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Pancreatic Cancer Fostered Immunosuppression Privileges Tumor Growth and Progression

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Abstract

The high progression rate of Pancreatic Ductal Adenocarcinoma (PDAC) depends on intrinsic genetic and epigenetic cancer cell aberrations and a profound imbalance in immune system cells infiltrating the PDAC stroma. Direct or exosome mediated shedding in the tumor microenviroment of different molecules (e.g. cytokines, chemokines, lectins) causes tumor, pancreatic stellate and inflammatory cells to recruit numerous immunosuppressive cells in the PDAC microenvironment and inhibit immune effector cells. $CD8^+$ T and dendritic immune effector cells (DCs) are reduced, while immunosuppressive T regulatory cells (T_{reg}), Myeloid Derived Suppressor Cells (MDSCs) and M2 Tumor Associated Macrophages (TAMs) accumulate in the PDAC stroma, mainly at the invasive front area. The imbalance in $CD4^+$ T cell subsets, with Th2 and Th17 prevailing over the Th1 effector arm, is associated with a worse PDAC prognosis that depends on the failure of immune system cells to destroy cancer cells, and the accumulation of immune cells in the PDAC stroma can have pro-neoplastic and pro-metastatic effects. $CD4^+$ T cells are indispensable for PDAC development; T_{reg} and M2 polarized TAMs favor neoangiogenesis and the epithelial to mesenchymal transition of PDAC cells, a pre-requisite for metastases; MDSCs favor metastases by releasing pro-metastatic inflammatory mediators such as S100A8/A9 proteins, and by creating pre-metastatic niches (in metastatic sites). Of the several treatment strategies aiming to abolish the immune cell imbalance in PDAC, and targeting the immune system, DCs manipulation, vaccination with tumor derived antigens and T_{reg} depletion appear to be of benefit, but still require validation before being recommended in the clinical setting.

Keywords: Pancreatic cancer; Lymphocytes; Myeloid derived suppressor cells; Dendritic cells; Tumor associated macrophages; Vaccines

Introduction

Pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive malignancies, is refractory to treatment, and the fourth leading cause of cancer related death in the developed world, with 46,420 new estimated cases and 39,590 estimated deaths in the US for the year 2014 [1]. The overall five-year survival rate of PDAC patients is lower than 20%, the dismal prognosis depending on late detection compounded by ineffective treatment options; only 15 to 20% of patients are eligible for radical surgery, the only known curative approach [2]. The first phase of the natural history of PDAC is longstanding, preneoplastic pancreatic intraepithelial neoplasias (PanINs) evolving into invasive cancer, often over several years; the second phase usually lasts a few months, with the rapid growth of established PDAC that invades the surrounding tissues and organs and metastasizes. Progression from PanINs to metastatic PDAC is characterized by the accumulation of cancer cells with several genetic and epigenetic alterations leading to a marked genetic heterogeneity, although mutations of four genes (*KRAS*, *TP53*, *SMAD4*, *CDKN2A*) are identified in the vast majority of PDAC, *KRAS* mutations occurring in more than 90% of cases [3,4].

PDAC development and progression depend on genetic and epigenetic tumor cell aberrations, and on the complex interplay between tumor cells and the surrounding stroma, with a pronounced desmoplastic reaction and the presence of several non-neoplastic infiltrating cell types, including mesenchymal-derived cells and cellular components of the vascular and immune systems [5]. These cells interact with each other and with cancer cells through direct contact or the release of cytokines and chemokines acting in an autocrine and/or paracrine manner to control and shape tumor growth. Recent research has focused on the role of the immune system in the development and progression of cancer, a defective immunological monitoring of tumors being considered an emerging hallmark [6]. According to the theory of immune surveillance, cells and tissues are continuously monitored by the immune system, which detects and eliminates incipient cancer cells, nascent tumors and nascent metastases. A highly immunosuppressive microenvironment is present in established PDAC, but the immune response is compromised in the early phase of development of this tumor type; this supports the belief that PDAC-associated immunosuppression originates in tumor inception. Immunosuppression is triggered when tumor cells and/or infiltrating inflammatory cells directly release inhibitory cytokines, followed by the recruitment of immunosuppressive cells in the tumor microenvironment and by the inhibition of immune effector cells [7].

The present review describes changes in the microenvironment in both PanINs and invasive PDAC, focuses on immune cell involvement and immune suppression, examines *in vitro* and *in vivo* experimental evidence of cell based treatments reported in the literature, and discusses immune modulating treatment strategies.

The Tumor Microenvironment Maintains Immunosuppression

Rather than merely reacting to cancer growth, the cancer microenvironment takes an active part in cancer development, often having cancer-promoting effects. PDAC is characterized by a dense desmoplastic reaction in which proliferating neoplastic cells co-exist with Pancreatic Stellate Cells (PSCs), Cancer Associated Fibroblasts (CAFs), hematopoietic and mesenchymal cells, and immune cells that permanently interact and influence each other. In the PDAC microenvironment, the prevalent immune cells tending to promote tumor progression via immunosuppression include T regulatory cells (T_{reg}), Myeloid Derived Suppressor Cells (MDSCs) and Type 2 (M2) Tumor Associated Macrophages (TAMs), while dampening immune effector cells [mainly $CD8^+$ and $CD4^+$ T cells, Dendritic Cells (DCs) and Natural Killer Cells (NK)].

Immunosuppressive Cells

T regulatory cells (T_{reg}): $CD4^+$ T cells generally defined as $CD4^+CD25^+FoxP3^+$ cells exerting an immunosuppressive function, T_{reg}, maintain immune tolerance against self-antigens, thus preventing autoimmunity. T_{reg} block Th1 differentiation by inhibiting the production of interferon (IFN)-γ and IL-2 and reduce antigen presenting cells (APCs)-induced Th1 cell activation; they also inhibit Th17 effector cells [8]. In cancer, T_{reg} produce a local immunosuppressive environment that enhances tumor growth. Malignant tumors can actively recruit, expand and induce a *de novo* generation of tumor antigen-specific T_{reg} able to suppress immune responses during disease progression. The expansion and accumulation of these immunosuppressive cells correlate with advanced tumor growth and indicate a poor prognosis [9].

In PDAC animal models and humans, T_{reg} accumulate in the tumor microenvironment mainly in close proximity (within 100 μm of the juxatumoral stroma) of tumor cells [10]. Prevalent in pre-neoplastic lesions, T_{reg} accumulate early at the tumor site, progressively increasing proportionate to disease progression as illustrated in Figure 1 [11,12]. T_{reg} accumulation in PDAC might involve tumor antigen specific clones, as described by Amedei et al. [13], who found increased enolase specific T_{reg} in patients with advanced PDAC. The relevance of T_{reg}-associated immunosuppression in PDAC progression is further supported by the observation that increased numbers of infiltrating and/or circulating T_{reg} correlate with more advanced disease, a lower chance of surgical resection and reduced survival after resection, while low circulating T_{reg} levels one year post-resection correlate with prolonged overall survival [11,14-16]. The role of T_{reg} in PDAC progression and resistance to immune mediated therapies has been recently supported by findings made in a series of studies using PDAC animal models, in which prolonged survival was achieved by using different treatments (immune stimulatory complexes, Listeria vaccine, IFN-α, aspirin) only when T_{reg} were depleted [17-20]. However, the inhibitory function of these cells was recently suggested to play a minor role with respect to other dominant immune suppressive mechanisms, such as those correlated with the release of CXCL12 by CAFs [21].

