the resistance to immunotherapy present in the treatment of pancreatic cancer.

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