Cancer Therapy: Preclinical

Inhibition of CD47 Effectively Targets Pancreatic Cancer Stem Cells via Dual Mechanisms

Michele Cioffi, Sara Trabulo, Manuel Hidalgo, Eithne Costello, William Greenhalf, Mert Erkan, Joerg Kleepf, Bruno Sainz Jr, and Christopher Heeschen

Abstract

Purpose: Pancreatic ductal adenocarcinoma (PDAC) is a cancer of the exocrine pancreas with unmet medical need and is strongly promoted by tumor-associated macrophages (TAM). The presence of TAMs is associated with poor clinical outcome, and their overall role, therefore, appears to be protumorigenic. The "don't eat me" signal CD47 on cancer cells communicates to the signal regulatory protein-α on macrophages and prevents their phagocytosis. Thus, inhibition of CD47 may offer a new opportunity to turn TAMs against nonmalignant cells in the pancreas. Targeting CD47 efficiently enhanced phagocytosis of a representative set of primary human pancreatic cancer (stem) cells and, even more intriguingly, also directly induced their apoptosis in the absence of macrophages during long-term inhibition of CD47. In patient-derived xenograft models, CD47 targeting alone did not result in relevant slowing of tumor growth, but the addition of gemcitabine or Abraxane resulted in sustained tumor regression and prevention of disease relapse long after discontinuation of treatment.

Experimental Design: We studied in vitro and in vivo the effects of CD47 inhibition on CSCs using a large set of primary pancreatic cancer (stem) cells as well as xenografts of primary human PDAC tissue.

Results: CD47 was highly expressed on CSCs, but not on other nonmalignant cells in the pancreas. Targeting CD47 efficiently enhanced phagocytosis of a representative set of primary human pancreatic cancer (stem) cells and, even more intriguingly, also directly induced their apoptosis in the absence of macrophages during long-term inhibition of CD47. In patient-derived xenograft models, CD47 targeting alone did not result in relevant slowing of tumor growth, but the addition of gemcitabine or Abraxane resulted in sustained tumor regression and prevention of disease relapse long after discontinuation of treatment.

Conclusions: These data are consistent with efficient in vivo targeting of CSCs, and strongly suggest that CD47 inhibition could be a novel adjuvant treatment strategy for PDAC independent of underlying and highly variable driver mutations. Clin Cancer Res; 1–13. ©2015 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most devastating cancers with a 5-year survival rate of less than 5% (1). Despite expanding research activities, there has been little therapeutic progress toward improving patients’ long-term survival. Gemcitabine (2), FOLFIRINOX (3), and more recently the addition of nab-paclitaxel (Abraxane; ref. 4) are able to moderately extend median survival, but eventually the vast majority of patients still succumb from progressive disease. Therefore, developing new and more effective anti-PDAC treatments represent an urgent and unmet medical need (5, 6). Because the cancer stem cell (CSC) hypothesis was functionally validated for leukemia in 1994 (7), convincing evidence has emerged for several solid tumors indicating that like adult tissues, tumors are sustained and promoted by cells that exhibit features of stem cells, including unlimited self-renewal (8). We and others have provided conclusive evidence for a hierarchical organization in human PDAC and, even more importantly, demonstrated that pancreatic CSCs, at the apex of the hierarchy, have exclusive tumorigenic and metastatic potential and are inherently resistant to chemotherapy (9–11). Indeed, the survival of such resistant CSCs during chemotherapy, despite initial tumor regression, represents a plausible explanation for the later fatal relapse of disease in most patients (3, 4).

