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Focused ultrasound for immuno-adjuvant treatment of pancreatic cancer: an emerging clinical paradigm in the era of personalized oncotherapy.

Ezekiel Maloney, MD,
University of Washington / Department of Radiology

Tanya Khokhlova, PhD,
University of Washington / Department of Medicine / Division of Gastroenterology

Venu G. Pillarisetty, MD,
University of Washington / Department of Surgery

George R. Schade, MD,
University of Washington / Department of Urology

Elizabeth A. Repasky, PhD,
Department of Immunology / Roswell Park Cancer Institute, Buffalo, NY 14263

Yak-Nam Wang, PhD,
University of Washington / Applied Physics Laboratory

Lorenzo Giuliani,
University of Rome “La Sapienza”

Matteo Spring, and
University of Rome “La Sapienza”

Joo Ha Hwang, MD, PhD
University of Washington / Department of Medicine / Division of Gastroenterology, Box 359773, 325 Ninth Avenue, Seattle, WA 98104

Abstract

Current clinical treatment regimens, including many emergent immune strategies (*e.g.* checkpoint inhibitors) have done little to affect the devastating course of pancreatic ductal adenocarcinoma (PDA). Clinical trials for PDA often employ multi-modal treatment, and have started to incorporate stromal-targeted therapies, which have shown promising results in early reports. Focused ultrasound (FUS) is one such therapy that is uniquely equipped to address local and systemic limitations of conventional cancer therapies as well as emergent immune therapies for PDA. FUS methods can non-invasively generate mechanical and/or thermal effects that capitalize on the unique oncogenomic/proteomic signature of a tumor. Potential benefits of FUS therapy for PDA include: 1) emulsification of targeted tumor into undenatured antigens *in situ*, increasing

Declaration of Interest

The authors report to conflicts of interest. The authors alone are responsible for the content and writing of the paper.

dendritic cell maturation, and increasing intra-tumoral $CD8^+$/ T regulatory cell ratio and $CD8^+$ T cell activity; 2) reduction in intra-tumoral hypoxic stress; 3) modulation of tumor cell membrane protein localization to enhance immunogenicity; 4) modulation of the local cytokine milieu toward a Th1-type inflammatory profile; 5) up-regulation of local chemoattractants; 6) remodeling the tumor stroma; 7) localized delivery of exogenously packaged immune-stimulating antigens, genes and therapeutic drugs. While not all of these results have been studied in experimental PDA models to date, the principles garnered from other solid tumor and disease models have direct relevance to the design of optimal FUS protocols for PDA. In this review, we address the pertinent limitations in current and emergent immune therapies that can be improved with FUS therapy for PDA.

Keywords

ultrasound; therapy; pancreatic; cancer; immune

Introduction

Pancreatic cancer responds poorly to many conventional and emergent immune therapies

Pancreatic ductal adenocarcinoma (PDA), commonly known as pancreatic cancer, typically presents as metastatic or unresectable disease and has an overall 5-year survival rate of less than 8% [1]. It has recently been estimated that by 2030, PDA will be the second most common cause of cancer-related mortality – indicating the limited impact of cancer therapy research to date on the clinical course of the disease [2]. The poor response of PDA to conventional therapies is thought to be in part due to a dense matrix of tumor stromal cells that fosters: 1) high interstitial fluid pressure (~99mmHg, versus 10mmHg in normal pancreas), that results in collapsed vasculature and hypoxia, and 2) an immunosuppressive microenvironment, impeding the endogenous immune system from eradicating the tumor [3–9]. Exogenous immune-modulating immunotherapies have garnered significant attention in recent years due to early successes in clinical trials for treatment of hematologic cancers and a limited number of solid tumors (*e.g.* melanoma, non-small-lung cancer, clear cell renal cell carcinoma, head and neck squamous cell carcinoma, urothelial cancer), but have unique obstacles to overcome in treatment of many other solid tumors, including PDA [10]. Clinical trials of many emergent immune strategies, such as immune checkpoint inhibitors and treatments targeting macrophages and myeloid-derived suppressor cells, have yielded few objective responses in PDA patients [11–16].

The importance of immune infiltrate, hypoxia, and the PDA stroma

Tumor-antigen specific, endogenous $CD8^+$ T cells are often present in the circulation and bone marrow of PDA patients, and $CD8^+$ T cells capable of T helper (Th)-1, anti-tumor functional response (*e.g.* IFN-gamma) dominate the immune cell infiltrate of resected tumor specimens [8, 16–20]. These latter findings contrast with those observed in genetically engineered mouse models of PDA, where $CD8^+$ T cells remain relatively scarce within the tumor compared to immunosuppressive cells [9, 21–24]. This discrepant picture of the immune response may be due to the relatively rapid tumor development after oncogene

activation in genetically engineered animals versus the prolonged genetic evolution of human PDA [25]. Regardless, despite the presence of an effector T-cell-rich infiltrate in human tumors, meaningful anti-tumor activity is most commonly not observed. For a graphical overview of the categorization of immune therapies employed in the treatment of PDA, please see Figure 1.