T_{reg} accumulation in the PDAC microenvironment correlates with $CD4^+$ and $CD8^+$ T cells alterations and depends on several mechanisms, most of which appear to be orchestrated by PDAC derived molecules, including chemokines, indoleamine 2,3-dioxygenase (IDO) and Transforming Growth Factor (TGF)-β1 (Table 1) [22-25]. Tumor-associated cytokines and chemokines effects on immune cells have been recently reviewed by Wörmann et al. [26]. IDO, upregulated in pancreatic cancer cells and implicated in suppressing T-cell immunity, is an IFN-γ induced immune regulatory enzyme that catabolizes tryptophan into kynurenin. Since tryptophan is a crucial metabolite for T cells undergoing antigen-dependent activation, its IDO-induced depletion can create an immunosuppressive environment due to T cell arrest, anergy or death and the induction of T_{reg} differentiation [22]. TGF- β1, significantly implicated in PDAC biology, has tumor promoting and tumor inhibitory effects. The signaling pathway of TGF- β1 is altered in PDAC [27]; TGF- β1 and TGF- β2, produced by both tumor cells and the surrounding inflammatory cells induce T_{reg} expansion in the PDAC microenvironment [23,28,29], although recently Shevchenko et al. [30] failed to confirm that TGF- β1 accumulated in the PDAC stroma expands T_{reg}.

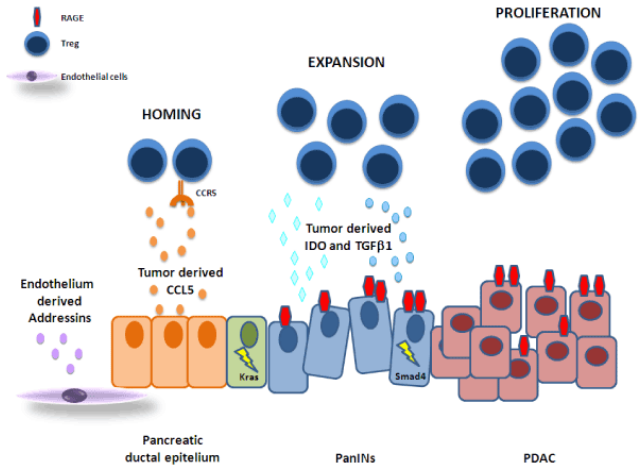

Figure 1: Schematic representation of T_{reg} accumulation in the PDAC microenvironment.

T_{reg} might first migrate into the PDAC stroma following chemotaxis. Tan et al. [31] demonstrated that PDAC cells direct T_{reg} homing to the tumor by releasing a CCR5 ligand, CCL5, which allows T_{reg} to migrate more efficiently to disease sites. The role of chemokines in inducing T_{reg} expansion in the PDAC stroma, has been emphasized by Kudu-Saito et al. [32], who demonstrated that CCL2 overexpression, in co-operation with Lipocalin 2, induces immunoregulatory DCs that expand T_{reg}. The transmigration of T_{reg} to the tumor microenvironment also appears to be driven by tumor endothelium addressins, such as VCAM-1, CD62-E, MAdCAM-1 and CD166 [33]. Using an orthotopic PDAC model, Shevchenko et al. [30] demonstrated that PDAC infiltrating T_{reg} undergo intense cell division and suggested that this elevated proliferation rate is a major mechanism underlying T_{reg} accumulation in PDAC stroma.

Since T_{reg} play a relevant immunosuppressive role and underlie the high progression rate of PDAC, the efficacy of cancer therapy might be enhanced by their modulation or depletion. In a murine PDAC model, Aida et al. used a monoclonal antibody anti glucocorticoid induced tumor necrosis factor receptor, constitutively expressed by T_{reg} at high levels, and found that tumor infiltrating T_{reg} and tumor growth were reduced [19]. Moreover, low-dose chemotherapy regimens emerged as a promising approach for selective T_{reg} depletion. For instance, low-

dose gemcitabine administered in tumor bearing mice, reduced Treg accumulation and improved survival [30], these effects being potentiated by aspirin [18]. Figure 1 summarizes the three main steps (homing, expansion and proliferation) underlying Treg accumulation in PDAC. PDAC cells favor T_{reg} chemotaxis and expansion in the tumor microenvironment through the release of chemokines, IDO and TGF-β1. Accumulated Treg might further proliferate in a TGF-β1 independent manner, thus contributing to making the PDAC microenvironment an extremely immunosuppressive milieau.

Myeloid Derived Suppressor Cells (MDSCs): Comprising a heterogeneous population of immature cells of myeloid origin, these cells can suppress T cell activation. Normally myeloid cells migrate from the bone marrow to peripheral organs, where they differentiate into mature myeloid cells, such as macrophages, DCs, or granulocytes; this process is usually blocked in the tumor microenvironment, immature cells frequently differentiating into MDSCs. CD11b/Gr1 surface markers clearly define MDSCs in mice; since Gr1 has no human homologous marker, any such definition in humans is more complex. Human MDSCs, which lack HLA-DR expression, are characterized by the membranal expression of the immature markers CD33 and CD11b, and by CD14 (monocytic MDSC or mMDSC) or CD15 (granulocytic or gMDSC). In several cancer types both MDSCs populations accumulate in the tumor microenvironment, in peripheral blood and in secondary lymphoid organs, and the prevalence of one subtype over the other appears to be cancer type-related. gMDSC accumulation has been described in patients with renal cell carcinoma, colon cancer and non-small cell lung cancer [34-36], whereas mMDSC has been detected in patients with melanoma, prostate cancer, hepatocellular carcinoma and head and neck cancer [37-39].

In human PDAC, MDSCs accumulate in tumor tissue, blood, bone marrow and secondary lymphoid organs [40-42], as occurs in animal models [12,41,43-46], gMDSCs appearing prevalent in tissue and blood [47]. Although MDSCs appear to accumulate at the cancer site at an early stage, they become a prominent component in established PDAC, increasing further during tumor progression, as demonstrated in animal models and illustrated in Figure 2 [12,43]. Unlike T_{reg} and TAMs, which infiltrate the tumor microenvironment at the pre-invasive stage, MDSCs increase most markedly during the transition from pre-invasive to invasive disease to become a dominant immune cell population infiltrating PDAC [48]. Of the several authors who investigated whether MDSCs accumulation parallels tumor stage in humans, some authors demonstrated that this was the case [40,42,49], but others did not [47]. This discrepancy probably reflects the complexity of MDSCs populations and functions, which might complicate their characterization in different settings. The contribution of MDSCs to cancer progression appears to be partly due to their accumulation within the tumor stroma and in metastatic sites, such as the liver, where they suppress T cell proliferation and cytotoxicity and trigger T_{reg} development, thus creating a favourable pre-metastatic niche [44]. In line with their important role in favouring PDAC progression and metastases, the circulating levels of MDSCs were shown to be an independent prognostic index, a unit increase in MDSCs percentage being associated with a 22% increased risk of death [40].

