Pancreatic Inflammation and Carcinogenesis

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Pancreatic ductal adenocarcinoma (PDAC) represents the quintessential example of an inflammation-driven cancer. This is supported by epidemiological data as well as evidence from preclinical models and clinical studies. In this brief review we discuss the recent advances pertaining to the link between inflammation and pancreatic cancer, and present our perspective on this topic.

Inflammation is relevant both as a risk factor for and as a consequence of oncogenesis in pancreatic cancer. In terms of risk of pancreatic carcinogenesis, patients suffering from chronic pancreatitis carry a 13-fold higher risk of PDAC development (45). Further, patients with hereditary autoimmune pancreatitis have an estimated lifetime risk for PDAC development of 40% (2). The duration of pancreatitis appears to correlate positively with the possibility of Kras mutations (29), which suggests a possible mutagenic role for repetitive bouts of inflammation. Nevertheless, the majority of PDAC cases develop in the absence of clinically evident overt pancreatitis (46). One can hence conclude that either subclinical, low-grade inflammation is sufficient to promote carcinogenesis, or that inflammation is a consequence of the earliest events in the stepwise process of pancreatic carcinogenesis. This low-grade inflammation that is a result of cellular stress and malfunction has been termed "parainflammation" and is hypothesized to either contribute to cellular adaptation to the noxious environment or to promote senescence in order to prevent malignant transformation (31).

Critical work done on genetically-engineered mouse models (GEMMs) in the past decade recapitulates the sequence of events occurring in human PDAC (30). Kras is mutated in more than 90% of human PDAC cases and constitutive activation within the pancreas results in PDAC development in mice in a fashion very similar to its human counterpart (12, 23). There appears to be a cooperative relationship between Kras activation and inflammation (1, 34). Specifically, Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$ ("KC") mice, which have mutant Kras expressed prenatally in all exocrine pancreatic lineages, exhibit pancreatitis as one of the earliest morphologic changes in their pancreas. In addition, treatment of these mice with only a few doses of caerulein – a cholecystokinin analog that hyperstimulates the pancreas and induces pancreatitis if injected repeatedly – dramatically accelerates the progression to advanced PanINs and invasive cancer within a few weeks (compared to months in untreated mice) (5).

A seminal discovery was made by Guerra *et al*, who used a Cre / Tet-off system to activate the Kras mutation in acinar cells of adult mice. Surprisingly, these mice only developed pancreatic cancer when chronic pancreatitis was

induced with caerulein, suggesting that Kras mutation alone – in absence of pancreatitis – is insufficient to induce pancreatic cancer in adulthood (22). The same group has shown that chronic pancreatitis enables Kras-driven carcinogenesis by thwarting oncogene-induced senescence – a homeostatic mechanism that diverts stressed cells prone to malignant transformation towards cell cycle arrest and quiescence (21). Concurrent treatment of the mice with a COX1/2 inhibitor not only prevented the progression of early PanINs to advanced PanINs and invasive cancer but also decreased the number of early PanINs, pointing to additional mechanisms through which inflammation promotes pancreatic carcinogenesis (21).

Logsdon and colleagues have studied the role of inflammation and its influence on Kras-driven carcinogenesis from a different perspective. They showed that even when Kras is mutated and constitutively active, it cannot reach the expected theoretical levels of activity and remains at levels close to the basal state (25). However, inflammatory insults such as caerulein and lipopolysaccharide (LPS) can hyperstimulate Kras, bringing its activity above the hypothetical threshold necessary for the initiation of the sequence of carcinogenesis (9, 25). Furthermore, constitutive activation of both Kras and either IKK2 (an activator of the NF-κB pathway) or COX2 (a downstream effector of the NF-κB pathway) in acinar cells dramatically accelerates carcinogenesis (9). Conversely, ablation of IKK2 mitigated the caerulein-induced inflammation and fibrosis, decreased the levels of active Kras, and protected against PanIN formation (9). Similar results were observed with inhibition of COX2 (9). Additional evidence was provided by another study which showed that mutant Kras induces the transcription of IL-1α through AP-1. IL-1α in turn activates the NF-κB pathway leading to production of more IL-1α as well as activation of the signaling adaptor p62 which prolongs the activity of this pathway (27). A very intriguing

finding was that, in the context of chronic pancreatitis and tissue injury, Kras mutation can give rise to PDAC originating from insulin-positive endocrine cells (20). The significance of this lies in the fact that chronic inflammation can induce de-differentiation of committed epithelial cells and thence promote carcinogenesis. In summary, inflammation synergizes with Kras through the establishment of a positive feedback loop that is dependent on NF-κB and COX2 and leads to sustained Kras activity; on the other hand, Kras activity promotes an IL-1α- and p62-mediated feed-forward loop that sustains NF-κB pathway activity.