Studies based on the inhibition of regulatory pathways that are crucially relevant for the self-renewal capacity of CSCs are promising (12, 13); however, the overly heterogeneous genetic background of PDAC may render larger populations of cells resistant to the targeting of single pathways. Consequently, we asked whether targeting pancreatic CSCs with broader immune-based therapeutic approaches could represent a more viable and potent alternative for eliminating these highly tumorigenic and chemoresistant cells. Macrophages play crucial roles in adaptive and innate immunity. In PDAC, tumor-associated macrophages (TAM) represent the major immune cell type present in the PDAC tumor microenvironment (14), and these cells are believed to drive cancer progression, presumably via promoting cancer cell proliferation, tumor angiogenesis, extracellular matrix breakdown, and subsequently tumor invasion and metastasis (15, 16). In addition, CD47, a transmembrane protein expressed on many cancer cells, serves as a ligand to signal regulatory protein-α (SIRPα), a molecule expressed on macrophages (17), resulting in the inhibition of phagocytosis by macrophages through a signaling

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most devastating cancers, and very few new treatments have revealed meaningful improvements in patient survival over the past decades. On the basis of our previous work demonstrating the existence of cancer stem cells (CSC) in pancreatic cancer and their strong resistance to standard chemotherapy, we now provide multiple lines of functional and mechanistic evidence for a treatment regimen, including inhibition of CD47 targeting both CSCs as well as their more differentiated progenies. Therefore, this new therapeutic strategy should be further explored in the clinical setting as its success bears the potential to improve the poor prognosis of patients with PDAC.

Materials and Methods

Primary human and mouse pancreatic cancer cells and macrophages

Human PDAC tissues were obtained with written informed consent from all patients and expanded in vitro as patient-derived xenografts (PDX), as previously described (12). For in vitro studies, PDX tissue fragments were minced, enzymatically digested with collagenase (STEMCELL Technologies) for 90 minutes at 37°C (12) and after centrifugation for 5 minutes at 1,200 rpm cell pellets were resuspended and cultured in RPMI supplemented with 10% FBS and 50 U/mL penicillin-streptomycin. For serialpassaging, 7-day-old cultures were kept in vitro by protein G.

Previous work in preclinical models of bladder cancer, leukemia, and lymphoma demonstrated that inhibiting the interaction between CD47 and SIRPα using anti-CD47 mAbs allows for increased phagocytosis of cancer cells in vitro and decreased tumor burden in vivo (19–21). Recently, CD47 was shown to be preferentially expressed in liver CSCs and inhibition of CD47 suppressed growth of hepatocellular carcinoma xenografts and had a chemosensitizing effect (22), suggesting that CD47 may also be a promising therapeutic target for hepatocellular CSCs. Here, we now demonstrate that CD47 is also highly expressed on pancreatic cancer (stem) cells and that anti-CD47 mAbs did not only enable phagocytosis of these cells by macrophages, but also directly induced apoptosis of the cancer (stem) cells, while exerting no effect on nonmalignant cells. Although CD47 targeting as a single treatment strategy was not effective in vivo, the combination with chemotherapy resulted in long-lasting tumor regression. Thus, our results demonstrate that targeting CD47 on PDAC cells changes the behavior of resident TAMs to inhibit, rather than promote tumor growth, and thus may evolve as a potent addition to our still sparse armamentarium against PDAC.

Flow cytometry

Cells were adjusted to a concentration of 10⁶ cells/mL in sorting buffer [1X PBS; 3% FBS (v/v); 3 mmol/L EDTA (v/v)] before analysis or sorting with a FACS Canto II or FACS Influx instrument, respectively (BD Biosciences). To identify distinct cancer (stem) cells, the following antibodies were used: anti–CD133/1-APC or PE (Miltenyi Biotec); CD47-APC, CXCR4-APC, SSEA-1-APC, or appropriate isotype-matched control antibodies (all from BD Biosciences). DAPI was used for exclusion of dead cells. Data were analyzed with FlowJo 9.2 software (Tree Star). For the assessment of apoptosis, cells were incubated with the DAPI and Annexin V FITC Staining Kit (BD Biosciences) according to the manufacturer’s instructions.
Figure 1.
CD47 is expressed in pancreatic cancer (stem) cells. A, quantification of CD47 expression in primary patient TMA–containing cores of normal pancreas, pancreatitis, PDAC, and metastases. Shown are the mean relative intensity values of CD47 staining within each core. (Continued on the following page.)
**In vitro phagocytosis assay**

For *in vitro* phagocytosis analysis, $5 \times 10^4$ monocyte-derived macrophages were plated in each well of 24-well tissue-culture plates and labeled with PKH26 according to the manufacturer’s instructions (Sigma). Macrophages were incubated in serum-free medium for 2 hours before adding $2 \times 10^5$ GFP-labeled live cancer cells. Anti-CD47 (B6H12) antibody (10 µg/ml) or IgG1 control antibody was added and incubated for 2 hours at 37°C. Macrophages were repeatedly washed and subsequently imaged using an inverted microscope (Leica DMi6000B). The phagocytic index was calculated as the number of phagocytosed GFP$^+$ cells per 100 macrophages.