Notable barriers to effective cytotoxic T-cell activity in PDA tumors include: 1) the poorly vascularized, profoundly hypoxic and acidic tumor microenvironment and advanced desmoplastic stroma that correlate with more aggressive tumor phenotypes [26–32]; 2) the immunosuppressive activity of $FOXP3^+$ regulatory T cells (Treg), $CD11b^+$ myeloid-derived suppressor cell (MDSC), and tumor-associated macrophages (TAM) within the PDA stromal matrix [33]. Increased hypoxia [34–36], and increased ratio of immunosuppressive versus effector memory $CD8^+$ T cells [17, 33] are poor prognostic indicators in PDA, and are closely inter-related. Hypoxic zones within tumors have been shown to: 1) foster localized accumulation and differentiation of immune inhibitory cell lines (*e.g.* Treg, TAM, MDSC) [37]; 2) promote immunosuppressive activity such as selective upregulation of PD-L1 expression on MDSCs and tumor cells [38, 39]; 3) decrease production of IFN-gamma and IL-2 [40, 41]; 4) diminish cytotoxic T-cell performance with tumor microenvironments *in vivo* [42–46]. Indeed, accumulation of intracellular hypoxia-inducible factors (*e.g.* HIF-1α) are correlated with poor tumor differentiation and fibrotic foci in PDA [47, 48] and have been shown to promote pancreatic cancer stem cell (CSC) expansion, including $CD133^+$ CSCs that are known to determine the metastatic phenotype of individual tumors through HIF-1α-dependent activation of the Notch signaling pathway [34, 49–53]. Treatments that decrease the hypoxic stress of the PDA microenvironment could significantly improve endogenous immune responses and immunotherapy efficacy, and have also been shown to improve response to radiotherapy and chemotherapy [53–55].

Tregs play a particularly important role in modulating the immune activity in the PDA microenvironment. There is debate and ongoing research regarding the most important features of Tregs and their interactions that help to enable tumor evasion of immune eradication; however expansion of Tregs in peripheral blood and tumor tissue has been shown to correlate with poor prognosis [56–58]. Th1 (T-bet^+), Th2 (GATA-3^+), and Th17 (ROR-gamma-t^+) have recently been shown to be not only T helper subtypes, but also dynamic phenotypes of Treg cells [59, 60]. GATA3 expression has been shown to be critical to Treg functions during inflammation, and PDA is associated with chronic inflammation [61, 62]. The ratio of $GATA3^+$/T-Bet^+ infiltrating-lymphoid cells in human PDAs has been shown to correlate inversely with survival, implying an association between Th2 dominance (increased IL-5 and IL-13 levels) and disease progression [63–65]. In addition, patients with PDA have expanded peripheral ROR-gamma-t^+ Tregs that induce both Th17 (*e.g.* IL-17, IL-6) and Th2 (*e.g.* IL-4, IL-13, IL-33) responses – effectively suppressing anti-tumor T cell activity while promoting chronic inflammation [60]. This finding correlates with resected human PDA specimens demonstrating expanded intra-tumoral Tregs and Th17 cells [17]. Thus the hybrid, dynamic, Th17/Th2-response-inducing Treg phenotype appears to be an important pro-carcinogenic driver of the PDA immuno-microenvironment [66].

PDA stromal remodeling strategies, such as selective depletion of specific subsets of immune-suppressor cells within the tumor stroma, have promoted endogenous T cell activity against PDA in genetically engineered pre-clinical models, and unmasked the benefit of complimentary checkpoint-inhibitor therapy [21, 22, 67]. These results are distinctly different from efforts to selectively ablate stromal fibroblasts, which appeared to evoke a more aggressive disease [68, 69], but similar to those achieved with targeted depletion of hyaluronic acid (HA; a naturally occurring glycosaminoglycan that is produced by PDA cells and is highly concentrated in the tumor extracellular space). Decreasing intra-tumoral HA concentration via intravenous recombinant enzyme administration lowered interstitial fluid pressure, increased intra-tumoral vessel diameter, improved chemotherapy delivery, and significantly prolonged survival in $Kras^{LSL\text{-}G12D/+};Trp53^{LSL\text{-}R172H/+};p48^{Cre/+}$ (KPC) mice (a genetically engineered model of PDA known to closely mimic the human disease) [5, 70]. In a recent phase 1B clinical trial, enzymatic HA depletion doubled overall survival when combined with chemotherapy in patients with high HA, stage IV PDA [4]. In addition, as described in greater detail below, a recent phase 1 clinical trial of relatively low-intensity focused ultrasound (FUS) therapy designed to minimize thermal effects, directed at the tumor and stroma in combination with chemotherapy, doubled median overall survival in patients with inoperable PDA versus chemotherapy alone [71]. Thus, stromal-targeted therapies appear to be an important component of evolving multi-modal PDA treatment regimens. The mechanisms underlying the efficacy of adjuvant stromal-targeted treatments are incompletely understood, but may be driven by reduction of intra-tumoral interstitial fluid pressure (*e.g.* targeted HA reduction), anti-tumor immune effects (*e.g.* selective immune- suppressor cell depletion), or a combination of these two (*e.g.* FUS therapy).