Experimental and animal studies have provided insight on MDSCs recruitment at the tumor site and its effects on tumor progression. Both PSCs and PDAC cells produce MDSCs-promoting cytokines, such as IL-6, VEGF, macrophage colony-stimulating factor (M-CSF) and chemokines (SDF-1, MCP-1), which promote MDSCs expansion by stimulating myelopoiesis and inhibiting mature myeloid cell differentiation in a Stat3- dependent manner [26,41,50]. PDAC probably promotes MDSCs expansion also by giving rise to high lactate levels in the surrounding medium [51,52]. The most relevant PDAC-derived molecules involved in MDSCs expansion are GM-CSF, IL-1β, IL-6, TGF- β1, and prostaglandin E2 [45,53-58]. The mechanism underlying MDSCs expansion following exposure to PDAC derived molecules involves the activation of several transduction pathways, mainly JAK/STAT kinase, TGFβ and RAGE [59], and the down-regulation of β-catenin, recently shown to lead to MDSCs-mediated tumor expansion in mice and humans [60]. In the expansion of MDSCs in PDAC-bearing mice, IL-6 and IL-1β activated Stat3 interacts with transcriptional factors such as C/EBPβ, which plays a key role in myeloid development and can also affect downstream targets, including the proinflammatory proteins S100A8 and S100A9 [45,61-63].

The more relevant mechanisms used by MDSCs to control antitumor immunity are high levels of inducible nitric oxide synthase (iNOS) and arginase 1, both enzymes acting on L-arginine, catalyzing the release of nitric oxides (NO) and converting l-arginine to urea and l-ornithine. MDSCs are also a source of the free radical peroxynitrite, which inhibits the binding of processed peptides to tumor cell-associated MHC, rendering tumor cells resistant to antigen-specific cytotoxic T cells [64]. The increased Stat3 and NADPH activity in the gMDSCs subset results in increased levels of ROS, while upregulation of Stat1 and iNOS expression in the mMDSC subset results in increased levels of NO. ROS and peroxynitrite induce the post-translational modification of T cell receptors and may cause antigen-specific T cell unresponsiveness, while NO suppresses T cell function via different mechanisms including the inhibition of Janus kinase 3, Stat5, MHC class II expression and the induction of T cell apoptosis. MDSCs-mediated immune suppression also includes the sequestration of cysteine, which is essential for T cells.

MDSCs create favorable conditions for tumorigenesis, tumor growth and metastasis, and neoangiogenesis. These closely related processes are governed by MDSCs-derived mediators, such as matrix metalloproteinases (MMPs), apoptotic factors (TNF-α, Api6), interleukins (IL-1, IL-6), growth factors (TGF-β1, VEGF, bFGF), and the hypoxia-induced factor (HIF)-1α, which was recently reviewed by Kumar et al. [65].

S100 proteins and MDSCs: S100A9 overexpression inhibits the differentiation of DCs and macrophages, and promotes MDSCs formation whereas S100A9 inhibition results in the reduction of MDSCs in the spleen of tumor bearing mice. Although the mechanism governing this phenomenon has not been identified, it has been suggested that the S100A8 and S100A9 heterodimers assist in the formation of the NADPH oxidase complex generating reactive oxygen species (ROS) in myeloid cells, which can interfere with their differentiation [66]. S100A8 and S100A9 inflammatory proteins have high expression not only in human MDSCs [67], but are also produced by PDAC cells and can induce MDSCs expansion [42]. The accumulation of S100A8/S100A9 producing MDSCs in the PDAC microenvironment probably contributes to the enhancement of PDAC progression, since these molecules, like TGF-β1, favor epithelial to mesenchymal transition (EMT) [68]. The main receptors of S100A8/S100A9 proteins are advanced glycation end products (RAGE) and TLR4 [69]. As demonstrated by Vernon et al. [70], RAGE plays a critical role in MDSCs accumulation in the context of pancreatic carcinogenesis: the targeted ablation of RAGE in mice expressing an

oncogenic variant of *Kras* limits the development of PanIN lesions and the associated accumulation of MDSCs. Interestingly, during pancreatic cancer progression in the absence of RAGE, the majority of immature myeloid cells (CD11b+) exhibit a more mature phenotype with the expression of the mature macrophage marker F4/80 and a loss of Gr1 expression. It has been suggested that RAGE overexpression within the PDAC tumor and stromal compartments undergo ligation by S100A8/S100A9 synthesized by MDSCs thus inducing a regulatory chemokine tumor gene profile and serving as a positive feed-back loop for the further recruitment of MDSCs [63]. The release of S100A8/S100A9 proteins by MDSCs accumulated in organs distant from the primary site might also play a role in establishing the pre-metastatic niche [67,71-75].

Exososomes and MDSCs: Researchers are paying increasing attention to the role of exosomes in the cross-talk between tumor and microenvironment cells, including MDSCs [76]. Exosomes are small vesicles (30-100 nm diameter) that can traffic from one cell to another and transfer their large cargo (proteins, RNA, microRNA, DNA and lipids), thus affecting cell function. Tumor-derived exosomes can alter myelopoiesis by modulating myeloid cells in the tumor microenvironment, in hematopoietic organs and in pre-metastatic sites; this leads to abnormal myeloid cell differentiation in favor of MDSCs [77]. On the other hand, MDSCs might also release exosomes, which are enriched with S100A8/S100A9 molecules [78]. Figure 2 summarizes the main mechanisms involved in PDAC-induced expansion of MDSCs.

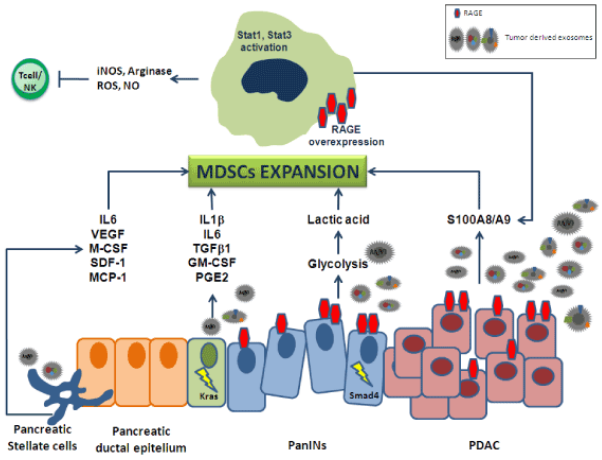

Figure 2: Schematic representation of MDSCs accumulation in the PDAC microenvironment.