**Immunohistochemistry**

For histopathologic analysis, FFPE blocks were serially sectioned (3-µm thick) and stained with hematoxylin and eosin (H&E). Additional serial sections were used for immunohistochemical (IHC) studies with anti-CD47 antibody (0.2 µg/ml; Abcam ab3283). Antigens were visualized using 3,3-diaminobenzidine tetrahydrochloride plus DAB®. Counterstaining was performed with hematoxylin. Histologic quantification of digitalized slides was performed using Pannoramic Viewer (3DHistech).

**Tissue microarrays**

Four human tissue microarrays (TMA) containing quadruplicate 1-mm cores from selected areas of paraffin-embedded pancreatic surgical specimens, including ducts, acini, pancreatitis, PDAC, and PDAC metastasis were constructed. A total of 42 tumors were included. Two xenograft TMAs containing quadruplicate 1-mm cores from selected tumor areas of 56 paraffin-embedded human PDACs grafted in nude mice were also constructed. The use of human tissue samples for the construction of the TMAs was approved by the Ethics Committee of the Hospital de Madrid Norte Sanchinarro. All sections were assessed and scored by an in-house pathologist (Maria Lozano).

**In vivo tumorigenicity assay**

Primary pancreatic cells were treated *in vitro* with anti-CD47 and serial dilutions of single-cells were resuspended in Matrigel (BD Biosciences) and s.c. injected into female 6- to 8-week-old NU-Fawn1™ nude mice (Harlan Laboratories). In some experiments, macrophages were depleted with the following treatment schedule: 200 µl of clodronate was injected i.v. twice a week. Tumor formation was evaluated after 2 months. Mice were housed according to institutional guidelines and all experiments were approved by the local Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (PA 34-2012) and performed in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals as stated in The International Guiding Principles for Biomedical Research involving Animals, developed by the Council for International Organizations of Medical Sciences.

**In vivo treatment**

Primary tumor tissue pieces of approximately 2 mm$^3$ were implanted s.c. into the flanks of NU-Fawn1™ nude mice, and once tumors were established mice were randomized to the respective treatment groups. Gemcitabine was administered twice a week (125 mg/kg i.p.), Abraxane was administered every 4 days (50 mg/kg i.v.), and anti-CD47 was administered daily (500 µg/mouse i.p.; ref. 20).

**Statistical analyses**

Results for continuous variables are presented as means ± SD unless stated otherwise and significance was determined using the Mann–Whitney test. All analyses were performed using SPSS 22.0 (SPSS).
AB

CD47– CD47+

Number of spheres

** P < 0.01

P < 0.05

**

P < 0.0001

Tumorigenicity

(4 tumors / 4 injections)

<table>
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Tumorigenicity

(4 tumors / 4 injections)

<table>
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<th>CSC Freq</th>
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Pancreatic CSCs are mostly confined to CD47+ cells