Passive Specific Immune Therapies

“Passive specific” immune therapies are exogenously engineered or expanded with tumor-specificity, and passively infused to mediate immediate anti-tumor immune activity. These include engineered or expanded tumor-specific T cells and antibodies. Ex-vivo expansion of endogenous anti-tumor T cells for adoptive transfer is discussed in greater detail below. Adoptive transfer of genetically engineered T cells, incorporating either a cloned T cell receptor (TCR) or synthetic chimeric antigen receptor (CAR), can improve anti-tumor immune function, and (with careful selection of target antigens) cause minimal off-target or on-target-off-tumor toxicity. Recent advances in high-throughput patient lymphocyte testing as well as sequencing of tumor transcriptomes and whole-exomes have enabled wide survey of tumor-specific antigens recognized by autologous T cells and facilitated a surge in clinical implementation of adoptive cell transfer [72]. CAR T cells directed against the B cell surface receptor CD19 have been effective in treating leukemia and lymphoma in early clinical trials [73–78], and research to translate this success to solid tumor models, including PDA, is gaining momentum.

MUC1 and mesothelin are two PDA-associated antigens that have been targeted with adoptive therapies. An early clinical trial in Japan employed adoptive transfer of peripherally-derived MUC1 targeted cytotoxic T cells for post-operative treatment of 20 patients with resectable PDA. They observed a 19.4% 3 year median overall survival, increased peripherally circulating cytotoxic T cells, and decreased peripherally circulating

Tregs ($p<0.05$) [79]. Similarly targeted CAR T cells have now been developed in the United States and were successfully employed in subcutaneous PDA mouse models [80]. Endogenous $CD8^+$ T cell titers with activity specific to mesothelin have been correlated with longer overall survival times in patients with advanced PDA [81, 82]. Engineered T cells with an affinity-enhanced TCR for mesothelin have been employed pre-clinically in KPC mice. Despite relatively transient anti-tumor activity, with bi-weekly, serial infusions, adoptive transfer of these cells in a blinded, placebo-controlled study in the KPC model led to: 1) stromal involution (including death of fibroblasts), 2) improved vessel patency, and 3) doubled overall survival time, without evidence of significant off-tumor tissue injury [23]. Results such as these have laid the foundation for multiple phase I/II clinical trials. Additional trials are currently recruiting patients to study the effect of tumor infiltrating lymphocytes in advanced cases of solid tumor-based malignancies that include pancreatic cancer [83, 84].

Active Specific Immune Therapies

"Active specific" immune therapies include vaccines and immunomodulatory agents designed to eventually lead to expansion of endogenous tumor-specific T cells. Numerous "cancer vaccines" have been employed for treatment of PDA – modest successes have been seen in early trials of whole-cell, dendritic cell and telomerase peptide vaccines (see two recent excellent reviews on this subject for more comprehensive discussion) [85, 86]. The most relevant example to the topic of this review is "whole cell" vaccine, designed to bolster the endogenous immune response by recruiting the host's antigen presenting cells (APCs) to the site of vaccination for T cell cross-priming / MHC class I presentation of a broad array of tumor antigens. For PDA treatment, these agents have had modest successes. In phase I/II clinical trials, intra-dermal vaccination of advanced-PDA patients with two irradiated, allogeneic pancreatic tumor cell lines, genetically engineered to secrete granulocyte-macrophage colony-stimulating factor to induce chemotaxis of APCs to the injection site has: 1) "Primed" T cells against a broad array of PDA antigens, including mesothelin [81, 82, 87, 88]; 2) significantly prolonged patient survival (from 4.6 to 9.7 months) when combined with low-dose cyclophosphamide and a "booster" of recombinant bacterial-vector for mesothelin expression in the cytosol of infected APC's [81]. A proposed mechanism for this latter study result from Le *et al* is induction of T cell trafficking from the periphery to central tissues by stimulatory cytokines released in response to the bacterial vector [81, 89]. A recent Phase 2b trial examining this same mesothelin "boosted" vaccine, however, revealed no significant difference in overall survival versus standard of care, and an ongoing trial is now examining its benefit in combination with a checkpoint inhibitor [90, 91]. While most human PDAs express mesothelin, and this is often used as a marker of tumor specific immune response, the degree of overexpression remains highly individualized. PDAs have relatively few coding mutations compared to more immunogenic cancers, and a recent study of genome-wide mutations from 99 informative PDA tumors revealed substantial heterogeneity [92, 93]. These factors provide significant challenge to the formulation of a single effective exogenous "vaccine" to bolster anti-tumor endogenous immune activity for PDA patients.

Non-Specific Immune Therapies and Immunomodulation

As early as the 1990's, clinical trials in Japan employed non-specific, activated peripheral lymphocytes for adoptive therapy in patients with advanced cancer, including PDA [94]. This practice has evolved with technologic advances enabling more tumor-specific adoptive therapies (as described above) but other non-specific immunomodulatory therapies, such as checkpoint inhibitors, have gained favor. As previously discussed, Th2/Th17 Treg activity represents a significant barrier to effective anti-tumor immune response and immunotherapies for PDA. Agents that promote Th1-type anti-tumor inflammatory activity (*e.g.* IFN-gamma) versus Th2-type immunosuppressive activity within the tumor microenvironment may greatly expand the therapeutic potential of PDA specific immunotherapies [16, 95–98]. By modulating the background $CD4^+$ T cell population response, "immune priming" strategies might allow for rescue of $CD8^+$ T cell anti-tumor activity, greater anti-tumor effect of passive specific therapies, and more durable immune response toward tumor eradication. For example, combining agonist CD40 monoclonal antibodies (designed to foster APC maturation and up-regulate Th1 chemokine expression) with chemotherapy and checkpoint inhibitors has improved endogenous $CD8^+$ T cell-mediated tumor rejection and long-term tumor free survival in both subcutaneous, and genetically engineered PDA murine models [67, 99].