Tumor-associated macrophages (TAMs): The mononuclear phagocytic system includes monocytes, macrophages and DCs. On migrating to tissues, circulating monocytes differentiate into resident macrophages that, in tumors, are named TAMs. M1 macrophages, or classically activated macrophages are pro-inflammatory, conferring an anti-tumor immune response through the release of Tumor Necrosis Factor α (TNF-α), IL-12, reactive nitrogen and oxygen intermediates; M2 macrophages are immunosuppressive and promote angiogenesis, matrix remodeling and metastasis, and induce a Th2 differentiation of T lymphocytes through the release of IL-10 [79]. M1 or M2 polarization depends on stimuli from PDAC cells and the tumor microenvironment, including cytokines and Reg3β lectin [80]. TNF-β and IL-1β are M1 polarizing cytokines, while IL-4 switches the differentiation from M1 to M2; this phenomenon, occuring in the PDAC setting, is associated with a worse prognosis [81]. While in PanINs macrophage abundance is reportedly fairly uniform at all stages, within the broader tumor microenvironment macrophage distribution is non-uniform, with a greater accumulation in regions of disrupted collagen where tumor invasion is mainly due to single cells [82]. M2 macrophages and one of their subsets expressing folate receptor beta accumulate mainly at the invasive front of PDAC and correlate with tumor stage and survival, probably because they favor angiogenesis by releasing VEGF [16,83-85]. By releasing VEGF, M2 macrophages infiltrating regional lymph nodes are also implicated in nodal lymphangiogenesis and occult nodal involvement in pN0 PDAC [86]. M2 and M1 macrophages may concur in favoring PDAC progression and metastases, also because they induce EMT as well as metalloproteinase (MMP)2 and MMP9 proteolytic activity in PDAC cells [87,88]. As recently demonstrated, the adverse effects of M2 TAMs on PDAC prognosis also depend on their ability to induce in cancer cells the expression of cytidine deaminase involved in the metabolism of gemcitabine thus preventing apoptosis during gemcitabine treatment [89]. Informations on the molecular pathways underlying M2 polarization in PDAC are scarse. Pancreatic cancer cell lines conditioned media were demonstrated to induce macrophage polarization through c-*MYC* expression, involved in controlling the transcription of key genes for tumor progression and metastases (MMP9, VEGF, HIF-1α, and TGF-β) [90]. Moreover, the embryonic homeobox transcription factor *CUX*1, highly expressed in PDAC upon TGF-β exposure, represses the M1 phenotype of macrophages by interacting with NF-κB [91]. Hyperglycemia, frequently found in PDAC, both the cause and consequence of diabetes mellitus, was shown to enhance *in vitro* tumor-driven macrophage enrichment and polarization [92].

In view of their relevance in PDAC and in the biology of other solid tumors, Adams et al. [93] evaluated whether TAMs identification in blood might be useful in diagnosing cancer. The authors found that, unlike controls but like patients with breast cancer, 93% of patients with PDAC have circulating cancer-associated macrophage-like cells (CAMLs). In the same study CAMLs were attached to circulating tumor cells in 10% of late-stage patients, this finding supporting the observation that interactions between tumor cells and immune cells are highly complex.

Immune effector cells

T cell lymphocytes: In cancer types including invasive colon cancer, melanoma, multiple myeloma, and PDAC, an increase in T cells (specifically activated CTLs and Th) is correlated with a better survival [94-96], although there is evidence that many T cell subsets present in solid tumors are involved in tumor promotion, progression and/or metastasis. Immune-mediated tumor rejection calls for a fully functional T-cell anti-tumor response, for which $CD8^+$ and $CD4^+$ T cells are considered the main effectors. The selective binding of $CD4^+$ T cells to class II MHC molecules ensures the response of $CD4^+$ cells to class II-associated peptide antigens, and that of $CD8^+$ T cells to class I-associated peptides. Because most solid tumors express MHC class I, but not MHC class II, it has been assumed that class-I restricted $CD8^+$ T cells are the main vectors of T cell tumor destruction. However $CD4^+$ T cells play a critical role in orchestrating the antitumor response. Accordingly, it has been found in animal models that T cell deficiency or disruption of specific cytotoxic mechanisms increase susceptibility to spontaneous or chemically induced carcinogenesis

[95,97]. In PDAC animal model, tumor-specific $CD4^+$ T cells are required for the full expression of the $CD8^+$ T cell antitumor effect [98]. Recent studies on animals have highlighted the dark side of $CD4^+$ T cells in PDAC, and have further evidenced the close relations between these cells and $CD8^+$ T cells: in $CD4^+$ T cell genetically depleted mice, *Kras*-driven pancreatic carcinogenesis is inhibited, unlike in $CD4^+$ non-depleted mice which rapidly develop PanINs and progress to higher grade lesions over time [99]. In the absence of $CD4^+$ T cells, $CD8^+$ T cells accumulate proximal to PanIN lesions, but also present an enhanced effector function by releasing IFN-γ and granzyme B; in the presence of $CD4^+$ T cells, few $CD8^+$ T cells infiltrate the neoplastic pancreas [99].

The CD4+ T cell population comprises Th1, Th2, Th17 and T_{reg} cells. The permissive effect of $CD4^+$ T cells on pancreatic carcinogenesis has mainly been attributed to $CD4^+$ T_{reg} and Th17 cells, which infiltrate the neoplastic pancreas and suppress $CD8^+$ T cell activity [99,100]. PDAC animal models and human tumors without $CD4^+$ T cell depletion, commonly contain a scarce and irregular $CD8^+$ T cell infiltration that is denser near the invasive front and scarcer at the tumor center [12,16,101]. As recently highlighted, the reduced migration of $CD8^+$ T cells to the PDAC stromal compartment depends not only on $CD4^+$ T cells, but also on activated PSCs [10]. These stromal cells and CAFs are primarily involved in creating the dense desmoplasia surrounding cancer cells, which might impede cell migration as it impedes drug delivery by causing increased interstitial fluid pressure and vascular collapse [102]. The reduced $CD8^+$ T cell infiltration in PDAC tumors implies a reduced trafficking of tumor specific $CD8^+$ T cells, this phenomenon being reflected, at least in part, by a reduction in the number of circulating $CD8^+$ T cells observed in PDAC, as well as in other solid tumors [42]. Moreover, the reduction in tissue and blood $CD8^+$ T cells associated with the concomitant accumulation of T_{reg}, indicates an adverse outcome [11,16], while an enhanced juxtatumoral $CD8^+$ T cell infiltrate correlates with a better survival in PDAC patients [10]. In line with the suggestion that $CD4^+$ T cells have potential tumor promoting effects, an increased tumor infiltration has been described in animals [12] and humans [16], although this finding has not been consistently made across studies [103-105]. This discrepancy may depend on the heterogeneity of $CD4^+$ T cells, the increase being mainly due to immunosuppressive rather than immune-effector subtypes, namely $CD4^+CD25^+$ T_{reg} but also to $CD4^+CD69^+$ T cells, a newly identified immunosuppressive $CD4^+$ T cell subset [105]. CD69 expression might affect the immune response by inhibiting $CD4^+CD69^-$ cell cytokine synthesis; this process seems to partly depend on TGF-β [106,107]. $CD4^+CD69^+$ T cells might also regulate the Th1/Th2 balance which, in several cancer types including PDAC, is reported to be driven toward Th2, which, unlike Th1 cells, favor a predominantly humoral response and do not secrete IFN-γ and TNF-α, responsible for activating and regulating the development and persistence of cytotoxic T cells [108,109]. Reduced levels of IL-2 and increased levels of IL-10 have also been implicated in the imbalance between Th1/Th2 [104]. The complex mechanism underlying the profound imbalance of T cells subsets, characterized by the expansion of immunosuppressive T cells with the dampening of immune effector T cells during the progression of pancreatic lesions from premalignant to malignant, is due to both soluble mediators and adhesion molecules (Table 1). By releasing soluble cytokines and chemokines, including TGF-β1, IL-8, GM-CSF, PDAC cells directly target $CD4^+$ T cells thus inhibiting their proliferation and migration and, interestingly, induce CD69 rather than CD25, expression [110]. The adhesion molecule L1CAM (CD171), which increases during PDAC progression in the ductal epithelium [111], also appears to be involved in reducing $CD4^+$ T cell proliferation and in inducing CD69 expression, probably because it mediates the release of soluble factors promoting the generation of $CD4^+$ T cells with a $CD69^+$phenotype [105]. Another PDAC-associated adhesion molecule, an ICAM-1 receptor $\alpha_L\beta_2$ integrin (also known as LFA-1 or CD11a/CD18), regulates $CD8^+$ T cell recruitment: only its knockout results in a marked impairment of $CD8^+$ T cell infiltration in experimental pancreatic tumors in mice, probably because its lack of expression impairs T cells activation and differentiation [112]. Overexpression and secretion by activated PSCs of β-galactoside-binding protein Galectin-1 (Gal-1), another molecule that mediates immunosuppression in PDAC by targeting T cell, increases Th2 and decreases Th1 cytokines, inducing $CD4^+$ and $CD8^+$ T cell apoptosis [113].

