

## REVIEW

# Stromal reengineering to treat pancreas cancer

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**Pancreatic ductal adenocarcinoma co-opts multiple cellular and extracellular mechanisms to create a complex cancer organ with an unusual proclivity for metastasis and resistance to therapy. Cell-autonomous events are essential for the initiation and maintenance of pancreatic ductal adenocarcinoma, but recent studies have implicated critical non-cell autonomous processes within the robust desmoplastic stroma that promote disease pathogenesis and resistance. Thus, non-malignant cells and associated factors are culprits in tumor growth, immunosuppression and invasion. However, even this increasing awareness of non-cell autonomous contributions to disease progression is tempered by the conflicting roles stromal elements can play. A greater understanding of stromal complexity and complicity has been aided in part by studies in highly faithful genetically engineered mouse models of pancreatic ductal adenocarcinoma. Insights gleaned from such studies are spurring the development of therapies designed to reengineer the pancreas cancer stroma and render it permissive to agents targeting cell-autonomous events or to reinstate immunosurveillance. Integrating conventional and immunological treatments in the context of stromal targeting may provide the key to a durable clinical impact on this formidable disease.**

## Introduction

Mutations in genes that regulate cell proliferation and survival drive malignancy (1). However, it is clear that cell-autonomous changes, while necessary, are not sufficient for solid tumor growth. Tumor cell-extrinsic factors also figure prominently in the pathogenesis of cancers, including pancreatic ductal adenocarcinoma (PDA), the most common cancer of the pancreas. PDA is lethal because of a propensity for metastatic spread, the advanced stage of disease at the time of diagnosis in most patients, and a lack of therapies providing durable clinical benefit. Even in the minority of patients who qualify for potentially curative surgical resection, the overall 5-year survival is <20%. Thus, treatment strategies to target this elusive disease are now extending beyond cell-autonomous targets to include non-cell autonomous mechanisms.

A PDA represents a complex tumor organ composed of epithelial, endothelial, mesenchymal and hematopoietic elements. Indeed, a distinguishing feature of PDA is the robust desmoplastic stroma that constitutes the bulk of the tumor mass (2). The stromal reaction includes myofibroblasts, endothelial cells, pericytes and various immune cell subsets that are embedded within a dense and complex extracellular matrix (ECM). The immune contexture (3) and components of the

**Abbreviations:** CAF, cancer-associated fibroblasts; ECM, extracellular matrix; FAP, fibroblast activation protein; GEMM, genetically engineered mouse model; HA, hyaluronic acid; IDO, indoleamine 2,3 dioxygenase; IFP, interstitial fluid pressures; HMW, high molecular weight; IL, interleukin; MDSC, myeloid-derived suppressor cells; PDA, pancreatic ductal adenocarcinoma; PSC, pancreatic stellate cells; SPARC, secreted protein acidic and rich in cysteine; TAM, tumor-associated macrophages; TEC, tumor epithelial cells; TGF, transforming growth factor; TLR, toll-like receptor.

ECM (4) can serve as prognostic factors in this disease. The biophysical properties of PDA have contributed to the limited success of cell-autonomous therapies thus far. PDA generates inordinately high interstitial fluid pressures (IFP) (5) that compress blood vessels and hinder passive transport processes of chemotherapeutics (5–7). Thus, at least some of the well-known resistance of PDA to a wide range of therapies stems from this biophysical barrier.

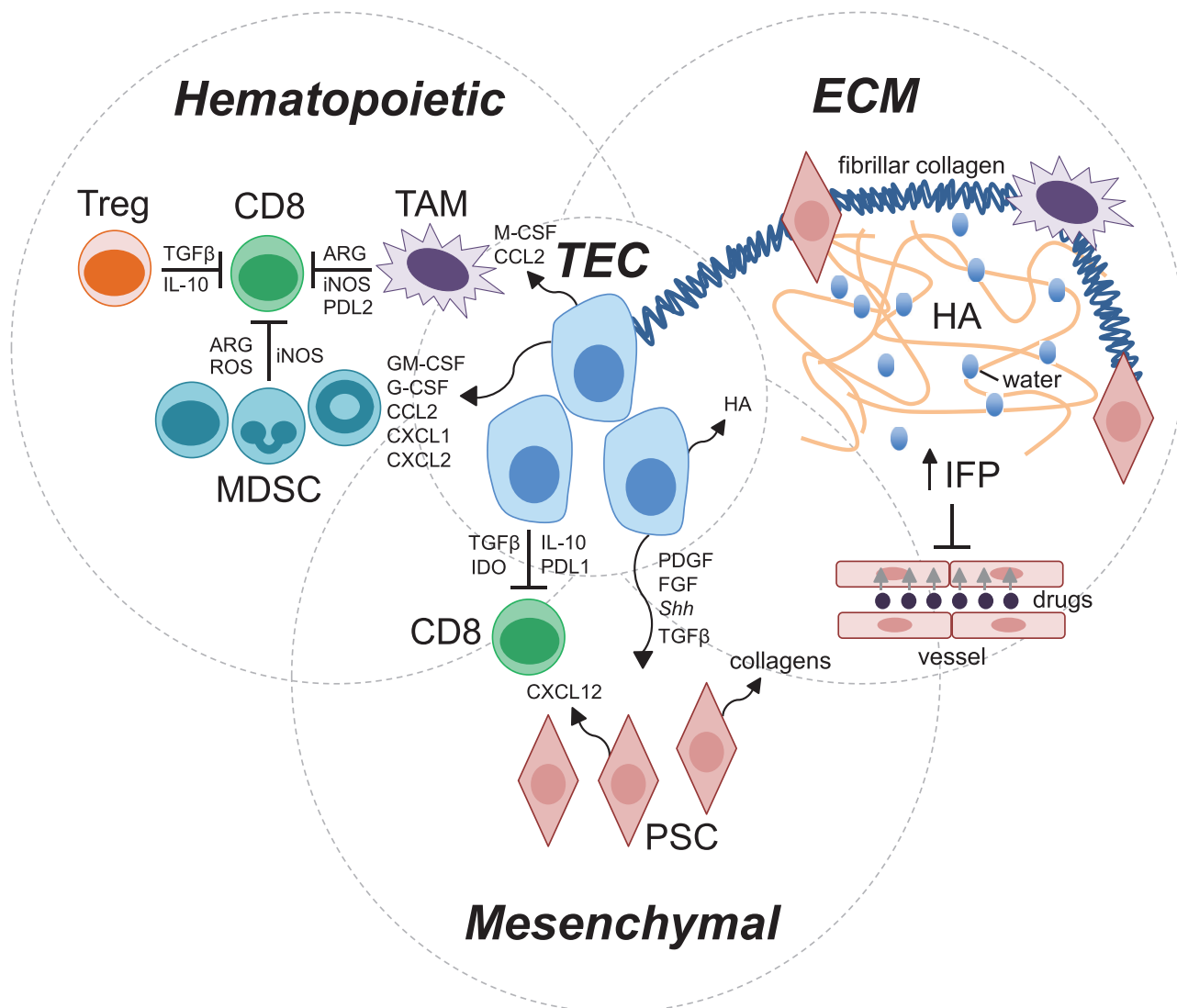
Stromal-targeted agents may also provide benefits independent of chemotherapy, including interventions designed to reinstate immunosurveillance (8,9). Complex treatment strategies to rationally modulate stromal components in combination with cytotoxic- and/or immune-based interventions will most likely be required to meaningfully impact survival of patients with PDA. In the following, we discuss multidimensional approaches to reengineer the pancreas cancer stroma for therapeutic benefit (Figure 1). The development of genetically engineered mouse models (GEMM) that faithfully recapitulate the genetic, histopathological and clinical trajectory of human PDA from inception to invasion (e.g. see refs. 10–15) has greatly aided fundamental studies of this cancer and identified potential vulnerabilities (reviewed in refs. 16,17). These models also provide rational preclinical platforms to rigorously test novel treatment strategies for translation to the clinic. To adequately assess some targets, such as those in the complex stromal environment, models of autochthonous disease may be essential.