Beyond direct cytotoxicity, both passive and active specific immune therapies can also elicit anti-tumor systemic immunomodulation. A recent meta-analysis of specific immunotherapy trials employed in treatment of pancreatic cancer revealed that circulating IFN-gamma levels were significantly higher post-treatment versus pre-treatment (4 trials with 81 patients; pooled mean difference of 3.75 IU/mL; $p=0.01$), and circulating IL-4 levels were significantly lower (2 trials with 55 patients; pooled mean difference of −1.85 IU/mL; $p<0.0001$) [100]. Anti-tumor immunomodulatory effects are most likely maximized with multi-modal non-specific and specific immune therapies. Indeed, many of the shortcomings of tumor specific immunotherapies (*e.g.* poor or heterogeneous expression of immunogenic antigens, local immunosuppressive mechanisms) may be overcome by appropriate combination with non-specfic therapies [101], or therapies that demonstrate both specific and non-specific effects, such as focused ultrasound.

Focused Ultrasound Treatment for Pancreatic Cancer

Thermal ablation—Please see Figure 2 for a schematic overview of the mechanisms that focused ultrasound (FUS) therapy employs to achieve anti-tumor effects. Thermally-ablative (coagulative-necrosis inducing) FUS has long been used clinically outside the United States as both primary and palliative therapy for pancreatic cancer. The majority of reported treatments have taken place in China since the late 1990's, and more recent case series have emerged from Korea, Japan, and European groups [103]. Typical study endpoints have included safety and feasibility confirmation, tumor volume reduction, and pain relief [104–110]. A comprehensive review of all clinical treatment series employing palliative FUS therapy for pancreatic cancer treatment is beyond the scope of this paper; but we direct the reader to an excellent recent meta-analysis by Dababou *et a*l that examined 23 studies on this topic and showed an overall pain reduction in 81% of treated patients (95% CI: 76–86) [111]. A recent, moderately powered (n=689) retrospective analysis from China revealed an

independent median overall survival benefit from ablative FUS treatment for patients with unresectable PDA who also received a variety of multi-modal treatment regimens, without severe adverse events (7.1 versus 5 months; p=0.005) [112]. A smaller retrospective series (n=38) from China found that an ablative FUS protocol with prolonged lower-power heat deposition had a greater median overall survival benefit versus a rapid heating protocol (10.3 versus 6.0 months; p=0.018) [113]. A small number of randomized clinical trials have examined the survival benefit of ablative FUS therapy. Li *et al* recently reported significant improvement in median overall survival time and progression-free survival (10.3 versus 6.6 months; n=120; p<0.001) in patients with unresectable PDA randomized to receive ablative FUS treatment and chemotherapy, versus chemotherapy alone [114]. A smaller randomized clinical trial by Lv *et al* found similar survival benefit (8.9 versus 5.5 months median overall survival; n=45; p<0.05) [115]. While these results are encouraging, multiple recent systematic reviews have found a relative paucity of randomized clinical trials evaluating the clinical survival benefit achieved with FUS ablative therapy for pancreatic cancer. Further studies are needed to improve the quality of evidence for its appropriate inclusion in multimodal treatment regimens [116–118].

In addition to the locally destructive thermal effects of FUS, recent data suggests potential immunomodulatory effects of FUS for PDA. Specifically, in a small series (n=15) of patients with advanced PDA by Wang *et al*, post-FUS-treatment blood samples showed increased percentages of circulating $CD3^+$, and CD4 T cells (in 66% of patients), a higher $CD4^+/CD8^+$ T cell ratio, and enhanced NK cell activity [119]. These findings were confirmed in a recent meta-analysis of 3022 clinical cases of FUS-thermally-ablated PDA [120]. One possible mechanism to explain these findings it that thermal ablation delivers sub-lethal heat exposure to peripheral, surviving tumor cells and can thus cause upregulation and surface expression of heat shock proteins (HSPs). HSPs are intracellular molecular chaperones that can bind tumor peptide antigens and have long been recognized as potent stimulators of tumor immunogenicity via antigen-presenting cells of the endogenous immune system (dendritic cells, macrophages, $CD4^+$ T cells) [121–127]. Although studies of HSP expression specific to pancreatic cancer cell lines have not yet been published, preliminary results have been presented, and this topic is being actively pursued [128].

The abscopal effect—Orsi *et al* recently presented preliminary results from a small clinical series where they observed diminished size in tumor metastases (the abscopal effect) following local thermally-ablative, palliative FUS treatment of patients with advanced PDA (4/46 patients) [129]. Similar effects have been observed by others [114, 130–132]. In all cases, patients received systemic chemotherapy concurrently with FUS treatments, but chemotherapy had been ineffective prior to initiation of FUS therapy. In theory, such systemic responses could be due to the above described favorable, local and systemic immunomodulatory effects observed following FUS therapy, however strong and durable immune responses have not been observed following tumor ablation as monotherapy in clinical trials.