PDAC and stromal-derived molecules	Effects on T cells	References
Adressins (e.g. VCAM-1, CD62E, Mad CAM-1, CD166)	Promote T_{reg} transmigration to the PDAC microenvironment	[33]
$\alpha_L\beta_2$ integrin	A reduced expression impairs $CD8^+$ T cell infiltration	[112]
Galectin-1	Increases Th2 and decreases Th1 cytokines	[113,143]
	Induces $CD4^+$ and $CD8^+$ T cell apoptosis	
	Induces IL-10 production in T_{reg}	
L1CAM	Reduces $CD4^+$ T cell proliferation	[105]
CCL2	Induces immunoregulatory DCs which in turn expand T_{reg}	[32]
CCL5	More efficient T_{reg} migration to PDAC site	[31]
CXCL12	Impedes T cells migration to the tumor site	[21]
TGF β1	Induces T_{reg} expansion	[23,28-30, 110,115]
	Inhibits $CD4^+$ T cell proliferation and migration	
	Induces Th17	
IL-1β and IL-6	Induce Th17	[115]
Fibroblast activation protein (FAP-α)	Suppresses effector T cells	[135]
Indoleamine 2,3-dioxygenase (IDO)	Induces T_{reg} differentiation	[22]
	Induces T cell arrest, anergy and death.	

Table 1: Tumor and stromal cells derived molecules acting on T cells and involved in PDAC immunosuppression.

The Th17 T cell subset plays a potent pro-inflammatory role in certain infections, tumors and autoimmune disorders [114]. Th17 cells, able to release the interleukins IL-17A, IL-21 and IL-22, develop from naïve $CD4^+$ T cells in the presence of TGF-β, IL-6, and IL-1β and are maintained long-term in the presence of IL-21 and IL-23 [115]. An increased infiltration of Th17 cells has been detected in PDAC murine

tumors [99,100], while increased Th17 associated serum cytokines have been reported in humans, high IL-17A and TGF-β1 levels being correlated with a worse prognosis [116]. The role of this $CD4^+$ T cell subset in the onset and progression of PDAC has not yet been completely clarified [115]. However, in an animal model it has been demonstrated that an enforced *Kras* driven IL-17 expression is required for the initiation and progression of PDAC [100] and experimental embelin treatment in tumor bearing mice reduces tumorigenicity and Th17 accumulation [62,117].

Natural killer cells (NKs): Large granular lymphocytes, NKs cooperate with adaptive immunity and rapidly detect and eliminate atypical cells. In PDAC there is a significant down-regulation of NKG2D, NKp30, NKp46 receptors, which participate in killing of tumor cells by recognizing specific ligands and perforin positive circulating NKs [118]. Lactate, which is overproduced in PDAC tumors, reduces both NKs cytotoxicity and NKp46 receptor expression [52]. The involvement of these cells in controlling tumor growth is borne out by the observation that, in a patient with regression of several pancreatic cancer metastases following the administration of the immune modulator Ipilimumab (anti-CTLA-4 antibody), NKs were increased and caused lysis of an autologous tumor as well as pancreatic cancer lines [119]. Moreover, the degree of NKs impairment appears to be directly related to the invasiveness of malignancy, the risk of recurrence after surgery and an unfavourable prognosis [120]. The concept that NKs act only in one-way by destroying tumor cells, thus exerting an anti-tumor effect, has been recently re-evaluated since these cells may acquire a pro-angiogenic phenotype and be potentially (pro-) tumorigenic [121]. Distinct NKs populations, anergized/regulatory NK cells (NK_{reg}) and activated NK may be found in the tumor inflammatory microenvironment. NK_{reg} can induce differentiation of the pancreatic cancer cell line MiaPaCa2, thus conferring a resistance to NKs-mediated cytotoxicity [122], while IL-15 activated NKs inhibit tumor growth and prolong the survival of tumor-bearing mice, being associated with tumor cell apoptosis, NKs and T-cell accumulation [123]. Interestingly, NKs may be able to eliminate cancer stem or cancer initiating cells, which have slow replication, are resistant to most chemotherapeutic agents and radiotherapy, and can give rise to rapidly proliferating cells.

Dendritic cells (DCs): like macrophages, DCs are APCs and, unlike other antigen-presenting cells, such as B cells and macrophages, can trigger secondary and primary immune responses (such as naive T cell activation) directed against specific antigens, thus linking the innate and the adaptive immune response. There are two functionally heterogeneous and distinct subsets: myeloid-DCs ($CD11c^+$ DCs) and lymphoid-DCs ($CD11c^-$ DCs) both of which express high levels of HLA-DR and lack the lineage markers CD3, CD14, CD15, CD16 and CD19. In PDAC, circulating myeloid DCs progressively decrease as tumor stage increases, while lymphoid DCs behave differently. DCs from PDAC patients demonstrate a significantly reduced ability to stimulate allogenic T cells, but both their number and their allogenic stimulatory capacity are restored following chemoradiotherapy and surgical resection [26, 42,124-128].

In agreement with the hypothesis that an impaired immune response to cancer cells contributes to favoring tumor progression, and in view of the role played by DCs in activating the immune response, it has been demonstrated that high levels of circulating DCs are an independent favorable prognostic factor in patients with resectable and unresectable PDAC [129,130]; similar findings were made on considering patients with tumor infiltrating DCs [131]. Of the numerous cytokines and chemokines released by pancreatic cancer cells and the surrounding stromal cells, IL-6 and G-CSF are believed to play an important role in inhibiting the maturation and activation of DCs [132].