Several other mechanisms contributing to inflammation-driven carcinogenesis in PDAC have been identified to date. A notable example is STAT3, which appears to be a central mediator in pancreatic carcinogenesis. STAT3 is activated in mice challenged with caerulein. In WT mice, the activation status reverts to baseline after a few days consistent with recovery from acute pancreatitis. By contrast, in KC mice STAT3 remains persistently activated (19). This is a consequence of communication between the epithelial cells and the surrounding stromal cells. Specifically, the Kras-mutant epithelial cells recruit myeloid cells which secrete IL-6 and activate the STAT3 pathway in the epithelial cells via IL-6 trans-signaling, thus completing a positive feedback loop (26). Persistent STAT3 activation drives pancreatic cancer progression through upregulation of anti-apoptotic and pro-proliferative proteins such as Bcl-X$_L$, Mcl-1, Survivin, c-Myc and Cyclin D1 (19, 26, 47). In incipient carcinogenesis, this may be critical for the evasion of oncogene-induced senescence, while later on it may be more important for proliferation under the adverse conditions of the hypoxic tumor microenvironment. Moreover, STAT3 activation in epithelial cells promotes the secretion of pro-inflammatory mediators which further recruit leukocytes; at the same time, epithelial STAT3 signaling induces the expression of MMP7 which supports tumor growth and metastasis (19).

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Genetic ablation of IL-6 or STAT3 , neutralization of IL-6 trans-signaling, as well as deletion of STAT3 exclusively in the epithelial cells dampens tumor-associated inflammation and protects from spontaneous and caerulein-induced PanIN formation and PDAC development (19, 26).

The tumor microenvironment in the pancreas is rife with factors that can attract inflammatory cells and entrain them to support the process of carcinogenesis and shield the cancer cells from the anti-tumorigenic arm of the immune system. Kras-mutant epithelial cells secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) to recruit myeloid cells that suppress CD8+ cytotoxic T cells (3, 35). The secretion of GM-CSF begins early on, and its neutralization depletes myeloid-derived suppressor cells and enables efficient tumor killing by CD8+ T cells (3, 35). In a similar fashion, pancreatic cancer cells produce the chemokine CCL2 that mobilizes inflammatory monocytes from the bone marrow and recruits them to the pancreas as well as to premetastatic niches such as the liver to promote tumor growth and metastasis, respectively (37). Cancer cells also secrete CCL5 and other ligands that recruit regulatory T cells (Treg) in a CCR5-dependent manner and thus contribute to an immunosuppressive tumor microenvironment (43).

Macrophages are the predominant immune cell type in the pancreas during pancreatitis, and promote de-differentiation and delayed pancreas regeneration after caerulein-induced acute pancreatitis (17). They infiltrate the pancreas early in the course of pancreatic carcinogenesis, and localize around pre-neoplastic ducts (7). Liou *et al* showed that macrophages directly act on nearby epithelial cells to promote acinar-to-ductal metaplasia (ADM) – the earliest pre-malignant lesion observed during sustained pancreatic inflammation (28). This ADM-promoting effect was contingent upon several macrophage-derived soluble mediators, especially TNF-α and CCL5/RANTES, which act through NF-κB to promote epithelial cell proliferation and MMP9-mediated remodeling of the extracellular matrix (28). Notably, depletion of macrophages significantly decreased the formation of ADM foci in mice undergoing caerulein-induced pancreatitis.

Manipulation of tumor-associated macrophages using an agonistic anti-CD40 antibody has been shown to have beneficial effects both in mice and in humans, by causing them to switch from a pro-inflammatory phenotype characterized by low MHC II expression and secretion of IL-10, TNF-α, and IL-6, to an anti-tumorigenic phenotype featuring upregulation of MHC II and CD86, elevated serum IL-12, TNF-α, and IFN-γ levels, enhanced tymor lytic capacity, and involution of tumor stroma and associated fibrosis (4). Notably, the anti-tumorigenic effects of CD40-primed macrophages were independent of CD4 and CD8 T cells (4).