## Reengineering the immune response

Similar to most solid tumors that invoke a sterile and persistent inflammation, the immune response plays conflicting roles in PDA. Inflammation is essential for Kras-driven malignant transformation (18). CD4<sup>+</sup>FoxP3<sup>+</sup> T regulatory (Treg) cells and tumor-associated macrophages (TAM) accumulate at disease inception in pancreatic intraepithelial neoplasms (PanIN), the most common histologic precursor to PDA (19); whereas myeloid-derived suppressor cells (MDSC), a heterogeneous population of immature myeloid cells that are immunosuppressive, infiltrate markedly during the transition to invasive disease (19,20). The distinct kinetics of these immune populations suggest the specific and chronologically definable construction of an immunosuppressive environment that shields tumor cells from immune detection and renders them resistant to immune-based therapies. Immature myeloid cells are significantly increased in the circulation of PDA patients and their frequency varies inversely with survival (21). Intratumoral accumulation of Treg similarly portends an unfavorable prognosis and the phenotype of intratumoral macrophages also predicts overall survival (22). In contrast to and underscoring the tumor-promoting role of regulatory immune cell subsets, intratumoral accumulation of CD8<sup>+</sup> cytotoxic T cells (CTL) is a favorable prognostic factor in PDA patients (23). These observations suggest that altering the immune contexture of PDA by decreasing or inhibiting immunosuppressive cell subsets while providing and/or inducing effective CD8<sup>+</sup> and T helper (Th1) CD4<sup>+</sup> T-cell responses may be beneficial.

### Immature myeloid cells

Many solid tumors are associated with dysregulated immune homeostasis. During malignant progression, increasing systemic levels of cytokines and growth factors expand multipotent, immunosuppressive myeloid progenitors (MDSC) (24,25). An understanding of the roles of immature myeloid cells in disease states is now rapidly evolving, along with a preferred nomenclature. For the purpose of consistency in the field, we will use the generic term MDSC to describe the heterogeneous population of immature myeloid cells that are immunosuppressive. MDSC contribute to the invasiveness and immune suppression of cancer and can serve as a surrogate marker for disease



**Fig. 1.** Complex network of stromal resistance in PDA. A number of distinct but often overlapping cellular and extracellular processes combine to create an immune-privileged and drug-free sanctuary that aids and abets tumor development and therapeutic resistance in pancreas cancer. These processes can be conceptualized as interacting modules that are largely driven by, and cooperate with, cell autonomous events (e.g. mutations in the *Kras* proto-oncogene) in tumor epithelial cells (TEC). This plethora of non-cell autonomous mechanisms also provides a novel landscape of therapeutic opportunities. (i) *TEC*: TEC produce cytokines [GM-CSF (granulocyte–macrophage colony-stimulating factor), G-CSF (granulocyte colony–stimulating factor), M-CSF (macrophage colony–stimulating factor)] that induce the expansion of immunosuppressive myeloid cells of both the granulocyte and monocyte lineage. TEC also produce chemokines (CCL2, CXCL1, CXCL2) that contribute to myeloid cell recruitment into the tumor parenchyma. TEC express PDL1 (our unpublished observations) and several immunosuppressive factors (TGFβ; IL-10; indoleamine 2,3-dioxygenase, IDO) that directly inhibit the functional activity of CD8 T cells. IDO is also implicated in the accumulation of Treg (data not shown). TEC produce TGFβ, fibroblast growth factor, platelet-derived growth factor, and *Shh* to influence PSC activation and proliferation. Lastly, TEC produce HA and collagen that profoundly alter fluid dynamics and pressures in the interstitium. (ii) *Hematopoietic compartment*: The immune contexture of PDA is biased toward immunosuppressive cell subsets that inhibit the recruitment, retention and activity of cytotoxic T lymphocytes. Typically, 50% or more of the cells in PDA are hematopoietic in origin. CD4<sup>+</sup>FoxP3<sup>+</sup> Treg and TAM are significantly increased in preinvasive disease and are maintained at high frequency during invasive disease. Treg deploy an immunosuppressive arsenal including IL-10 and TGFβ. TAM are potentially immunosuppressive in PDA via production of T-cell inhibitory factors [iNOS, arginase, and IL-10] and also express inhibitory ligands, such as PDL2. Immature myeloid cells with suppressive activity (MDSC) represent a heterogeneous population of immature granulocytic and monocytic progenitors that utilize a variety of immunosuppressive mechanisms including reactive oxygen species, arginase, iNOS, peroxynitrates, TGFβ and potentially IDO. (iii) *Mesenchymal compartment*: Activated PSC are a major source of inflammatory factors and ECM proteins. The secretion of CXCL12 may sequester CD8 T cells away from TEC and may also promote the migration of monocytes and/or Mo-MDSC. Activated PSC are also a principal source of fibrillar collagen. (iv) *ECM*: The inordinately high IFP in PDA is largely generated by a complex and dysregulated ECM. An inverted oncotic pressure gradient recruits fluid from the vasculature that binds HA in the interstitium. The swelling pressure generated by hydrated HA strains tethered collagen fibrils bound to surface receptors on TEC, PSC and immune cells. This applied tension triggers active contractile forces as the cells attempt to maintain tensional homeostasis. These combined passive and active forces contribute to the elevated IFP in PDA, presenting a primary biophysical barrier to cytotoxic drug delivery and perfusion. The impact of the specific ECM components on cell signaling in various cell types is incompletely understood, but targeting HA and other ECM components may alter critical biochemical signaling to infiltrating and resident cells to therapeutic advantage.