The desmoplastic stroma

The immune infiltrate of cancer is but a part of the tumor microenvironment. CAFs, cells of mesenchymal origin, combined with extra cellular matrix (ECM) proteins and tumor associated vasculature, are also important components. By providing a physical framework and acting as a reservoir for soluble mitogens, the ECM influences the growth, differentiation, survival and motility of cancer cells in various malignancies. In addition, the various effects that malignant and non-malignant components of the cancer mass can have on each other alter angiogenesis, ECM components, epithelial-mesenchymal interactions, substratum adhesiveness, and cancer-directed immune responses, all of which influence cancer behavior [133]. A dense desmoplastic stroma reaction, long recognized as a hallmark of PDAC, promotes tumorigenesis and resistance to therapy. This concept is corroborated by the observations made by Provenzano et al. [102], who identified hyaluronic acid as the principal ECM component involved in causing high interstitial fluid pressure, vascular collapse and resistance to chemotherapy. By interacting with the immune system CAFs promote tumor progression, attracting TAMs to the tumor microenvironment (NF-κB related mechanism) and causing increased fibrosis and tumor growth [134]. Moreover, CAFs by secreting the fibroblast activation protein (FAP-α) and the chemokine ligand 12 (CXCL12) further suppress effector T cells and impede T cells migration to the tumor site [21,135].

The histological assessment of the pancreata of patients with PDAC or mice engineered to express oncogenic *Kras* in the epithelial compartment of the pancreas reveals that even the early stages of PanIN development are associated with a stromal reaction characterized by a robust desmoplastic response and the recruitment of immune cells. Based on the composition of the immune infiltrates surrounding PanINs, it has been shown that the stromal constituents surrounding PanINs form an inflammatory and immune suppressive environment thereby allowing the precursor lesions to escape immune surveillance [136]. The main pancreatic cancer-associated stromal fibroblasts, PSCs, are key players in the development of desmoplasia [137], and predominantly secrete gelatinases (MMP2, MMP9), which degrade the basement membrane collagen (type IV) and are associated with inflammation, fibrosis, angiogenesis, and cancer invasion. In various cancers including PDAC, basement membrane breaching, a critical step in cancer progression, brings malignant cells into direct contact with ECM proteins such as collagen type-1, thus supporting their growth, contributing to their chemo-resistance, and paving the way for invasion and metastasis.

Changes in Tumor-Associated Antigens (TAAs)

An effective anti-tumor immune response involves recognition of TAAs by the immune system and the generation of T or B cell responses that kills the tumor cells but leave life-sustaining normal tissue intact. This mechanism, however, might induce a selective pressure, killing tumor cells with TAAs expression and favouring the proliferation of tumor cells with fewer immunogenic surface antigens. TAAs include mucins, oncogenes and tumor suppressor gene products. Muc1, an epithelial cell membrane-bound glycoprotein that is approximately 80% carbohydrate, is expressed in normal pancreas

but it is overexpressed and aberrantly glycosylated in >90% of metastatic PDAC; its aberrant expression has been associated with increased metastasis and the poor prognosis of PDAC and other cancers [138].

In agreement with the concept that mucins play a functional role in PDAC progression, Rachagani et al. have shown that there is a progressive increase correlated with the increased expression of the mucin inducer inflammatory cytokines IFN-γ, CXCL1 and CXCL2, in the expression of mucins particularly Muc1, Muc4 and Muc5AC in the pancreas of mice with advanced PanIN lesions and PDAC both at mRNA and protein levels [139]. Accordingly, knock-down in Muc1, Muc4 or Muc5AC was shown to decrease the growth and metastatic potential of pancreatic cancer cells [140-142]. This suggests that mucins expression may play an important role in the development of PDAC and may be a potential tumor specific target for treatment.

Known to contribute to the immunosuppressive tumor microenvironment and evasion of immune responses, galectins are soluble immunomodulating glycoproteins involved in T- cell homeostasis, the suppression of autoimmunity, survival and inflammation. Gal-1 promotes a Th2 cytokine profile in PDAC, induces IL-10 production in T_{reg}, activates DCs and regulates immune cell trafficking [143]; this glycoprotein, overexpressed by pancreatic tumor cells, has been identified as a proteomic biomarker closely correlated with disease stage [144]. Gal-1, also expressed on PSCs, contributes to stellate cell activation and maintenance of the immunosuppressive microenvironment. Genetic ablation of Gal-1 in a murine PDAC model dampened tumor progression by inhibiting proliferation, angiogenesis and the desmoplasic reaction, and also by triggering a tumor-associated immune response, thus leading toa 20% increase in relative lifespan [145]. However, Gal-1 overexpression is found on the stromal tissues of long term pancreatic cancer survivors [113], indicating that the prognostic significance of Gal-1 expression is not entirely clear. Blocking molecules for Gal-1 is a potential strategy for cancer treatment.

The analysis of antibodies in patients' cancer sera that recognize TAAs has been suggested as a useful tool in identifying new diagnostic indicators and/or new potential targets for therapy [146]; antibodies identified include those elicited against mutated tumor suppressor genes (p53, p16) or other key pro-survival molecules (e.g. survivin), the epithelial cell adhesion molecule (EpCAM) and mucins such as the well-known CA19-9 and CD44 [147]. However, since these antibodies are detectable at a low frequency, they are not recommended for diagnosis, although they do indicate a down-regulated immune response to these antigens.

Tumor antigens are processed and presented to T cells by HLA class I and class II molecules on the surface of APCs. The resulting T cells kill tumor cells expressing specific tumor antigens in class I molecules. In PDAC, tumor cells down-regulate or lose expression of HLA class I and its associated β2-microglobulin [148]. Reportedly HLA class I expression can be re-induced in PDAC cell lines in vitro by IFN-γ treatment [149], thus making it possible to alter the balance between cellular and humoral immunity by promoting Th1/cell-mediated immunity.

Treatment Strategies: Focus On the Immune System

The front-line approaches in PDAC treatment are surgery and chemotherapy. Recently, immunotherapy has proven promising although more effective when used in anadjuvant setting for patients with operable disease at a high risk of post-operative recurrence than in patients with advanced disease [150]. The aim of immunotherapeutic strategies against PDAC is to amplify the immune reaction to cancer. The present paper focuses on vaccination, DCs based therapies, interference with co-stimulatory and inhibitory receptors, approaches designed to increase the number of tumor specific cytotoxic T cells (adoptive T cell immunotherapy), chimeric antigen receptors (CARs) and T_{reg} depletion.

Vaccination strategies

The development of vaccination strategies was prompted by the finding that TAAs administration induces a specific T-cell response in both animals and humans. Tumor antigens might be normal proteins expressed at much higher concentrations by PDAC cells than normal cells (e.g. Muc1, VEGF-R) or mutated proteins expressed exclusively by PDAC cells (e.g. mutated K-ras). The antigens can be administered as whole proteins or peptide fragments. Human clinical phase I/II trials have been conducted to verify safety, immune activation and clinical response to different vaccines including cancer cells transduced to express GM-CSF, mutant K-ras peptide, telomerase peptide GV1001, Muc1 peptide, and synthetic peptides derived from the cancer-testis antigens KIF20A and CDCA1 [151-164]. Although vaccines are safe, and trigger the specific immune response, clinical responses have been weak.