Because our data suggested that CD47 is preferentially expressed in pancreatic CSC (i.e., CD133+ cells), we aimed to assess whether CD47+ cells were more "stem-like." We first FACSorted primary pancreatic cancer cells for CD47 (Fig. 2A) and then determined their self-renewal capacity using sphere formation as a readout. We observed that CD47+ cells isolated from 185, 215, and 354 primary cells formed significantly more and larger spheres compared with CD47− cells (Fig. 2B), suggesting that CD47+ cells are indeed enriched in CSCs. However, to obtain conclusive evidence for the latter, we performed in vivo limiting dilution tumorigenicity assays. Ten weeks after injection, CD47+ cells had formed more tumors, indicating that CSCs are mostly contained in the CD47+ cell population (Fig. 2C). To further evaluate the function of CD47 in the CSCs context, we sorted four populations based on CD133 and CD47 expressions and observed that CD47+/CD133+ cells possessed the highest sphere formation capacity (Fig. 2D). Building upon the latter, we sorted cells for both CD133+ and CD47+ and injected them into nude mice depleted for macrophage by means of treatment with clodronate. Macrophage depletion was performed to more definitively demonstrate that the tumorigenic capacities observed in vivo were indeed due to differences in functional CSC content and not related to the inherent resistance of a cell to macrophage phagocytosis based on cell surface CD47 expression. Not only did we confirm that in vivo tumorigenicity is indeed mostly confined to the CD47+ population, as previously seen (Fig. 2C), but we also show that CD47+CD133+ cells are more highly enriched for CSCs, and thus more tumorigenic, whereas CD47−CD133− cells bear the least tumorigenic potential (Fig. 2E). Taken together, these data suggest that targeting CD47 should achieve a major reduction in CSC activity.

Anti-CD47 treatment enables phagocytosis of pancreatic CSCs

It has been previously demonstrated that blocking CD47-mediated SIRPa signaling using targeted mAbs induces phagocytosis of leukemia, lymphoma, and bladder cancer cells by human and mouse macrophages (19, 21, 28). Using primary PDAC cells stably infected with a lentivirus-expressing GFP (green) and PKH26 dye (red)–labeled primary human monocyte-derived macrophages isolated from healthy donors (ratio cancer cells:macrophages:4:1), we show in contrast with cells treated with an isotype-matched mouse IgG control antibody, primary PDAC cells treated with the blocking anti-human CD47 (hCD47) mAb B6H12.2 were efficiently phagocytosed by macrophages. This effect was observed for adherent cells, which mainly contain non-CSC, and sphere-derived cells or CD47+CD133+ sorted cells, which are enriched for CSCs (Fig. 3A and B) and was independent of the method of macrophage polarization (Supplementary Fig. S2A).

We next attempted to mimic the tumor in vivo microenvironment conditions by polarizing macrophage cultures toward an "M1" phenotype with GM-CSF and an "M2" phenotype with M-CSF, respectively (26), or by exposing them to CSC-conditioned media from primary cultures of PDAC spheres (29). We first confirmed that GM-CSF–treated macrophages possessed a classic M1 circular morphology, whereas M-CSF–treated and CSC-conditioned macrophages both showed a more elongated shape typical of M2-polarized macrophages (Fig. 3C, top). In the absence of anti-CD47-blocking antibodies, CSC-conditioned M2+ macrophages had the lowest phagocytic index levels compared with the other macrophage subtypes (0.9 vs. 2.8 for M2-polarized macrophages), consistent with a truly protective and protumorigenic role for these macrophages. Treatment with the blocking mAb B6H12.2, however, significantly enhanced phagocytosis of cancer cells across all macrophage subtypes, with a more pronounced increase for M2 (6.7-fold increase) and CSC-conditioned (13-fold increase) macrophages (Fig. 3C, bottom). Importantly, primary monocyte–derived murine macrophages, regardless of their initial polarization, were also capable of phagocytosing human PDAC cells when CD47 was blocked (Fig. 3D).

Importantly, we evaluated the percentage of CD133+ CSCs following anti-CD47 treatment and observed a significant reduction compared with isotype-treated cells (Fig. 3E). Moreover, we observed a consistent and significant reduction in the sphere formation capacity of surviving/nonphagocytosed cells, indicating that anti-hCD47 treatment indeed eliminated the CSCs pool (Fig. 3F). Using a more stringent ratio of macrophage:caner cells (1:1), we observed similar effects in terms of phagocytosis as well as significant reduction in sphere formation and CD133+ CSC content (Supplementary Fig. S2B–S2D). In contrast, for nontransformed cells no significant induction of phagocytosis was found (Supplementary Fig. S2B, right), which might be attributed to the lack of "eat-me" signals on these cells. Finally, to validate the specificity of anti–CD47-induced phagocytosis, we tested a different antibody that also binds a large fraction of PDAC cells (i.e., anti-CD44). However, treatment with anti-CD44 did not induce phagocytosis whereas in the same experiments treatment with anti-CD47 showed strong induction of phagocytosis (Supplementary Fig. S2F). These data demonstrate that induction of phagocytosis by anti-CD47 is likely independent of FcR stimulation of macrophages. It is worth noting, however, that blocking CD47 using a Fab molecule would be necessary to definitively demonstrate that Fc receptor is not required. Nonetheless, the data suggest that CD47 is a legitimate therapeutic target for PDAC.