Cancer cells can also influence the immune response indirectly by manipulating other stromal cells. Cancer-associated fibroblasts (CAFs) – which in pancreatic cancer may originate from pancreatic stellate cells (PSCs) – are induced to express an NF-κB-dependent pro-inflammatory gene signature that enhances tumor growth, vascularization and macrophage recruitment (15). Additionally, inflammatory mediators such as IL-1β and TNF-α released by the cancer cells instruct CAFs to release thymic stromal lymphopoietin (TSLP), which in turn signals to myeloid dendritic cells (DCs) to skew CD4+ T cells towards T_H2 polarization (10). T_H2-deviated CD4+ T cells have a pro-carcinogenic role in the pancreas through the perpetuation of pancreatic fibro-inflammation and the recruitment of M2 macrophages (10, 11). Further, activated PSCs upregulate adhesion molecules and secrete chemokines – the most prominent being CXCL12 – that sequester CD8+ T cells around them and prevent them from attacking the cancer cells (14). Another study validated these results and further

showed that fibroblast activation protein (FAP)-expressing CAFs secreting CXCL12 are the limiting factor for the efficacy of T cell checkpoint inhibitors (16). Inhibition of CXCL12 reverses the immunosuppressive effects of PSC/CAFs and synergizes with anti-PD-L1 immunotherapy leading tumor elimination (14, 16).

Contrary to the above, two recent studies showed conflicting data regarding the role of CAFs in pancreatic cancer. Özdemir *et al* used the Ptf1a$^{Cre/+}$;LSL-Kras$^{G12D/+}$;Tgfrb2$^{fl/fl}$ mouse model (PKT) and crossed it with αSMA-tk transgenic mice to selectively deplete α-smooth muscle actin (α-SMA)-positive myofibroblasts upon ganciclovir administration (48). Surprisingly, myofibroblast depletion early and late during the life of these mice led to less differentiated tumors and significantly decreased the survival of these mice. Similar findings were observed in KPC;αSMA-tk mice, suggesting that α-SMA+ myofibroblasts are protective, contrary to the notion that desmoplasia is detrimental for PDAC. Further, low α-SMA immunostaining in human PDAC specimens correlated with worse survival. From a mechanistic point of view, the authors showed that depletion of myofibroblasts led to suppression of angiogenesis, enhanced tumor hypoxia, promoted epithelial-to-mesenchymal transition (EMT) and cancer stem cell-like phenotype, reduced type I collagen content, altered the ECM organization, and decreased stiffness and elastic modulus of tumors in PKT mice. Moreover, myofibroblast-depleted tumors exhibited decreased overall immune cell infiltration but an increased percentage of Tregs and higher expression of CTLA-4. Administration of anti-CTLA-4 antibody rescued the phenotype of myofibroblast-depleted tumors in PKT mice, improved overall survival, and led to a transcriptional profile that resembled that of control non-depleted tumors. Interestingly, the number of FAP+ cells was not affected in the above depletion model, suggesting that heterogeneity may exist among CAFs, and raising questions on whether the aforementioned results are attributable to selective depletion of subsets of CAFs that have tumor-protective effects.

In the second study, Rhim, Oberstein, Thomas *et al* employed the PKCY (a modification of the KPC mouse model harboring a YFP reporter) and by negating paracrine Sonic Hedgehog (SHH) signaling through genetic deletion or pharmacologic inhibition, they achieved significant attenuation of the stromal component of the tumors (36). This depletion of the tumor stroma led to decreased infiltration of leukocytes, enhanced tumor cell proliferation, augmented EMT signature, accelerated formation of premalignant lesions, more undifferentiated tumors, and increased metastasis, similarly to Özdemir *et al*. However, SHH ablation resulted in increased angiogenesis, in contrast to the former study. Moreover, in the context of deficient SHH signaling, anti-VEGFR2 blocking antibodies significantly improved survival.

Instead of using a stroma-depleting strategy, Sherman *et al* adopted a different approach for CAF manipulation (39). By comparing quiescent, activated, and cancer-associated PSCs using massively parallel RN sequencing, they found that the latter two express high levels of the Vitamin D Receptor (VDR). Activation of VDR with the agonist calcipotriol during caerulein-induced acute and chronic pancreatitis resulted in reversal of the PSC phenotype to the quiescent state, leading to attenuated inflammation and fibrosis, as well as lower phospho-STAT3 levels. When administered in vivo to KPC mice, calcipotriol had no measuarable beneficial effects. however, combined with gemcitabine chemotherapy, VDR agonization led to markedly increased intratumoral gemcitabine levels, decreased tumor size, and significantly prolonged overall survival, compared to gemcitabine alone.