DCs based vaccines

Based on the observation that DCs maturation and function is impaired in PDAC and in view of the fact that these cells are readily available *in vitro* following stimulation of monocytes with GM-CSF and IL4, several studies have aimed to obtain DCs-precursor cells from the blood or the bone marrow of cancer bearing mice or patients, differentiate and activate them in culture, load them with tumor antigens, and re-inject the cells in the tumor bearing mice or the patient. Different tumor antigens and protocols have been used in this DCs vaccination strategy, which has achieved activation of anti-tumor immune response, delayed tumor growth and improvement in survival, as shown in Table 2 [165-184].

Immune checkpoint blockade

A complex balance of multiple stimulatory and inhibitory receptors on the surface of T cells ensures proper functioning of the immune system. Receptors such as cytotoxic T lymphocyte-associate protein-4 (CTLA-4) and programmed death 1 (PD-1) expressed on the surface of activated T cells inhibit T cell activation upon binding to ligands CD80/CD86 and PD-L1/PD-L2, respectively. CTLA-4 and/or PD-1 overexpression by immune and/or tumor cells might dampen local anti-tumor immunity that, in turn, might favour tumor progression. PD-1 expression by cancer cells in PDAC is associated with reduced cytotoxic T-cell infiltration, advanced stage disease and a poor prognosis [185]; these co-inhibitory molecules might also be expressed by immature myeloid cells, probably contributing to limiting their immunosuppressive effects. Following this hypothesis, we demonstrated a reduced CTLA4 expression in immature myeloid cells

in PDAC patients, and found that this reduced expression is associated with an immunosuppressive phenotype [42].

Type of study	DCs source	Pulse antigen	Verification model	Results	References	
					No.	Year
In vitro	PB of patients with PDAC	CA 19-9	Cytotoxicity in co-culture with pancreatic cancer cells	Higher cytotoxic activity against pancreatic cancer cells using CA 19-9 pulsed DCs	[165]	2000
In vitro	PB of blood donors	Pancreatic cancer cell lines lysates	Cytotoxicity in co-culture with pancreatic cancer cells	Higher cytotoxic activity against pancreatic cancer cells using pulsed DCs	[166]	2001
In vitro	PB or buffy coats of blood donors	Lysates of apoptotic and non apoptotic pancreatic cancer cell lines	Cytotoxicity in co-culture with pancreatic cancer cells	Higher cytotoxic activity against pancreatic cancer cells using DCs pulsed with apoptotic pancreatic cancer cell lysates	[167]	2002
In vivo animal model	Syrian hamster BM	Pancreatic cancer cell lines lysates	Hamsters inoculated s.c. with a piece of tumor and treated with s.c. injection of unpulsed or pulsed DCs	Inhibition of tumor growth in hamsters treated with pulsed DCs	[168]	2002
Human study	Autologous PB monocytes	mRNA encoding CEA	Three patients with resected PDAC following neoadjuvant chemoradiotherapy received DCs monthly for 6 months	All three alive without evidence of disease more than 2.5 yr from the original diagnosis	[169]	2002
Human study	Autologous PB monocytes	Autologous tumor cell lysate for 10 vaccinations and lysate of the tumor cell lines AsPc-1 and BxPc-3 for a further five vaccinations.	One patient with stage IV PDAC given pulsed DCs in three-week intervals injected into a growing lymph node for a total of fifteen vaccinations	Stable disease for six months	[170]	2003
In vivo animal model	Syngenic BM	RNA derived from a pancreatic cancer cell line	Intratumor injections of DCs in orthotopic PDAC	DCs administration induced significant antitumor immunity and significantly reduced tumor volume	[171]	2003
In vivo animal model	Syngenic BM	Heat-treated tumor lysate (HTL-DC) and tumor lysate (TL-DC) from a pancreatic cancer cell line	Immunocompetent C57BL/6 mice inoculated s.c. with PANC02 cells at −18 day. At day 0, 7, and 15, s.c. immunization	The group treated with HTL-DC had significantly smaller tumors at 30 days	[172]	2006
In vivo animal model	Syngenic BM	Alpha-galactosylceramide	Immunocompetent C57BL/6 mice inoculated s.c. with PANC02 cells. At day 0, 14, and 28, s.c. immunization	Pulsed DCs strongly decreased tumor growth and increased the percentage of tumor-free mice	[173]	2006
In vivo animal model	Syngenic BM	Panc02 cells	Immunocompetent C57BL/6 mice inoculated s.c. with PANC02 cells. Mice received either no treatment, Panc02-pulsed DCs, gemcitabine (Gem) or Panc02-pulsed DCs plus Gem	DC-based vaccination alone was almost as equally effective as Gem treatment in preventing death. Survival could be significantly increased in the combined treatment arm	[174]	2007
Human study	Autologous PB mononuclear cells	Unpulsed	Seven patients unsuccessfully treated (Gem) for unresectable PDAC received intratumoral injection of 10 billion or more immature DCs	Safety: unpulsed DCs delivered into the pancreatic cancer using endoscopic ultrasound-guided fine needle injection is safe	[175]	2007
Human study	Autologous PB mononuclear cells	MUC1 peptide	20 pts with stage III – IV PDAC received 2 to 15 times intradermal injection of pulsed DCs	One patient with multiple lung metastases had a complete response. Five patients had stable disease. The long survival group (more than 6 months) showed significantly higher expression of mature DC ($CD83^+$)	[176]	2008
Human study	Autologous PB mononuclear cells	OK432 (a penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus pyogenes*)	5 non metastatic inoperable PDAC treated with Gem and with endoscopic ultrasound-guided	1 patient had partial response and 2 were long-standing survivors (more than 6 months). Induction of tumor antigen-specific CTLs	[177]	2009

			fine needle injection of OK432-pulsed DC			
Human study	Autologous PB mononuclear cells	Autologous tumor cell lysate	12 patients with advanced disease treated with Gem and DCs injected intradermally next to an inguinal lymph node. Patients were treated in bi-weekly intervals for the first 6 weeks, then once every 4 weeks	1 patient had a partial remission, 2 had stable disease, and 5 survived 1year or more after diagnosis of advanced disease	[178]	2011
Human study	Autologous PB mononuclear cells	MUC1	7 patients with recurrent lesions or metastasis after surgery underwent intradermal vaccinations at 2 week intervals	Safety: DCs is non-toxic and capable of inducing immunological response to tumor antigen MUC1 in advanced pancreatic cancer patients. The vaccination did not prolong patients' survival time or stabilize their disease	[179]	2012
Human study	Autologous PB mononuclear cells	None	9 PDAC received (DC group) while 15 PDAC did not received (non-DC group), preoperative endoscopic ultrasound-guided tumor inoculation of iDCs and OK432	Two DC group patients, one of whom was stage IV with distant lymph node metastasis, survived more than 5 years without requiring adjuvant therapy	[180]	2012
In vivo animal model	Syngenic BM	None	Mice received Panc02 cells s.c. and were treated with PBS, Gem alone, DCs alone, or combination therapy with DCs and Gem	Gem therapy alone delayed tumor growth. Combined therapy with Gem and DC vaccination delayed tumor growth, this combination leading to significantly prolonged survival in Panc02-bearing mice	[181]	2013
Human study	Autologous PB mononuclear cells	CA 19-9	Retrospective analysis of 134 patients subjected to long antigen exposition DCs therapy	Median survival was significantly higher in group of patients who started immunotherapy within 2 months of diagnosis or repeated immunotherapy	[182]	2013
In vivo animal model	Syngenic BM	OVA protein	Mice were implanted with orthotopic PancOVA tumors and treated with combinations of DC-OVA i.p. and/or Gem	Mice treated with OVA-DC showed highly efficient tumor control, 9/13 mice having complete remission leading to long-term survival of more than 150 days	[183]	2014
Human study	Autologous PB mononuclear cells	WT1 and/or MUC1 peptide antigens according to patient's HLA-A type	255 patients were injected 5 or more times intradermally with DCs in close proximity to axial and/or inguinal lymph nodes, biweekly. 12 patients received DC vaccines simultaneouswith first-line chemotherapy; the other 243 patients began receiving DC vaccines after first- or second-line chemotherapy	Erythema after vaccination > 3 cm was an independent and treatment-related prognostic factor for better survival	[184]	2014