Anti-CD47 treatment induces apoptosis of pancreatic CSCs

Antibodies directed against CD47 have also been shown to directly induce apoptosis of several hematopoietic malignancies (30–32). We therefore incubated sphere-derived cells with anti-hCD47 mAb B6H12.2, but in the absence of macrophages, and

Figure 2.
Pancreatic CSCs are mostly confined to CD47+ cells. A, representative flow-cytometry plots of CD47 staining showing the gating strategy for sorting. B, representative images of spheres (left) and quantification of spheres (right) in 185, 354, and 215 primary pancreatic cancer cells sorted for CD47. C, In vivo tumorigenicity of FACSorted 185, 354, and 215 primary pancreatic tumor cells for CD47. D, sphere formation capacity for cells FACSorted for CD47 and CD133. E, In vivo tumorigenicity of cells FACSorted for CD47 and CD133 injected in mice depleted for macrophages by treatment with clodronate (twice a week).
Figure 3.
Anti-CD47 enables phagocytosis of pancreatic CSCs. A, representative confocal images (top) and phagocytic index (bottom) of human peripheral blood (PB)-derived macrophages (red) phagocytosing patient-derived CSCs (green) in the presence of blocking anti-CD47 mAb (B6H12) or IgG1 isotype control Ab. (Continued on the following page.)
subsequently assessed apoptosis 2 and 12 hours after treatment by Annexin V staining. Although we observed no induction of apoptosis in nontransformed human cells (Fig. 4A) or in primary murine PDAC tumor cells (Supplementary Fig. S3A), we did detect a significant increase in apoptotic cells across several primary human PDAC cell lines following treatment with the anti-CD47 antibody compared with IgG mAb-treated controls (Fig. 4B). Importantly, no apoptosis was observed in any of the samples tested following 2 hours of treatment (Supplementary Fig. S3B). Thus, because phagocytosis of CSCs by macrophages was detected as early as 15 minutes after incubation with the anti-CD47 antibody (data not shown), we identify two distinct mechanisms of action, the first being phagocytosis whereas the second being an apparent PDAC-specific elimination of CSCs via direct induction of apoptosis without involvement of macrophages.

**Anti-CD47 treatment inhibits in vivo tumorigenicity and tumor progression, preventing relapse**

To test the efficiency of CSCs elimination in vitro, we tested the ability of surviving/nonphagocytosed cells after anti-CD47 treatment to form tumors in vivo. We observed a significant reduction in the tumorigenicity of anti-CD47-treated cells compared with isotype-treated cells (Fig. 5A), and the few tumors that formed from the anti-CD47-treated cultures were significantly smaller in size compared with isotype control mAb.

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(Continued.) B, the phagocytic index of macrophages phagocytosing human PDAC cells FACSorted for CD47 and CD133 in the presence of blocking anti-CD47 mAb or IgG1 isotype control Ab. C, phagocytic index of human unpolarized, M1, M2, and CSC media polarized macrophages. D, the phagocytic index of murine M1- and M2-polarized macrophages in the presence of blocking anti-CD47 mAb or IgG1 isotype control Ab. E, flow-cytometry analysis of CD133 cell surface expression on surviving cells following incubation with primary human macrophages and treatment with anti-CD47 mAb (B6H12) or IgG1 isotype control mAb. F, sphere formation quantification of cells after treatment with anti-CD47 mAbs, compared with IgG1 control treated cells.
CD47 Targets Pancreatic Cancer Stem Cells

A

Tumorigenicity (tumor take rate in %)

B

Tumor weight (g)

C

Surface expression (% of IgG1-Iso) CD133

D

Experimental setup:

Day 0 14 28 35 56 70 100

Implantation of xenografts

Abraxane
Gemcitabine
Anti-CD47
Assessment of CSC content

Tumor volume (mm³)

* P < 0.05 vs. single treatment
** P < 0.01 vs. control

E

Control | GEM | Anti-CD47

HE

CK 19

GEM + Anti-CD47 | ABX | ABX + Anti-CD47

HE

CK 19

F

CD133 surface expression (%) * P < 0.05

Control

GEM

Anti-CD47

GEM + Anti-CD47

ABX

ABX + Anti-CD47

CD133
tumors (Fig. 5B). In addition, although a similar amount of EPCAM expression was observed across all samples regardless of the treatment, anti-CD47-treated tumors contained a significantly lower percentage of cells expressing the CSC surface markers CD133 and SSEA1 (Fig. 5C). Again, when we used a more stringent ratio of macrophage:cancer cells (1:1), we observed that tumorigenicity following injection of surviving cells was essentially abrogated (Supplementary Fig. S2E).

Encouraged by these promising in vivo tumorigenicity data, we next performed in vivo therapeutic intervention studies with human-derived PDAC xenografts expressing intermediate levels of CD47. Once tumors had formed (~100 mm³), mice were randomized to one of the following six treatment groups: Diluent control; gemcitabine (biweekly 125 mg/kg i.p.) from day 14 to 56; Abraxane (every 4 days 50 mg/kg i.v.) from day 14 to 28; anti-CD47 (daily 500 μg/mouse i.p.) from day 14 to 35; gemcitabine + anti-CD47; and Abraxane + anti-CD47. Interestingly, for both used PDX models no significant differences were observed for chemotherapy and anti-CD47 single treatments; however, tumors treated with a combination of chemotherapy + anti-CD47 were significantly reduced compared with control tumors and single treatment tumors. Specifically, for PDAC-185, treatment with Abraxane plus anti-CD47 significantly stalled tumor growth. Although mice previously treated with Abraxane alone showed similar initial response, tumors eventually relapsed. No relapse (i.e., de novo growth of tumors) however, was observed when mice were treated with both Abraxane and anti-CD47 (Fig. 5D).

PDAC-185 tumors in the gemcitabine + anti-CD47 treatment group had to be harvested early due to ulcerations. Of note, tumors did not differ in gross morphology, as assessed by H&E and CK19 immunohistochemistry (Fig. 5E), and were also similarly vascularized and contained M2 macrophages (Supplementary Fig. S4A). Importantly, flow-cytometry analysis of digested tumors showed a significant decrease in the percentage of cells expressing the CSC marker CD133 only in mice that had received anti-CD47 treatment (Fig. 5F), suggesting that anti-CD47 treatment effectively targeted the CSC population. In the PDAC-215 PDX model, similar treatment benefits were observed when anti-CD47 was combined with a chemotherapeutic, although in this PDX model gemcitabine was more effective than Abraxane when used in combination with anti-CD47 as evidenced by the lack of tumor relapse during long-term follow-up (Supplementary Fig. S4B and S4C).

Discussion

Macrophages can undergo specific differentiation/polarization depending on the environment and surrounding cellular context. Two distinct states of macrophage polarization have been defined (33): (i) the classically activated (M1) macrophage that plays an important proinflammatory effector role in Th1 cellular immune responses, including the secretion of cytokines and phagocytosis of target cells or (ii) the alternatively activated (M2) macrophage that is involved in type II helper T-cell processes, such as wound healing and humoral immunity. In cancer, protumorigenic M2 TAMs enhance neoplasia via matrix remodeling, angiogenesis and the secretion of protumor growth factors, such as TGFβ (34). In contrast, M1 macrophages are believed to inhibit tumor growth via antitumor-adaptive immunity mechanisms that include phagocytosis. The latter, however, mainly depends on macrophage recognition of phagocytic (“eat me”) signals on target cells, but can be inhibited by simultaneous expression of anti-phagocytic (“don’t eat me”) signals, such as CD47. In the context of cancer, CD47 has been found to be strongly overexpressed on different tumor cells, conferring an anti-phagocytic benefit to these cells (35, 36). Importantly, inhibition of CD47 using mAbs efficiently induces phagocytosis of cancer cells by macrophages in experimental models of leukemia, lymphoma, and bladder carcinomas (19–21); however, the relevance of this molecule and its therapeutic targeting in PDAC (stem) cells had yet to be studied.