Beyond thermal ablation—FUS treatment settings (*e.g.* pulse duration and repetition frequency, acoustic power, focal pressure, transducer frequency) can be customized to

achieve a variety of effects in target tissues. Indeed, the versatility of FUS is what sets it apart from alternative methods for targeted coagulative necrosis (*e.g.* radiofrequency, microwave, laser). Predominantly pre-clinical study of FUS treatment for pancreatic cancer has advanced beyond thermal ablation. Modern FUS transducers can precisely (at ~1mm focus), non-invasively generate targeted, sub-ablative hyperthermia, as well as mechanical and pressure-related effects (such as cavitation of gas and vapor bubbles, acoustic radiation force, and microstreaming) to achieve several desirable results in the treatment of PDA, including those summarized in Figure 2 and Table 1. Of note, not all of these results (facets of immunomodulation, mechanical tumor homogenization, exogenous antigen delivery) have been specifically studied in PDA models to date. However, assuming some degree of independence of FUS effects from tissues targeted, the general principles garnered from other tumor and disease models have direct relevance to the design of optimal FUS protocols for PDA treatment. See Figure 3 for a graphical depiction of these mechanisms in compliment to other PDA therapies.

Pulsed focused ultrasound—Pulsed FUS protocols have been used in early clinical trials [71] and pre-clinical studies [133–136] for PDA treatment to augment tumor drug delivery, and recently presented pre-clinical results show favorable immunomodulatory effects from similar treatments [128]. “Pulsed” protocols typically include a relatively short pulse duration (*e.g.* ~1ms) and low pulse repetition frequency (*e.g.* 1 Hz; duty cycle 0.1%) to minimize temperature effects while harnessing the mechanical effects of FUS. Specifically, pulses often employ spatial-average pulse-average acoustic intensities sufficient to generate peak focal pressure levels to achieve cavitation, or utilize lower intensities combined with microbubble contrast agents. Pulsed FUS treatments in combination with doxorubicin chemotherapy in the KPC model have: 1) disrupted the stromal collagen architecture of treated PDA; 2) increased intra-tumoral doxorubicin concentrations up to 4.5-fold versus intravenous chemotherapy alone [135]. An early phase I clinical trial from Norway by Dimcevski *et al* examined 10 patients with inoperable PDA who were treated with a pulsed FUS protocol at low intensity, with exogenously administered microbubbles, designed to minimize thermal deposition while facilitating stable cavitation. This treatment, in combination with gemcitabine infusion, doubled the median overall survival compared to gemcitabine treatment alone (17.6 months versus 8.9 months) [71]. Preliminary results from pre-clinical studies in PDA models, as well as studies applying pulsed or low-intensity FUS treatment protocols to non-PDA tumors and tissues support an acute post-treatment immunomodulatory effect toward Th1-type inflammation, upregulation of localized cell recruitment factors and tumor-cell-surface immunogenic proteins, and increase in local $CD8^+$/T regulatory ratio [128, 137–143]. Anti-tumor effects achieved with pulsed FUS protocols in pre-clinical studies are summarized in Table 1.

Boiling and cavitation-cloud histotripsy—“Histotripsy” is a specific type of non-thermal, pulsed FUS method designed to non-invasively, mechanically homogenize target tissue into subcellular debris without thermal denaturation of proteins through gas or vapor bubble activity – a mechanism distinct from thermally-induced coagulative necrosis [144, 145]. Targeted histotripsy treatments of tumors could thus uncover large quantities of undenatured tumor antigen *in situ*. Histotripsy can be accomplished through application of:

1) repetitive, short duration pulses of FUS with shock fronts to targeted tissues, generating transient, millimeter-sized vapor bubbles that mechanically disrupt tissues (a.k.a. “boiling” histotripsy – a misnomer of sorts, as thermal contribution to the tissue effect is negligible) [146, 147]; 2) application of extremely high magnitude peak negative pressures to induce abundant cavitation events (a.k.a. “cavitation cloud” histotripsy) [148, 149]. To our knowledge, the use of histotripsy FUS methods on pancreatic tumors has not previously been reported. However, our group has recently successfully employed this technique in feasibility tests in the KPC model (unpublished work). In addition, preliminary work from our institution has suggested Th1-type immunomodulatory effects in a rat model of renal cell carcinoma with boiling histotripsy treatment associated with release of the damage-associated molecular pattern (DAMP) HMGB1 into the plasma *in vivo* (an important factor in inciting the acute inflammatory response in the context of acute tissue trauma) and increased $CD8^+$ T cells in both the treated and contralateral kidneys [150–152]. Pre-clinical studies exploring the anti-tumor effects of histotripsy treatments are summarized in Table 1.