Table 2: Dendritic cells (DCs) vaccination strategy. Precursor DCs obtained from peripheral blood (PB) or bone marrow (BM) were pulsed with different tumor derived antigens before testing their ability to activate anti tumor T cell based cytotoxicity or their effects on tumor growth and disease prognosis. In vitro, in vivo animal models and human studies are reported.

Various monoclonal antibodies targeting CTLA-4, PD-1 and their ligands, considered critical immune check-points, have been developed for cancer therapy. Monoclonal antibodies that bind and inhibit CTLA-4, ipilimumab and tremelimumab, are currently being tested in multiple clinical trials (ClinicalTrials.gov). The clinical response to ipilimumab in patients with advanced PDAC is poor: in one phase 2 trial, only 2/15 had disease stabilization after an initial period of progression [186], and only 1/ 27 patients had a delayed response [187].

Monoclonal antibodies targeting PD-1 or its ligand (PD-L1) trigger significant antitumor responses and inhibit tumor growth in mice with PDAC by inducing $CD8^+$ T cells tumor infiltration [188]. Antibodies against PD-1 and PD-L1 have entered clinical trials with great success in patients with advanced melanoma and advanced non-small cell lung cancer [189,190]. In their multicenter phase 1 trial, Brahmer et al. [191] administered intravenous anti-PD-L1 antibody to patients with different types of cancer. The antibody-mediated blockade of PD-L1 induced durable tumor regression and prolonged stabilization of disease in some patients with non-small-cell lung cancer, melanoma, and renal-cell cancer but not in patients with PDAC. Therefore, while immune check-point blockade evokes clinical responses in several tumor types, no well-defined benefit was observed in PDAC. This failure probably depends on the complexity of the immune alterations occurring in PDAC and on the dual face nature of CTLA-4 and PD-1, the inhibition of which might enhance anti-tumor immunity in

immune effector cells, while the same inhibition might reduce anti-tumor immunity in immature myeloid cells. The findings made by Zhu et al. [192] in their study on a PDAC animal model, support this hypothesis: CTLA-4 and PD-1 blockade significantly benefitted PDAC bearing mice only when the treatment was associated with the inhibition of CSF1/CSF1R signaling, relevant for MDSCs. This suggests that combined therapeutic regimens may be necessary for the successful treatment of PDAC.

Adoptive T cell immunotherapy

Adoptive T-cell immunotherapy, which involves the transfer of tumor infiltrating lymphocytes (TILs) derived from ex vivo expanded tumor biopsies, aims to enhance the immune system response. After being removed from the tumor tissue expanded and manipulated, a massive number of TILs are returned to the patient to allow the cells to prime to tumor antigens, or transfection with recombinant DNA encoding for T cell receptors specifically directed towards tumor-antigens. The expanded TILs are then re-infused in combination with immune-adjuvant therapy, such as IL-2. Results with this approach have been very encouraging in selected melanoma patients. Since abundant patient tumor material is not always available, genetic modification of autologous isolated peripheral lymphocytes is another option. Rather than being isolated from the tumor, lymphocytes are obtained from the peripheral blood, after which they are exposed to retroviral vectors encoding for specific genetically modified TCR designed to target specific tumor antigens. In the PDAC setting, Muc1-specific autologous T cells isolated from patients'PBMCs were expanded by incubation with a Muc1-presenting cell line prior to administration to eight patients with unresectable and 20 patients with resectable PDAC postsurgically [193]. This treatment reduced post-surgical hepatic recurrence and improve survival with respect to surgery alone, although the overall benefit was minimal (median survival: 17.8 *vs.* 14.0 months).

Chimeric antigen receptor (CARs)

Another approach uses lymphocytes genetically engineered to carry chimeric antigen receptors (CARs) that are transmembrane proteins typically comprising the extracellular domain of CARs (an Fab fragment of an antibody specific for a tumor antigen), a spacer, a membrane spanning element and the intracellular domain of CARs (signaling portion containing intracellulardomains of the T-cell receptor–CD3); T-cells thus target specific tumor antigens and are activated upon binding. This approach was successful in the treatment of relapsed or refractory acute lymphoblastic leukemia [194]: treatment with autologous T cells transduced with a CD19-directed chimeric antigen receptor (CTL019) was followed by complete disease remission in 27/30 cases. PDAC treatment with this specific CAR is likely to be ineffective, since the CD19 antigen is not PDAC associated, but this emerging therapy was effective in a PDAC murine model when using T cells engineered to express a CAR for CEA [195]. Prostate stem cell antigen (PSCA), a small extracellular glycoprotein of unknown function that is overexpressed in PDAC cells, was recently used in adoptive immunotherapy using CAR-transduced T cells [196]; in this study, *in vivo* antitumor efficacy was improved, but several hurdles must be overcome before this treatment can be employed in patients.

T_{reg} depletion

Given the importance of T_{reg} in attenuating anti-tumor immune response, one strategy is to target T_{reg} directly. Two different types of T_{reg} depletion strategies have been developed and tested in clinical trials, particularly in melanoma [197]; both target the IL-2 receptor alpha chain (CD25), highly expressed by T_{reg}, by an IL-2-diptheria toxin fusion protein (denileukin diftitox) or by a monoclonal antibody directed against CD25. T_{reg} depletion obtained with the latter strategy reduced progression of early stage, but not late-stage, PanINs in a murine model after immunization with Listeria monocytogenes (which induces $CD4^+$ and $CD8^+$ T-cell immunity) [20]. This approach enhances lesion infiltration with inflammatory cells, and it may prove possible to design immunotherapies against PanIN lesions to slow or prevent progression to PDAC.

Conclusion

PDAC induces a profound imbalance between immune effector and immune suppressor cells. The resultant immunosuppressive microenvironment favours tumor growth and metastases not only because it underlies tumor immune tolerance, but also because it shapes a more aggressive tumor cell phenotype through soluble factors, cell to cell contact and exosomes shuttle.

Although the overall efficacy of immune based therapies evaluated in the PDAC setting appears to be limited, the altered immune response occurring in cancer growth and progression is relevant, with evolving alterations paralleling tumor plasticity from the pre-invasive to the invasive phase. For this reason it is likely that different immune therapies approaches should be applied in different clinical setting: early PDAC will probably benefit from T_{reg} targeted therapies, while advanced PDAC will probably benefit from combined treatments aimed to induce tumor-specific DCs while dampening MDSCs.

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