Herein, we show that CD47 is overexpressed in the majority, but not in all primary PDAC patient samples tested using multiple tissue microarrays (>150 patients represented). Although CD47 was significantly overexpressed in about two thirds of neoplastic tissues, we did not observe a correlation between high CD47 protein expression and poor clinical outcome (Supplementary Fig. S1C), which is in contrast with what has been shown for other cancers including, AML, HCC, glioma, and ovarian cancer (19–21, 36). The analysis of tissue microarrays may not be sufficient to capture the general CD47 expression profile for each individual tumor. Indeed, we observed significant variation between different cores that were available from the same patients (Supplementary Fig. S1A and S1B). Thus, analysis of complete sections of primary patient PDAC samples is likely needed before a correlative connection can be definitively determined for PDAC. Notably, although CD47 was not “clinically predictive” in our tissue microarrays, we did note that CD47 expression increased in sphere-derived CSC-enriched cultures, was expressed at even higher levels in CD133⁺ cells, and CD47⁺ PDAC cells exhibited higher self-renewal and tumorigenic properties compared with CD47⁻ cells. Thus, like leukemia, bladder cancer, and HCC (19–21), CD47 expression is strongly expressed on pancreatic CSCs; however, it is likely not a suitable surrogate CSC marker as its strong expression on a large fraction of non-CSCs limits the level of enrichment for CSCs in CD47⁺ cells, and thus would require the use of other CSC markers (e.g., CD133) in combination.

Using a neutralizing antibody for CD47, we found that inhibiting the anti-phagocytic function of CD47 allowed for both human and mouse macrophages to phagocyte CSCCs cells in vitro, and the non-phagocytosed surviving PDAC cells exhibited significantly reduced expression of CSC markers and functional phenotypes, such as self-renewal and in vivo tumorigenicity. Importantly, this phenotype was independent of the polarization state of the macrophage, as M1 and M2 macrophages were equally capable of phagocytosing CD47⁺ CSCCs cells.
of phagocytosing PDAC cells treated with anti-CD47 mAbs compared with unpolarized macrophages. In the context of the tumor microenvironment, M1 macrophages infiltrate the tumor during immune surveillance, but once recruited into tumor sites, M1 macrophages can differentiate into M2 macrophages upon exposure to cytokines released by tumor cells and tumor stromal cells (e.g., TGFβ, IL4, IL13, and IL10; ref. 33). Therefore, our finding that MCSC-polarized M2 macrophages as well as CSC-conditioned M2-like macrophages were able to phagocytose PDAC cells treated with anti-CD47 mAbs highlights the potential of M2 macrophages, the predominant macrophage subtype present with the PDAC tumor microenvironment, as biologic tools to target CSCs and their more differentiated non-CSCs progenies.

From a therapeutic perspective, we additionally show in two xenotransplantation models of PDAC that treatment with mAbs for CD47 in combination with gemcitabine or Abraxane significantly reduced primary tumor growth. Specifically, we found that anti-CD47 therapy alone only marginally reduced the size and rate of tumor growth, which again contrasts with previous findings for other epithelial cancers (19–21), and may be attributed to the more aggressive growth nature of PDAC, which cannot be completely controlled by phagocytophagic macrophages. Alternatively, untreated tumors with their dense stroma may represent too strong a barrier for the CD47 antibodies to reach the cancer cells. Importantly, however, in mice treated with gemcitabine or Abraxane, the addition of anti-CD47 therapy resulted in efficient growth control of tumors and prevented relapse after discontinuation of treatment. The later was particularly apparent when Abraxane was used in combination with anti-CD47. Specifically, tumors in mice treated with both Abraxane and anti-CD47 mAbs diminished in size, such that one tumor was completely eliminated, and the remaining tumors failed to relapse as compared with mice treated with Abraxane alone where relapse was evident in all mice by day 77. Regarding the tumor CSC content, we observed that only anti-CD47 therapy was able to reduce the percentage of CD133+ cells in the tumor, which confirms our in vitro results and indicates that anti-CD47 mAbs preferentially target CSCs. Taken together, these results strongly suggest that anti-CD47 therapy could be an effective means of treating primary PDAC tumors, but combination with other anticancer therapeutic agents, such as Abraxane, is needed.