Considering that inflammation is present very early in the stepwise process of pancreatic carcinogenesis, it is reasonable to assume that it would be an attractive target for chemoprevention.

Indeed, multiple approaches have been adopted using either natural compounds with diverse effects or synthetic compounds that have specific targets (41). For example, inhibition of cyclooxygenases has been investigated in multiple studies using both non-steroidal anti-inflammatory drugs (aspirin, nimesulide, etodolac, sulindac etc) and COX2-specific inhibitors (e.g. celecoxib) (8, 41). Several of those have been associated with decreased risk of pancreatic cancer (8, 41). Curcumin – an agent with pleiotropic effects on the tumor stroma and the associated inflammation – also seems to have a protective effect in pancreatic carcinogenesis (8). In light of the aforementioned study by Sherman *et al*, VDR agonists may also represent an attractive mode of chemoprevention, which has already been suggested by previous studies. For example, in two cohort studies with long-term follow-up, Skinner *et al* found that higher intakes of vitamin D were associated with lower risks for pancreatic cancer (40). Prospective studies in larger cohorts are required to address the efficacy of such compounds in preventing pancreatic cancer.

Innate immune receptors have an emerging role in driving inflammation within the pancreatic tumor microenvironement. Toll-like receptors (TLRs) represent the best described family of pattern-recognition receptors. They are present on most types of immune cells and they bind a variety of microbe-associated molecular patterns (MAMPs, such as LPS) as well as byproducts of dying cells and sterile inflammation denoted DAMPs (damage-associated molecular patterns) (42). Upon ligand binding, they recruit either the MyD88 or the TRIF adaptor molecules (depending on the specific TLR) to transduce activation signals to the NF-κB and MAPK pathways. Recent reports showed that TLR4 and TLR7 are upregulated within the tumor microenvironment of pancreatic cancer (32, 33). Further, TLR activation can fuel pancreatitis and can synergize with Kras to dramatically accelerate pancreatic carcinogenesis in mice (9, 13, 32, 33, 38). These pro-

carcinogenic effects of TLRs can be prevented through inhibition of either NF-κB or MAPK pathway (33). Furthermore, mice deficient in several TLRs are protected from acute pancreatitis and direct inhibition of TLR4 as well as TLR7 protects KC mice from pancreatic carcinogenesis (32, 33).

Other pattern recognition receptors and associated signaling molecules have been implicated in the pathogenesis of inflammation-driven cancer. Nucleotide-binding domain and leucine-rich repeat containing molecules or NOD-like receptors (NLRs) are cytoplasmic pattern-recognition receptors. When engaged by their ligands, they can activate the NF-κB pathway but they also associate with other molecules to form large oligomeric complexes called inflammasomes (18). Caspase-1 is a key component of activated inflammasomes and is responsible for the proteolytic cleavage and maturation of the pro-inflammatory cytokines IL-1β and IL-18 (18). NLRs and their downstream effectors are necessary for keeping the intestinal microbiota under control. For example, mice deficient in several NLRs, Caspase-1, or IL-18 exhibit alterations in the gut microbiome and dysbiosis, and are highly susceptible to colorectal carcinogenesis (6). Consistent with their pro-inflammatory role, the inflammasomes have been found to contribute to the pathogenesis of pancreatitis (24, 44). Specifically, administration of a NOD1 agonist synergized with caerulein in the induction of acute pancreatitis (44). This was a result of NOD1 activation in acinar cells, which promoted acinar NF-κB and STAT3 signaling, and CCL2-mediated recruitment of CCR2+ pro-inflammatory cells (44). Furthermore, genetic ablation of NOD1, Nlrp3, Caspase-1, or ASC (another component of the inflammasome) protects from caerulein-induced acute pancreatitis (24, 44). However, a definitive role for NOD1, Nlrp3, Caspase-1, or ASC in pancreatic carcinoma is still investigational.