burden (25). GM-CSF, a prominent cytokine produced by PDA, can promote the generation of MDSC and is required for the establishment and/or growth of transplanted preinvasive ductal cells and PDA allografts (26,27); primary and metastatic pancreatic ductal cells also secrete a panel of myeloid-centric factors including G-CSF, M-CSF,

CCL2, CXCL1 and CXCL2 that may contribute to MDSC expansion and recruitment (20).

MDSC have been shown to inhibit T cells via an arsenal of mechanisms, potentially reflecting distinct states of MDSC differentiation. Commonly described MDSC inhibitory mechanisms include the

production of iNOS, arginase, reactive oxygen species and peroxynitrate; some of these mechanisms, such as iNOS and reactive oxygen species, can induce T-cell death (reviewed in ref. 28). MDSC are currently segregated into two subsets: granulocytic (Gr-MDSC) and monocytic (Mo-MDSC) based on phenotypic markers consistent with distinct ontogenies in mouse models (25,29). In mice, Gr-MDSC are distinguished from Mo-MDSC by the expression of Ly6G and include granulocytes and their progenitors. Mo-MDSC (CD11b<sup>+</sup>Ly6G<sup>-</sup>Ly6C<sup>high</sup>) phenotypically overlap with inflammatory monocytes arising from macrophage dendritic progenitors and include monocytes and their progenitors. Both subsets have also been associated with numerous human malignancies. However, because of different strategies used to identify the cells and the intrinsic heterogeneity across cancers, clear distinctions between these subsets are not yet resolved. Indeed, the complexity and plasticity of the myeloid lineage may reflect a continuum of a similar population at distinct stages of differentiation. In human solid tumors including PDA, the frequency of MDSC (Lin<sup>-</sup>CD11b<sup>+</sup>CD33<sup>+</sup>) in the circulation correlates directly with clinical stage of disease (30). Gr-MDSC, as defined by expression of CD15 (CD11b<sup>+</sup>CD33<sup>+</sup>CD15<sup>+</sup>), are also significantly increased in PDA patients and their frequency correlates with more advanced disease (31). We have recently shown (20) that both the Gr- and Mo-MDSC subsets are significantly expanded systemically and intratumorally in a GEMM of autochthonous PDA that faithfully reflects the natural history and the cardinal features of the human disease (10,12). Gr-MDSC predominate in cell number and frequency compared with Mo-MDSC (CD11b<sup>+</sup>Ly6G<sup>-</sup>Ly6C<sup>high</sup>) and, similar to the human corollary, are associated with malignant progression (20). Targeted depletion of Gr-MDSC in autochthonous PDA induced endogenous T-cell activation, proliferation and infiltration into established tumors, consistent with reinstating immunosurveillance (20). A decrease in ECM deposition and an increase in mean vessel diameter corresponding to regions of mononuclear infiltrates suggested that this immune-based strategy can also remodel the tumor stroma, reminiscent of the effects of anti-CD40 that also operates by modulating the myeloid lineage (8) (discussed in greater detail below). Thus, abrogating Gr-MDSC can overcome aspects of tumor-dependent immune tolerance in PDA, encouraging efforts to identify a safe and effective means to target this population in patients. Of note, depletion of Gr-MDSC in these studies caused a concomitant increase in the Mo-MDSC subset—both systemically and intratumorally—revealing homeostatic regulation between these two populations. The consequent rise in Mo-MDSC has potential therapeutic implications as these cells are also immunosuppressive in PDA (20) and, as mentioned, share an overlapping phenotype with inflammatory monocytes, a cell population that is significantly increased and associated with advanced disease in PDA patients (21). Thus, targeting one subset could have unintended consequences on other immature and suppressive myeloid subsets. A recent phase I study (NCT00892242) using zoledronic acid, shown previously to inhibit Gr-MDSC in transplantable tumor mouse models (31), was not effective at decreasing the frequency of Gr-MDSC in patients (32). Specifically targeting MDSC without compromising the integrity of normal innate responses remains somewhat of a clinical conundrum, and a plethora of strategies are being tested in mouse models and in clinical trials including aborting MDSC egress from the bone marrow, preventing MDSC migration into tumor sites, targeting mechanisms of immunosuppression and/or promoting maturation into immunostimulatory antigen presenting cells (25). A better understanding of the ontogenies, fates and relationships of the MDSC subsets to resident myeloid cells in PDA may reveal novel approaches to manipulate MDSC therapeutically. Furthermore, identifying if chemotherapy, or novel stromal targeted therapies, alters myeloid cell differentiation and function (suppressive versus stimulatory) could inform combinatorial strategies.