Although the main acute mechanism of action of anti-CD47 therapy relies on macrophage-mediated phagocytosis of CSCs, we did observe that long-term treatment of PDAC cells with anti-CD47 mAbs induced a prominent and cancer cell–specific induction of apoptosis. It has previously been shown that ligation of CD47 triggers caspase-independent programmed cell death in normal and leukemic cells (31, 32, 37); thus, in addition to blocking the antiphagocytic CD47 ligand on PDAC cells, anti-CD47 mAbs may also function to eliminate CSCs via a separate apoptotic-specific mechanism of action. Additional studies are still needed to resolve whether anti-CD47 therapy induces apoptosis of tumor cells in vivo. In addition, it is important to note that during cell death, phagocytic (”eat me”) signals such as calreticulin are shuttled to the cell membrane (38). Thus, it is also logical to hypothesize that anti-CD47 mAb-induced apoptosis may also facilitate macrophage-mediated apoptosis via upregulation of the phagocytic (”eat me”) signal calreticulin. Therefore, anti-CD47 therapy may have multiple mechanisms of action, each of which likely facilitates macrophage phagocytosis of CSCs.

Is CD47 targeting in PDAC suitable and ready for further clinical exploration? Although our in vitro study provides proof-of-concept for CD47 targeting in PDAC, indeed several open questions remain to be addressed in further preclinical studies, but could not be tackled in the present studies based on the currently prohibitive costs of low scale antibody production. Once high-affinity clinical grade antibodies or high-affinity SIgRPs monomers are available in larger amounts (39), it should be determined whether the abundant stroma in PDAC tumors represents a relevant physical barrier for the antibodies to reach the cancer cells. Therefore, it should be tested whether coadministration of a stroma targeting agents leads to better response rates for CD47 antibody treatment. However, a cautionary note comes from recent studies demonstrating that stroma targeting alone could result in adverse outcomes. Specifically, mouse studies demonstrated that loss of stroma leads to dedifferentiation of cancer cells rendering them more aggressive (40, 41). In addition, a recent clinical trial on hedgehog pathway inhibition was prematurely stopped on the basis of excessive death rates in the treatment group. Whether enhanced delivery of CD47 antibodies to stroma-depleted tumors and subsequently enhanced treatment response will outweigh these putative adverse effects of stroma-targeting remains to be determined in carefully designed preclinical studies. Second, we observed considerable variation in CD47 expression across a large panel of primary PDAC samples. Specifically, 10% of patients showed no detectable or very low levels of CD47 staining. Those patients may not gain significant therapeutic benefit from anti-CD47 treatment. Such stratification could be based on CD47 expression on circulating tumor cells as these have been shown to also express CD47 (42). Third, it seems reasonable to explore the possibility of combining anti-CD47 mAb therapy with treatments that either target TAM recruitment (e.g., anti-CSF1 therapy) or their polarization toward M2 macrophages (e.g., anti-TGFβ therapy).

In conclusion, we have found that CD47 is expressed on primary PDAC cells and we have demonstrated that inhibiting CD47 function using mAbs is an effective method of treating PDAC in vitro and in vivo, thereby forming the rationale for evaluating the clinical efficacy of anti-CD47 therapy in more comprehensive preclinical studies, which may eventually lead to first trials in human patients with PDAC. Although further mechanistic studies are still needed to determine how anti-CD47 treatment reduces tumor growth (i.e., phagocytosis and/or apoptosis), the data presented herein add to the growing repertoire of tumors that can be potentially treated with anti–CD47 mAb-based therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Cioffi, S. Trabulo, M. Hidalgo, E. Costello, W. Greenhal, M. Erkan, J. Kleeff, B. Sainz Jr
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Cioffi, S. Trabulo, E. Costello, M. Erkan, B. Sainz Jr, C. Heeschen
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