In summary, pancreatic dysplasia arises in the context of inflammation **(Figure 1)** and is driven toward carcinogenesis by a network of pro-inflammatory signaling mechanisms mediated by the interaction of an array of immune competent cells in the tumor microenvironment – including T cells subsets, M1 and M2 macrophages, dendritic cells, mast cells, neutrophils, and inflammatory monocytes – with each other and with the genetically at-risk epithelial and acinar compartments. Therapeutic targeting of inflammation and strategies to upwardly modulate adaptive immunity hold promise for the prevention of pancreatic carcinoma in at-risk individuals as well as for the treatment of established carcinoma in combination with approaches which directly address the genetically transformed cells.
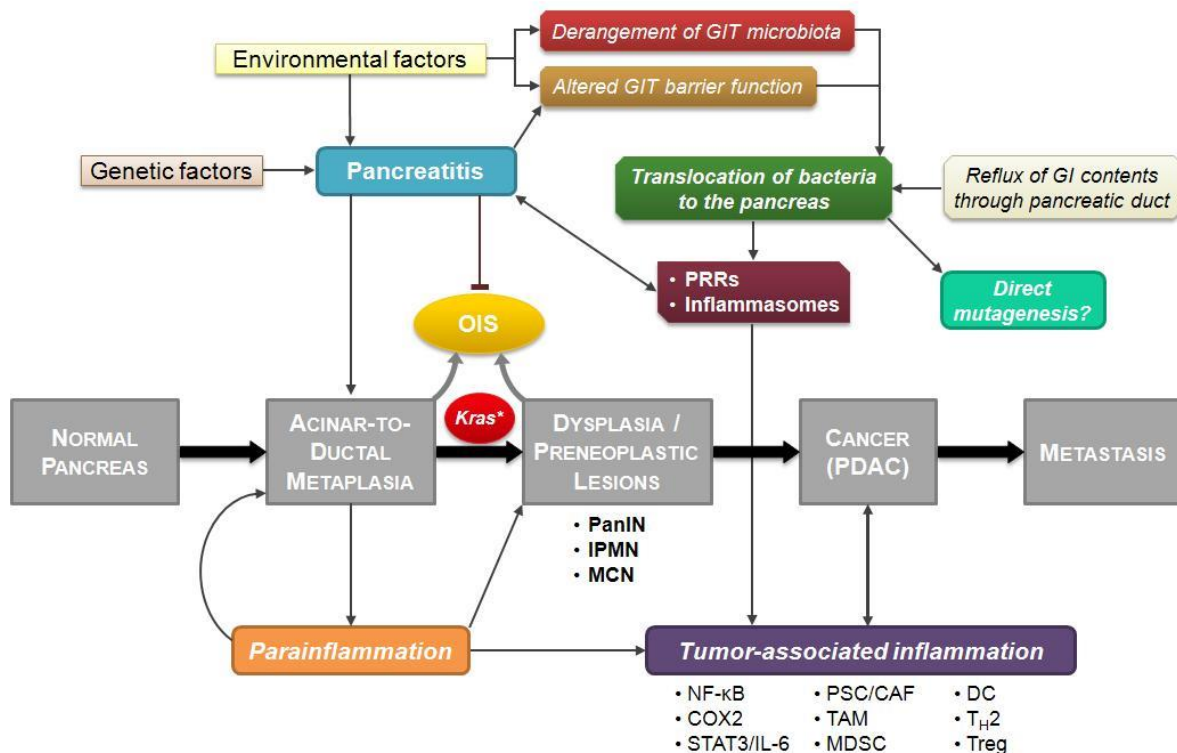

Figure 1. Interplay between tumor-associated inflammation and pancreatic carcinogenesis. Pancreatic cancer progresses through a series of defined stages that involve acinar-to-ductal metaplasia (ADM) in response to repetitive injury, development of pre-neoplastic lesions, and eventually invasive cancer. Pancreatitis can be caused by genetic and environmental factors (e.g. alcohol), and can promote pancreatic carcinogenesis by inducing ADM while inhibiting oncogene-induced senescence (OIS). Noxious stimuli to the pancreas result in a low-grade maladaptive inflammatory response termed "parainflammation" that can synergize with mutant Kras in tumor development and evasion of OIS, and recruit immune cells that promote cancer-associated inflammation. Environmental factors, such as antibiotics and alcohol consumption, as well as pancreatitis can cause derangement of the gut microbiome and compromise the intestinal barrier function to promote translocation of bacteria to the pancreas. Translocated dysbiotic bacteria (pathobionts) can stimulate pathogen recognition receptors (PRRs) and inflammasomes, perpetuating tumor-associated inflammation. Tumor-associated inflammation is further exacerbated by activation of pro-inflammatory signaling pathways including STAT3, NF-κB, COX2. In parallel, tumor-associated immune suppression is exerted by a variety of cellular subsets including regulatory T-cells (Tregs), T-helper (Th2) cells, myeloid derived suppressor cells (MDSC), and tumor associated macrophages (TAMs).

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