### Treg

During development in the thymus, T cells that express high-affinity self-reactive T-cell receptors are deleted in a process called central tolerance. T cells with minimal affinity for self-antigens are not deleted and instead exit into the periphery (33). Although this process minimizes the

frequency of high-affinity self-reactive T cells, it is imperfect and some self-reactive T cells that are capable of eliciting autoimmunity escape thymic deletion. Such self-reactive T cells are kept in check by a variety of peripheral tolerance mechanisms including CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg). Treg are critical for immune homeostasis and tolerance (34) and may become an obstacle to achieving antitumor immunity as the majority of tumor antigens described to date represent aberrantly expressed self-antigens. Tumor-derived factors such as IDO (35,36), transforming growth factor- $\beta$  (TGF $\beta$ ) (37), interleukin-10 (IL-10) (37) and prostoglandin E<sub>2</sub> (38,39) most likely contribute to Treg accumulation in malignancy. Treg are elevated in patients with solid tumors and are, with few exceptions, associated with poor prognosis (40). In humans (36) and in our GEMM of PDA (19,20), Treg accumulate in preinvasive lesions potentially undermining effector T-cell activity at the earliest stage of disease. Treg are increased in the blood, draining lymph nodes and primary tumors of patients with PDA (22,41), and the intratumoral ratio of Treg to CD4<sup>+</sup> T cells is significantly associated with shorter survival (3).

Treg possess various suppressive mechanisms, enhanced by the expression of inhibitory ligands such as CTLA-4 and LAG3 (34,42) and the secretion of immunosuppressive cytokines such as TGF $\beta$ , IL-10 (40) and IL-35 (43). These molecules can directly and indirectly—through altering antigen presenting cell activity—mitigate functional immune responses. In addition to thymic-derived Treg, there is an inducible population (iTreg) that can be converted from CD4<sup>+</sup>Foxp3<sup>-</sup> T cells. These Treg populations may differ in how they suppress. Identifying the relative contribution of each of these subsets in tumors will be of use for developing clinical agents. Currently, several strategies to target Treg in malignancy are under investigation. Cyclophosphamide (Cy) transiently decreases Treg (44–46) and is often combined with adoptive immunotherapy to enhance the efficacy of tumor-reactive T cells (47,48). Targeting the high-affinity receptor expressed on most Treg, IL-2R $\alpha$  (CD25), with monoclonal antibodies such as basiliximab and daclizumab (49,50) or a recombinant IL-2 diphtheria toxin (51) is being tested to deplete Treg. However, the caveat that CD25 is transiently expressed on effector T cells following their activation complicates the application of such strategies. Therapies designed to interfere with Treg function [antibodies to CTLA-4 (Ipilimumab), glucocorticoid-induced tumor necrosis factor-related receptor] (52); trafficking (blockade of CCR5/CCL5 (53) or CCR4/CCL22) (54); or peripheral conversion (inhibition of IDO) (55) also have promise but will probably be insufficient as stand-alone agents in PDA and also affect the immune system independently of Treg. Although anti-CTLA-4 has yet to show significant promise in PDA (56), combining anti-CTLA-4 with an allogeneic irradiated tumor cell vaccine [granulocyte-macrophage colony-stimulating factor gene-transfected tumor cell vaccine (GVAX)] may enhance endogenous T-cell responses in patients by not only improving T-cell activation but also interfering with Treg activity at the tumor site (57). Treg depletion can cause undesirable autoimmunity in normal tissues and strategies designed to modulate the intratumoral Treg:effector T-cell ratio, when possible, may be preferable to overt systemic Treg ablation. Alternatively, there may be distinct pathways governing Treg during inflammatory settings such as cancer that are distinct from normal homeostasis. Indeed, neuropilin-1 (Nrp1) is a receptor expressed on Treg that has been suggested to specifically impact Treg function in settings of inflammation and malignancy (58). Intriguingly, neuropilin-1 binds Semaphorin 3A, a receptor-ligand that is increased in pancreas cancer and associated with invasiveness and decreased survival (59), suggesting that this pathway may impact intratumoral Treg in PDA and provide a means for a more tailored intervention.

### Tumor-associated macrophages

TAM are major cellular constituents of the tumor stroma. TAM secrete cytokines, chemokines, proteases and angiogenic factors that contribute to neoplastic progression and ECM remodeling (60). Macrophage expression of inflammatory factors can promote acinar-ductal metaplasia, a dedifferentiated state potentially more permissive to malignant transformation (61). Macrophages are also intimately associated with preinvasive lesions (19,20). TAM isolated from autochthonous



PDA are potently immunosuppressive via production of iNOS and arginase (ref. 20 and our unpublished observations).

Complex tissue-specific signals, as well as potentially distinct progenitors, instruct TAM differentiation resulting in a marked *in vivo* heterogeneity. *In vitro* studies have defined two extreme and simplified subsets of tissue macrophages based on functional characteristics that follow triggering of distinct inflammatory pathways. Toll-like receptor (TLR) signaling or interferon- $\gamma$  promotes “classical” (M1) macrophages that have increased capacity to phagocytose, present antigen to T cells and secrete proinflammatory cytokines (IL-12, IL-1 $\beta$ , tumor necrosis factor- $\alpha$ ). M1 macrophages are often considered analogous and complementary to tumor-antagonistic Th1 responses, as Th1 cytokines induce M1 macrophages. In contrast, Th2 cytokines such as IL-4 and IL-13 induce “alternatively” activated (M2) macrophages that are involved in tissue repair/fibrosis, produce antiinflammatory cytokines and are suppressive toward T cells. Th2-biased immune responses and M2 macrophages (CD163<sup>+</sup> or CD204<sup>+</sup>) are associated with shorter survival, whereas increased frequency of M1 macrophages (HLA-DR<sup>+</sup>CD68<sup>+</sup>) predicts longer survival in patients with PDA (3,62). An agonistic antibody to CD40 has demonstrated therapeutic activity independent of chemotherapy in a subset of patients with PDA by programming systemic macrophage progenitors toward a cytotoxic M1 phenotype and subsequent dissolution of the tumor stroma (8). These effects of anti-CD40 were also independent of the endogenous T-cell response, a surprising finding as anti-CD40 conditions dendritic cells to activate T cells in most other contexts (63–65).