Endogenous T-cell therapy and focused ultrasound—“Endogenous T-cell therapy” relies on isolation and expansion of often low-frequency endogenous T cells that are reactive to tumor antigens from patient’s peripheral blood for ex-vivo expansion and adoptive transfer back into the patient. This method of adoptive cell transfer therapy is appealing given: 1) its flexible, personalized tumor-antigen result (particularly appealing for PDA patients, who are more likely to need rare, personalized peptide targets due to the relatively sparse and heterogeneous coding mutations described above); 2) minimally invasive method of cell acquisition; 3) ability to generate both effector T cells and central memory type T cells; 4) lack of regulatory hurdles and logistical barriers associated with clinical application of T cell receptor engineering [153]. FUS has already been employed pre-clinically as an *in situ* method of priming the endogenous immune system to generate more effective, tumor-specific cytotoxic T cells for ex-vivo expansion and adoptive transfer. Two recent studies employed a thermally ablative FUS method to generate endogenous tumor-antigen-primed cytotoxic T cells in a subcutaneous murine model of hepatocellular carcinoma and observed: 1) increased Th1-type inflammatory response (*e.g.* increased levels of TNF-alpha and IFN-gamma) and cytotoxicity (*e.g.* percentage of LDH release above baseline from co-culture of effector and target tumor cells, greater frequency of MHC class I tetramer/$CD8^+$ cells) from lymphocytes collected 14 days post-treatment from the spleens of ablative-FUS treated animals versus sham and control groups); 2) significantly prolonged overall survival rate at 60 days in subsequent HCC-animals infused with T cells derived from the randomly assigned FUS-treated animals versus sham or control (86% versus 33% and 16%, respectively; log-rank $p<0.0001$) [154, 155].

Predominantly mechanical FUS methods may be more effective than thermal in promoting systemic anti-tumor endogenous immune response. Xing *et al* found that melanoma metastasis rates were decreased and overall survival increased in both thermally and mechanically FUS-treated animals versus control, when allowing 2 days between treatment and amputation of the limb bearing the primary tumor (sufficient time for dendritic cell infiltration and migration), but found greater benefit in the mechanical-FUS treatment group versus the thermal-FUS group [156]. Similar results were reported by Hu *et al* in a murine

colon adenocarcinoma model – where mechanical FUS treatment resulted in significantly greater dendritic cell activation versus thermal treatments [157]. In combination with the above-described endogenous T cell activation, these results demonstrate: 1) the importance of the APC-to-cytotoxic-T-cell mechanism in FUS-induced anti-tumor endogenous immune effects; 2) that mechanical FUS methods may have greater immune benefit – either for use in generation of effective endogenous T cell subsets for subsequent *ex vivo* expansion and adoptive transfer, or for complimentary systemic endogenous immune activity to other therapies. The relatively recently developed histotripsy FUS methods may prove to be an integral component of optimized PDA FUS treatment protocols in this regard.

Concluding Remarks—Clinical cancer immunotherapy has rapidly progressed over the past decade, with several new agents approved by the FDA (including immune checkpoint therapy to promote cytotoxic T cell-mediated anti-tumor activity) and the first and most promising CAR T cells for treatment of hematologic malignancy pending FDA review. Even so, efficacy in treatment of PDA has been marginal, likely based on the elaborate immunosuppressive barriers encountered in the tumor microenvironment. Non-invasive FUS treatment methods designed to maximize non-thermal effects have shown promising early results at improving drug delivery to PDA and prolonging overall clinical survival in combination with chemotherapy. In addition, FUS treatment has the potential among similarly effective PDA stromal-directed therapies to directly and favorably modulate the endogenous immune response to the tumor. Thus, FUS methods may provide both beneficial changes to the treated PDA microenvironment and systemic endogenous immune effects that improve response to metastatic lesions and recurrent disease. Future research will help to precisely define optimal FUS protocols for augmentation of multi-modal PDA treatment regimens, including immune checkpoint inhibitors and adoptive cell transfer.

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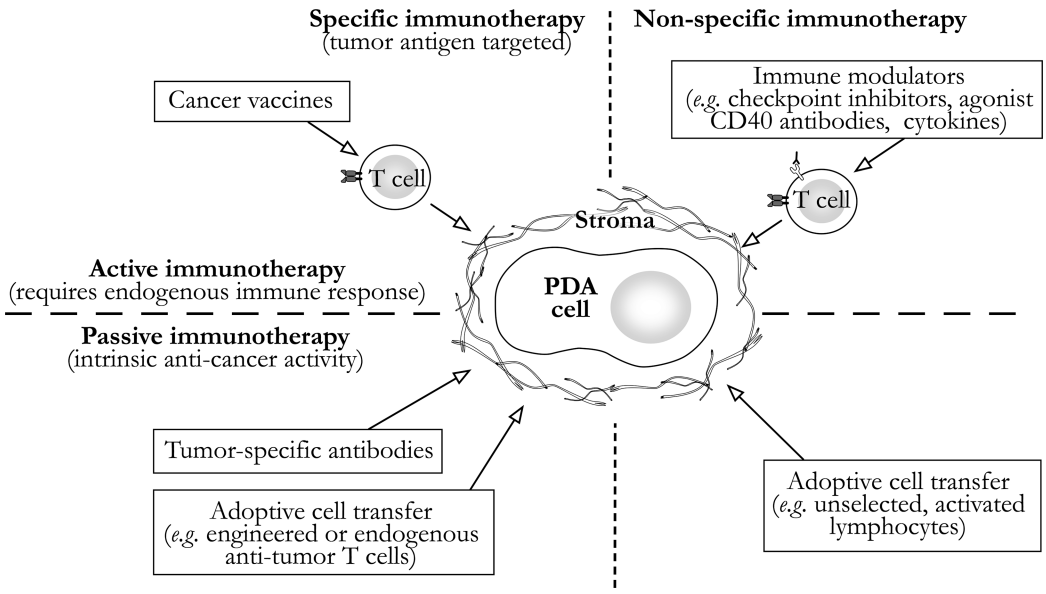

Figure 1.