TAM influence responsiveness to chemotherapy (reviewed in ref. 66). In an orthotopic pancreas cancer model, gemcitabine increased TAM accumulation; CCR2 or CSF1 (M-CSF) antagonists decreased TAM and improved gemcitabine response (67). In this study, the depletion of macrophages, which were immunosuppressive, was required to institute an endogenous CD8<sup>+</sup> T-cell response. In another orthotopic study, chemotherapeutic resistance was attributed to TAM upregulation of cytidine deaminase, an enzyme that metabolizes gemcitabine (68). However, intratumoral macrophages can also increase the efficacy of chemotherapies. Cytotoxic agents can induce immunogenic cell death, which requires professional antigen-presenting cells including macrophages and dendritic cells (69). Integrating macrophage targeting with chemotherapy in this setting could be counterproductive. Refining these approaches will clearly require further investigation in autochthonous tumors, as well as confirmation in clinical samples, as the evolution of a cancer through a preinvasive state within the tissue of origin could alter TAM differentiation and programming in distinct and critically important ways from transplantable models. Identifying the source(s) of TAM, which could arise from resident macrophages and/or from recruited immature myeloid progenitors, will also help direct appropriate systemic therapies to impact the intratumoral population.

#### CD8 T cells

PDA has relatively few coding mutations compared with malignancies such as melanoma and lung cancer, resulting in a limited neoantigenic spectrum (70–73). This limited antigenicity of PDA could explain, at least in part, why checkpoint blockade inhibitors such as anti-CTLA-4 and anti-PD1/PDL1 have been minimally effective in PDA thus far, despite achieving some clinical benefit in more overtly antigenic tumors (74,75). Thus, approaches that depend on endogenous T-cell activity for efficacy may be inadequate. However, several observations suggest cause for some optimism. First, the intratumoral frequency of CD8<sup>+</sup> cytotoxic T cells is a favorable prognostic factor in PDA patients (23). Second, clinical trials with an irradiated allogeneic tumor cell vaccine engineered to secrete GM-CSF (GVAX) expanded CD8 T cells specific to the aberrantly expressed self/tumor antigen, mesothelin, that correlated with improved clinical outcomes (76). Perhaps most surprisingly, alleviating distinct aspects of immune suppression, either through targeted depletion of Gr-MDSC (20) or with a CXCR4 antagonist

in combination with anti-PDL1 (77), unmasked a latent immune response demonstrating the potential of endogenous T cells to engage tumor epithelial cells (TEC).

Perhaps the most direct way to reengineer immunity is to infuse the cells that have the greatest potential to kill malignant cells and/or the surrounding stroma. Genetically engineering T cells to express enhanced affinity tumor antigen-specific receptors, while more technically cumbersome, may overcome some of the limitations to engendering effective endogenous T-cell responses in cancer (78). This approach is designed to overcome the limitation of low-affinity responses, a major obstacle as tumor cells often have defects in antigen processing and presentation. Such strategies may be particularly effective in PDA if combined with modalities to alter the tumor environment or, alternatively, if the donor T cells are engineered in such a way that renders them reactive to the tumor stroma. The advent of genetic engineering of T cells *ex vivo* allows for the possibility to reprogram additional cell-intrinsic T-lymphocyte functions. For example, the infused T-cell product could be rendered refractory to inhibitory pathways, such as TGF $\beta$  receptor and/or PD1 signaling by gene-specific small interfering RNA or gene-specific DNA-targeted nucleases (78). Moreover, because the migration of cells is not dependent on passive transport processes and is therefore less susceptible to the biophysical barriers that diminish cytotoxic drug penetration into PDA, the infused cells could be engineered to produce additional antitumor factors and serve as a Trojan horse. For example, engineering tumor-reactive T cells to produce IL-12, a cytokine typically produced by M1 tumor-antagonistic macrophages, modulated the tumor microenvironment in a mouse model of melanoma to therapeutic benefit (79). Increasing IL-15 expression in a fibrosarcoma prior to transplant induced T-cell-dependent tumor elimination that was surprisingly independent of cognate antigen (80). Such intriguing approaches will require a deeper investigation in autochthonous models in combination with T cells expressing tumor-specific antigen receptors that target naturally expressed tumor antigens, to not only establish a basis for efficacy but also uncover potential undesirable toxicities when targeting antigens that are expressed at a lower level in normal tissues.

#### Reengineering the stromal mesenchyme

The pronounced desmoplastic reaction in pancreas cancer is mediated in large part by cancer-associated fibroblasts (CAF), which include the conversion of resident pancreatic stellate cells (PSC) to an activated state (activated PSC are referred to as myofibroblasts). PSC normally account for ~4% of the pancreas and are activated during injury and inflammation to become major sources of ECM, cytokines and growth factors. Pancreatic carcinoma cells secrete such factors as TGF $\beta$ , fibroblast growth factor and platelet-derived growth factor that signal to PSC to produce ECM components and promote fibrogenesis (81). Activated PSC in human PDA secrete increased levels of CXCL12, a chemoattractant for many immune cells, including immature myeloid cells and some T-cell populations, via binding to the chemokine receptor CXCR4. CXCL12 production by activated PSC has been proposed to cause the focal sequestration of CD8 T cells by inducing the migration toward PSC and away from tumor cells (82). Thus, therapeutic targeting of PSC may have multiple and non-overlapping benefits in PDA by modifying the immune response, ECM content, and growth-promoting paracrine signaling to TEC.

#### Paracrine signaling to mesenchymal cells

Initial insights into the role of the tumor stroma in impeding chemotherapy penetration in PDA emerged with administration of a sonic hedgehog (Shh) inhibitor, saridegib, to *KPC* mice (7). Saridegib inhibits paracrine signaling between tumor cells and CAF and was found to deplete CAF and transiently increase intratumoral vessel diameter and drug perfusion in *KPC* tumors. Although results from a clinical trial targeting the Shh pathway in PDA patients have not

yet been published, the trial was prematurely terminated suggesting unanticipated outcomes. There are a number of potential reasons why saridegib failed to improve survival in combination with gemcitabine. One possibility, the relatively rapid emergence of resistance, was anticipated by the preclinical experiment (7). Within 10 days of treatment, the degree of hypoperfusion and vascular collapse essentially returned to pretreatment levels. From that point on, saridegib could only potentially add to the side-effect profile without improving efficacy. Another possibility is that sustained inhibition of Hh signaling could have induced the evolution of a more aggressive disease. Indeed, in an important follow on study, either prolonged exposure to Hh inhibition or genetic ablation of Shh in *KPC* mice resulted in less well-differentiated, more aggressive tumors (83). These Shh-deficient tumors were also more angiogenic and more responsive to vascular endothelial growth factor inhibition, suggesting a way to treat a subset of PDA with similar characteristics. These results emphasize the need to carefully consider inclusion/exclusion criteria in preclinical studies and to ensure sufficient treatment time on study.

#### *Differentiation states of mesenchymal cells*

Attempts to revert activated PSC toward a quiescent state represent another therapeutic strategy to undermine PDA growth and survival. For example, all-trans retinoic acid can restore PSC quiescence and reduce ECM deposition in the autochthonous *KPC* GEMM of PDA (82,84). All-trans retinoic acid binds to ligand-responsive nuclear receptors that regulate transcription (85). Intriguingly, all-trans retinoic acid can cause the maturation of immature myeloid cells to become stimulatory antigen presenting cells and may enhance cancer vaccine T-cell responses in patients with non-small lung cancer (86). Vitamin D analogues bind another nuclear receptor, the vitamin D receptor, and have the potential to induce quiescence in myofibroblasts. Vitamin D receptor ligands inhibit hepatic stellate cell activation, the putative equivalent of PSC and associated liver fibrosis (87,88). Vitamin D signaling also inhibits renal fibrosis by inhibiting TGF $\beta$ -SMAD (small mothers against decapentaplegic) signal transduction (89). Promoting vitamin D signaling may revert fibrosis in multiple contexts by disarming fibroblasts.

#### *Elimination of mesenchymal cells*

Fibroblast activation protein (FAP), a serine protease selectively produced by CAF, is associated with poor prognosis in PDA patients (90). FAP enzymatic activity may modify the stromal ECM to promote tumor cell invasion via engagement of  $\beta$ 1-integrins expressed on TEC (91). Depleting FAP-expressing cells in combination with a cancer vaccine in mouse models of lung carcinoma or transplantable PDA controlled tumor growth that was dependent on immunomodulatory cytokines, tumor necrosis factor- $\alpha$  and interferon- $\gamma$  (9), suggesting immune-mediated control. The adoptive transfer of T cells engineered to express a chimeric antigen receptor (CAR) reactive with FAP caused cachexia and lethal bone marrow toxicities in mice through targeting of FAP<sup>+</sup> bone marrow stromal cells. FAP<sup>+</sup> cells are also present in human bone marrow (92), although such toxicities were not observed in studies of human xenografts and CAR T-cells reactive to human FAP (93) as this chimeric antigen receptor may not cross-react with mouse FAP. These results again emphasize the importance of faithful preclinical modeling to reveal the safety and long-term effects of stromal targeted therapies, in particular. A CXCR4 antagonist (AMD3100) (94) that blocks binding of CXCR4 to its ligand, CXCL12, in combination with the checkpoint blockade inhibitor, anti-PDL1, caused a decrease in tumor volume within 2 days of treatment that was maintained for the 6 day follow-up (77). Although overall survival was not assessed, and mice were enrolled with end-stage disease and a tumor doubling rate that is more reminiscent of transplantable tumors, such remarkable short-term results do encourage further investigations into the mechanisms of CXCR4 targeting. CXCR4 is implicated in the migration of Mo-MDSC in patients with ovarian cancer (95) and may be expressed on monocytes that can differentiate into fibrocytes, a population of bone marrow-derived cells that secrete fibrillar collagen and hyaluronan, promoting fibrosis and

tissue repair (96,97). The induction of CXCL12 in ovarian cancer and CXCR4 expression on Mo-MDSC was attributed to tumor-derived prostaglandin E<sub>2</sub>, providing a complementary approach to potentially interfere upstream of this pathway (95). Because this same CXCR4 antagonist also causes the rapid mobilization and egress of myeloid progenitors from the bone marrow in patients with other malignancies (98), it will be necessary to understand the long-term impact of interfering with this axis in PDA to fairly predict clinical potential.

Another recent study highlights the potential conundrums in attempting to target cells with dichotomous roles in disease. In contrast to the role of FAP<sup>+</sup> CAF in contributing to immunosuppression, genetic ablation of another myofibroblast population (characterized by  $\alpha$ Sma expression) caused the development of poorly differentiated, more hypoxic tumors with increased intratumoral Treg (99). Of note, these *KPC* mice were additionally engineered to lack Tgfr2 expression on epithelial cells. These tumors responded to anti-CTLA-4 at baseline in correlation with a reduction in intratumoral Treg. Although the contribution of the loss of TGF $\beta$  signaling is not known, these studies indicate that distinct modalities of targeting the tumor stroma, and even distinct subpopulations of tumor fibroblasts, will not necessarily yield similar results. The heightened sophistication of both the genetic tools and immune-based therapies will help to reveal the ideal sequence and combination of such multipronged strategies to safely promote patient survival.

#### **Reengineering the ECM**

The interstitium, through its complex ECM, provides biophysical and biochemical cues that determine cell responses in both development and disease. PDA presents its own characteristic ECM signature that not only has the potential to provide diagnostic value but also is intimately involved in the malignant phenotype. Matrix composition and complexity evolve throughout cancer progression and excess deposition of key components predicts poor prognosis. The cellular constituents in PDA contribute to, and operate within, this dynamic and dysregulated ECM, which promotes tumor invasion and impacts response to therapy.