Classification systemof immunotherapies for pancreatic ductal adenocarcinoma (PDA). The tumor stroma impedes these therapies withmultiple immunosuppressive mechanisms described in the text. Stromal-directed treatments, such as focused ultrasound, have shown promising results in early clinical trials and may provide substantial clinical benefit as immuno-adjuvant therapy.

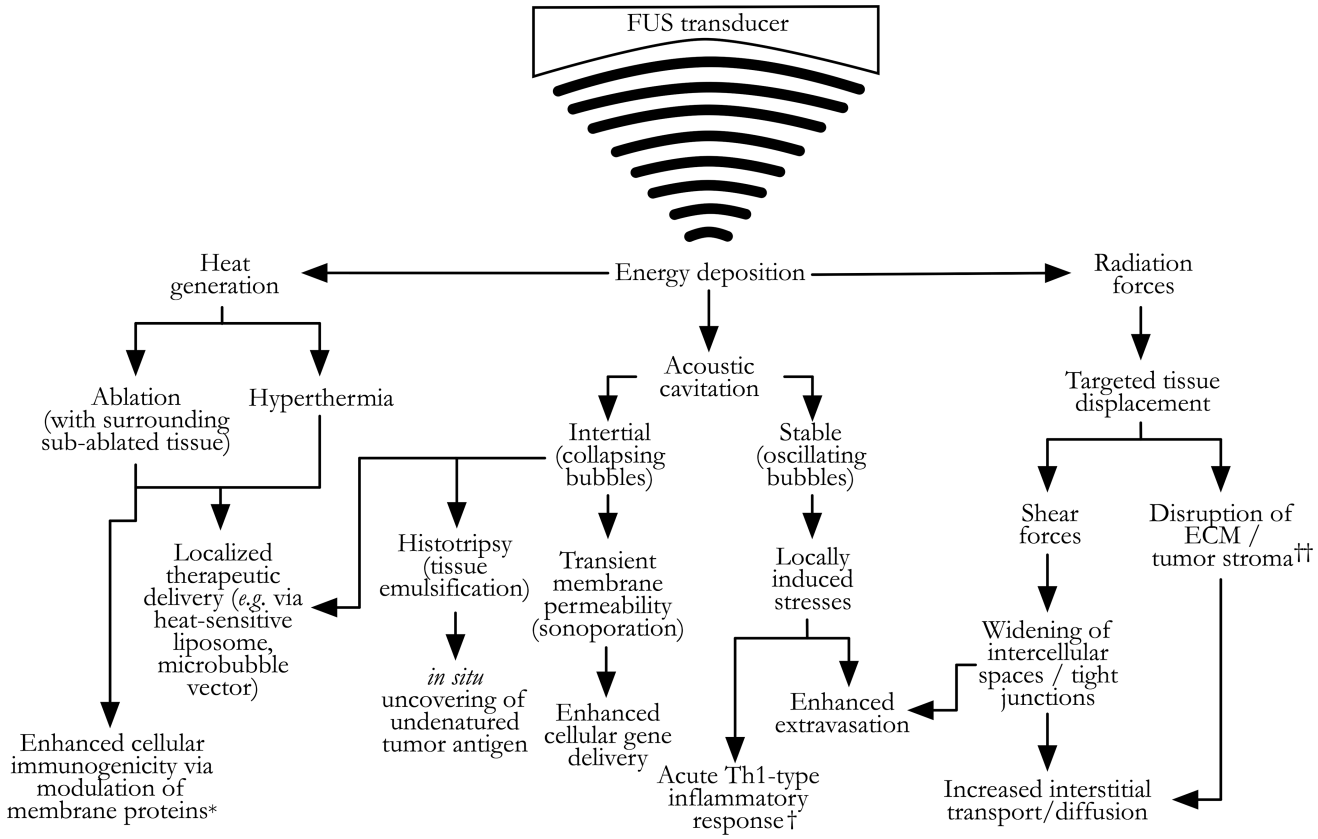

Figure 2.

Schematic depiction of selected mechanisms underlying the anti-tumor effects of non-invasive energy deposition via focused ultrasound (FUS). These mechanisms are not mutually exclusive, and indeed, co-exist on a continuum; however, FUS treatment protocols can be adjusted to maximize some mechanisms and minimize others. Most commonly, this involves minimizing heat generation relative to cavitation and radiation forces. This figure is adapted with significant revision froma review by Victor Frenkel, [102], where the reader will find a more comprehensive discussion of these mechanisms as they relate to localized delivery of therapeutics to solid tumors, beyond the immuno-adjuvant effects reviewed here. *Immunogenic cellular membrane protein modulation has also been observed with lowintensity/minimal-thermal protocols. †Acute T-helper (Th)1-type inflammatory responses have also been observed with thermal-emphasis protocols. ‡Disruption of the extracellularmatrix (ECM) / tumor stroma is likely the sequela of both radiative forces and acoustic cavitation activity. See text and Table 1 for details.

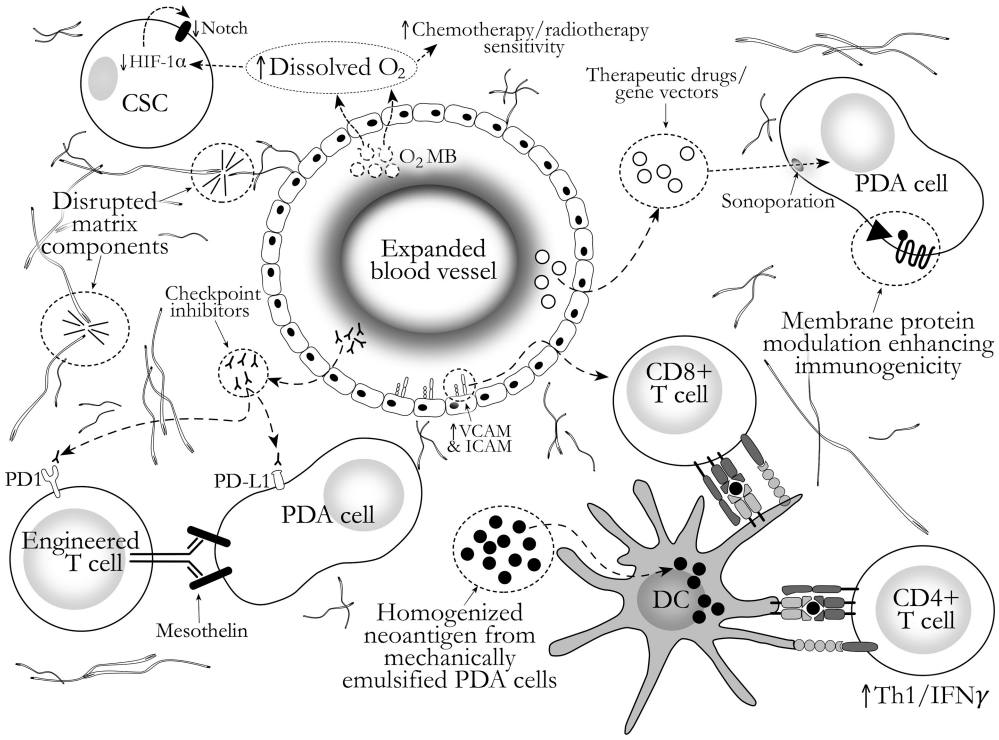

Figure 3.

Conceptual diagram of focused ultrasound effects for augmentation of pancreatic ductal adenocarcinoma (PDA) treatment. See text for details. CSC = Cancer stem cell; DC = dendritic cell; HIF = hypoxia-inducible factor; O2 MB = Oxygen-gas-loaded microbubble.

Table 1 –

Summary of selected pre-clinical focused ultrasound effects relevant to immuno-adjuvant PDA treatment

Focused Ultrasound Effect	FUS Protocol(s)	Tissue(s) Targeted	References
• Increase penetration and diffusion of drugs into tumor	Pulsed	PDA	[134–136]
• Disrupt tumor stroma	Pulsed	PDA	[135]
• Reduce intra-tumoral hypoxic stress via augmented oxygen delivery (*e.g.* oxygen-gas-loaded microbubbles)	Pulsed	PDA	[54, 55]
• Modulate tumor cell membrane protein localization to enhance immunogenicity (*e.g.* immediate downregulation of CD47, a suppressor of phagocytic activity; upregulation of HSP70, calreticulin, CD40, CD80 and CD86)	Thermal (CD47), low-intensity	PDA (CD47), lung / breast / prostate cancer	[128, 140]
•Modulate local and systemic cytokine milieu toward a Th1-type inflammatory profile (*e.g.* increase in IFN-gamma, TNF-alpha)	Pulsed, thermal, low-intensity	RCC, prostate cancer, dystrophic muscle, HCC	[137–139, 154, 155]
• Up-regulate local chemoattractants (cytokines, chemokines, trophic factors, VCAM and ICAM)	Pulsed[†], low -intensity	Normal, ischemic and dystrophic muscle	[137, 141–143]
• Mechanically homogenate / liquefy targeted tissue into cellular debris – releasing large quantities of undenatured tumor antigen *in situ*	Histotripsy, pulsed	Muscle, liver, prostate cancer, colon adenocarcinoma, melanoma	[144–148, 150, 158–160]
• Increase local dendritic cell maturation	Pulsed, thermal	HCC, prostate cancer, colon cancer	[157, 161, 162]
• Increase intra-tumoral $CD8^+$/ T regulatory cell ratio and $CD8^+$ T cell activity	Histotripsy, pulsed, thermal	RCC, prostate cancer, colon cancer, HCC	[139, 152, 154, 155, 161]
• Facilitate delivery of exogenously packaged immune-stimulating antigen or genes (*e.g.* via microbubbles, liposomes, sonoporation)	Pulsed, low-intensity	Colon cancer, HCC, prostate cancer, ovarian cancer, melanoma, lymphoma	[163–170]

[*] FUS = focused ultrasound; PDA = pancreatic ductal adenocarcinoma; RCC = Renal cell carcinoma; HCC = hepatocellular carcinoma; VCAM = vascular cell adhesion protein; ICAM = intercellular adhesion molecule.

[†] Aicher et al. [142] applied targeted ultrasound to muscle via a shockwave lithotripter device using a low duty cycle, similar in some regard to pulsed protocols used with FUS transducers.