#### *Altered biophysics in PDA: causes, consequences and remedies*

*Hyaluronan: biophysical barrier to drug delivery.* PDA is characterized by extensive deposition of hyaluronan, or hyaluronic acid (HA), a naturally occurring, negatively charged, megadalton glycosaminoglycan (100,101). Large molecular weight HA is secreted into and trapped within the interstitium in PDA and reaches concentrations among the highest observed in nature, rivaling those found in the umbilical cord and joint spaces (102). HA contributes to hydrostatic and oncotic fluid pressures in the interstitium through a combination of electrostatic repulsion and Donnan and van't Hoff forces, respectively (103,104). These properties enable HA to imbibe large amounts of water, creating an immobile fluid phase that also provides turgor to normal tissues. Indeed, it has long been appreciated that interstitial fluid is comprised of both mobile and immobile fluid with the latter predominating (105). In addition to resisting compression, its natural function in the joint space, HA can expand considerably when hydrated. These unique properties also enable HA to demonstrate viscoelastic behavior in solution; nevertheless, it behaves as a Newtonian fluid over a wide range of concentrations and shear rates, becoming non-Newtonian only at the extremes (106).

The high concentrations of HA in PDA generate a substantial swelling pressure that stresses collagen fibrils tethered to surface receptors that contract in response in the effort to maintain tensional homeostasis. The large immobilized fluid phase created by HA, together with the tensile load on stressed collagen fibers, results in inordinately high IFP in PDA, which, in turn, causes widespread vascular collapse and explains the previously described hypoperfusion (5,7). The extent of these fluid pressures had been underappreciated because of the model systems and the methods used to measure them (reviewed in ref. 107). The majority of studies on IFP have been performed in engraved

tumors and tumor explants that possess well-perfused, hypervascular beds comprised of 'leaky' vessels; the relatively modestly elevated fluid pressures measured in these settings result from equilibration with the hydrostatic pressure in the artificial neovasculature (108). However, autochthonous PDA appear not to support significant angiogenesis and instead have hypovascular tumor beds with structurally and functionally intact vessels (6). In addition, the classical methods used in most prior studies to assess IFP, including micropipette (109), wick-in-needle (110,111) and implanted capsule (112), can only measure pressures associated with the free-fluid phase (reviewed in ref. 105). More modern methods such as the piezoelectric pressure catheter (113,114) can measure pressures associated with both mobile and immobile fluid, uncovering the major barrier to perfusion in PDA.

This understanding of the unusual physiology and biophysics of autochthonous PDA suggested a potential remedy, namely to target and remove HA in order to mobilize the complexed fluid and relieve the associated pressures. Indeed, systemic administration of pegylated hyaluronidase (PEGPH20) can deplete intratumoral HA, causing a sharp decrease in IFP, increased vessel patency and perfusion and increased delivery of small-molecule therapeutics (5). When combined with gemcitabine in a randomized, placebo-controlled preclinical trial in *KPC* mice, PEGPH20 significantly increased objective response rate, decreased metastatic tumor burden and prolonged median survival (5). Intriguingly, the decrease in HA content and vascular perfusion frequently persisted even weeks after ceasing combination therapy, suggesting a permanent remodeling of the ECM (5). In a parallel study, PEGPH20 + gemcitabine also increased survival of *KPC* mice with near-terminal disease and was shown in ultrastructural studies to cause vessel fenestrations and interendothelial junctional gaps that would be expected to enhance macromolecular permeability as well (6). The combined PEGPH20 + gemcitabine regimen has completed early-phase trials in patients with promising results and two randomized phase II trials are currently underway testing distinct combination chemotherapy regimens together with the enzyme (NCT01839487 and NCT01959139). These studies also raise the possibility that incorporating hyaluronidase may reveal an antitumor activity of other drugs that have previously failed in clinical trials, perhaps, due to the biophysical barrier to delivery.

**HA signaling to immune and tumor cells.** HA can signal diversely to cells depending upon its molecular weight and the specific receptor to which it binds. HA is generated by synthases (HAS1, -2 and -3) and degraded by hyaluronidases (115). HA and HAS2 are independently associated with poor survival following PDA resection (4). Catabolism and oxidative stress in response to injury and inflammation can degrade high molecular weight (HMW) HA into low molecular weight fragments that further promote inflammation (116,117). HA fragment size can determine signal specificity to immune cells via binding to various HA receptors including CD44, TLR2 and TLR4 (118,119). For example, HMW-HA binding to monocytes induces their differentiation into fibrocytes, a bone marrow-derived lineage of cells that share many overlapping features with stromal fibroblasts including the expression of HA and collagens I and III (97). In contrast, low molecular weight-HA signals monocytes to differentiate into immunostimulatory dendritic cells (120). HMW-HA binding to the pattern recognition receptors, TLR2 and TLR4 dampens cytokine expression, whereas binding of smaller HA fragments to TLR2 and TLR4 induces inflammatory gene expression (121,122). Similar to the immunosuppressive role on the myeloid lineage, HMW-HA promotes the persistence and function of natural Treg via CD44 signaling (123,124) and induces the conversion of effector memory CD4<sup>+</sup> T cells to IL-10 producing iTreg (125). Thus, degradation of HA by PEGPH20 may promote immune surveillance, providing a separate potential mechanism of therapeutic benefit.

HA receptors, such as CD44 and RHAMM (receptor for hyaluronidase-mediated motility receptor), are also expressed on TEC and putative pancreatic cancer stem cells and can activate proliferation, adhesion and migration pathways (reviewed in ref. 126). CD44 expression is significantly associated with poor survival in PDA patients (127). Treatment of patient-derived PDA xenografts with an anti-CD44 antibody reduced tumor growth, metastasis and postradiation recurrence

of pancreas cancer (128). Overexpression of RHAMM in a mouse model of islet cell tumorigenesis promoted liver metastases (129). However, because CD44 is expressed on a variety of immune cells in addition to TEC, the net effects on autochthonous tumor response and recurrence remain to be determined.

#### *Additional ECM targets in PDA*

**Collagen.** Fibrillar collagens are also dramatically overexpressed in PDA (130). Myofibroblasts are the primary source of collagens, but TEC can produce collagens as well. Fibrillar collagen can bind to surface integrins expressed on tumor epithelial, mesenchymal and immune cells and provide tensile strength and resistance to force. In normal structures, collagen is typically found in a 'curly' conformation; cancer-associated collagen is thicker, linearized and frequently crosslinked by lysyl oxidase (131). As described above, we have proposed that immobilized fluid complexed to HA stresses a tethered collagen network that contracts in response further increasing the associated IFP (104,107). Thus, we would predict that removal of collagen or inhibition of contractile forces that counteract HA swelling would cause an incremental or a stepwise drop in pressure but not complete normalization. Consistent with this idea, collagenase treatment of human colorectal tumors grown in nude mice decreases IFP by 34% (132). Collagen, too, can mediate cell signaling. The increase in matrix stiffness with excess collagen deposition can induce force-dependent integrin clustering and downstream signaling resulting in cancer cell migration, in addition to providing the physical 'tracks' for cancer cells emigrating from and immune cells trafficking into the stroma (131).

The diverse and ubiquitous roles of collagens in normal cellular and tissue biology may limit their potential as direct therapeutic targets. However, inhibiting cellular contractile forces at discrete points along their signaling pathways, including potentially perturbing collagen-cell surface interactions by targeting integrins, may provide a sufficient therapeutic window to at least partially alleviate the biophysical barriers to drug delivery.

**Secreted protein acidic and rich in cysteine (SPARC).** A recent phase III trial of gemcitabine plus *nab*-paclitaxel, an albumin-bound formulation of paclitaxel, significantly prolonged survival in patients with metastatic PDA (133). The enhanced therapeutic activity of *nab*-paclitaxel in PDA has been attributed in part to stromal depletion.

*Nab*-paclitaxel has a long *in vivo* half-life (estimated at >24h), rendering it more capable of overcoming the biophysical barrier posed by the stroma and accessing tumor cells. Also, PDA cells upregulate macropinocytosis to acquire nutrients, including albumin (134), potentially resulting in preferential uptake of the drug by TEC. It has also been proposed that *nab*-paclitaxel homes to SPARC, a glycoprotein expressed primarily on myofibroblasts whose expression correlates inversely with patient survival (135). This would provide an alternative explanation for the enhanced efficacy of the agent. However, studies in GEMM of PDA in both SPARC (+/+) and SPARC (-/-) backgrounds suggested no impact in sequestering *nab*-paclitaxel intratumorally nor in the preferential apoptotic cell death induced in tumor epithelial versus stromal cells (136). One possible explanation for this result is that the death of TEC diminished critical paracrine signaling to fibroblasts, leading to dissolution of the stroma.

**Additional ECM components and targets.** A number of other ECM components may be of therapeutic interest in PDA. Connective tissue growth factor (CTGG) is highly expressed in both human and murine PDA. Connective tissue growth factor antagonism with a therapeutic monoclonal antibody (FG-3019) in combination with gemcitabine decreased tumor size and increased survival in *KPC* mice; these effects were associated with decreased expression of X-linked inhibitor of apoptosis (137). Combining FG-3019 with gemcitabine and erlotinib is currently under clinical investigation in pancreas cancer patients (NCT01181245). The large proteoglycan versican is increased ~27-fold in the ECM in PDA (138) and may facilitate tumor invasion and metastasis (139,140) by inhibiting cell adhesion to the ECM (141). Fibronectin, which binds  $\beta$ 1 and  $\beta$ 3 integrins, is also upregulated in PDA and promotes tumor cell migration *in vitro*



(142). It should be mentioned that some ECM components may have antitumorogenic properties. Decorin, a small proteoglycan also abundant in PDA (138,140), can inhibit tumor cell proliferation (143,144). A recent study showed that higher levels of laminin in the basement membrane of PDA correlated with longer survival in PDA patients (145). Identifying which components to inhibit and which to augment in the stromal matrix biology of PDA will be part of the challenge in developing novel treatment regimens.

## Conclusions

The increasing appreciation of a burgeoning pancreas cancer as a coordinately evolving neo-organ that invokes an entirety of cellular and non-cellular elements has generated a deeper appreciation for the challenges we face while also providing a broadened array of potential vulnerabilities. Thinking beyond the cancer cell to the co-conspirators complicit in its ambitions presents a new therapeutic landscape to consider. GEMM of PDA represent a potential advance over transplantable tumor models by, among other things, faithfully recapitulating the robust desmoplasia; vascular architecture, structure and function; immune contexture; and response to chemotherapy seen in the human disease (reviewed in refs. 146,147). Models of autochthonous PDA provide a means to study the complicated interactions among these components and contributions to malignancy at each stage of disease progression. Inhibiting immunosuppressive subsets while providing effective T-cell responses may reinstate immunosurveillance, reengineering the immune system to effectively target the tumor. Strategies that focus on the stromal mesenchyme may eliminate critical protumorogenic paracrine signaling of myofibroblasts to tumor cells and relieve additional immunosuppressive influences. Eliminating physical barriers posed by the non-cellular ECM will lower IFP to improve drug delivery, as well as remove potential survival signals. A number of complex treatment strategies that exploit the stromal compartment in combination with cytotoxic and/or epithelial cell-targeted agents can be envisioned to meaningfully impact the prognosis of patients with pancreas cancer.

Perturbing the homeostasis of the pancreas cancer neo-organ is not without risk, however. The recent results from the saridegib trial speak to the need to ensure not only that preclinical studies accurately reflect the anticipated clinical trial design but also that the correct lessons are discerned and appropriately applied. Studies in near-terminal, moribund animals do not permit extended treatment exposure and may not accurately reflect the majority of human patients seen in the clinic. Patients with metastatic disease—certainly ones considered for clinical trials—typically have good performance status at presentation and life expectancies on therapy of 6–12 months (148,149). Particularly as we begin to target non-cell autonomous mechanisms, which may have secondary unanticipated sequelae, allowing sufficient time for these changes to manifest themselves is prudent before translation to the clinic. The challenge now will be to identify and rigorously scrutinize those combinations and schedules destined to matter most while successfully navigating the potential consequences of further unleashing the disease we seek to contain.

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