Inhibition of CD47 effectively targets pancreatic cancer stem cells via dual mechanism

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Short title: CD47 targets pancreatic cancer stem cells

Conflict of Interest: The authors have no conflict of interest to declare.
STATEMENT OF TRANSLATIONAL RELEVANCE

Pancreatic ductal adenocarcinoma remains one of the most devastating cancers, and very few new treatments have revealed meaningful improvements in patient survival over the past decades. Based on our previous work demonstrating the existence of cancer stem cells 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 cancer stem cells 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 pancreatic ductal adenocarcinoma.
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 (TAMs). The presence of TAMs is associated with poor clinical outcome and their overall role therefore appears to be pro-tumorigenic. 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 PDAC cells including cancer stem cells (CSCs) as the exclusively tumorigenic population.

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 non-malignant 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 xenografts 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.
BACKGROUND

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 towards improving patients’ long-term survival. Gemcitabine (2), FOLFIRINOX (3), and more recently the addition of nab-paclitaxel (Abraxane) (4) are able to moderately extend median survival, but eventually the vast majority of patients still succumbs from progressive disease. Therefore, developing new and more effective anti-PDAC treatments represent an urgent and unmet medical need (5, 6). Since 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 the disease in most of the patients (3, 4).

Studies based on the inhibition of regulatory pathways that are crucially relevant for the self-renewal capacity of CSC 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 (TAMs) 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.
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 cascade mediated via phosphorylation of the immunoreceptor tyrosine-based inhibitory motif present on the cytoplasmic tail of SIRPα (18).

Previous work in pre-clinical models of bladder cancer, leukemia, and lymphoma demonstrated that inhibiting the interaction between CD47 and SIRPα using anti-CD47 monoclonal antibodies (mAbs) allows for increased phagocytosis of cancer cells \textit{in vitro} and decreased tumor burden \textit{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 non-malignant cells. Although CD47 targeting as a single treatment strategy was not effective \textit{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.
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 vivo as patient-derived xenografts (PDX), as previously described (12). For in vitro studies, PDX tissue fragments were minced, enzymatically digested with collagenase (Stem Cell Technologies, Vancouver, BC) for 90 min at 37°C (12) and after centrifugation for 5 min at 1,200 rpm, cell pellets were resuspended and cultured in RPMI supplemented with 10% FBS and 50 units/ml penicillin/streptomycin. These primary cultures were used in vitro only until passage 10. Murine PDAC cells were derived from the K-Ras+/LSL-G12D;Trp53LSL-R172H;PDX1-Cre mice (KPC) as a model of advanced PDAC (23). KPC-derived tumors were minced, mechanically (gentleMACS Dissociator; Miltenyi, Bergisch-Gladbach, Germany) and enzymatically dissociated with collagenase (Stem Cell Technologies, Vancouver, BC) and subsequently cultured in vitro as previously detailed (24). Epithelial clones were picked, pooled, and further expanded to heterogeneous primary cancer cell cultures (AAU77G and CHX6).

Human peripheral blood-derived mononuclear cells were obtained from healthy donors with informed consent. Monocyte-derived macrophage cultures were established in IMDM supplemented with 10% human AB serum as previously described (25). Sixty ng/ml GM-CSF or M-CSF (R&D, Minneapolis, MN) was added to the cultures to generate M1 and M2 monocyte-derived macrophages, respectively (26). Murine monocytes were isolated from mechanically disrupted spleens, passed through a 40µm mesh filter, and differentiated into macrophages under adherent conditions on non-tissue culture-treated 100 mm dishes in RPMI supplemented with 10% FBS and 10 ng/ml of murine M-CSF (PeproTech, London, UK). To generate M1- and M2-polarized murine macrophages, 10 ng/ml of IFN-γ (PeproTech) and LPS (Sigma, St. Louis, MO) (M1) or 10 ng/ml IL-4 (M2) (PeproTech) were added to the cultures.
**Sphere formation assay.** Spheres were generated by culturing $2 \times 10^4$ pancreatic cancer cells in suspension in serum-free DMEM/F12 supplemented with B27 (1:50, Invitrogen, Karlsruhe, Germany), 20ng/ml bFGF and 10units/ml pen/strep for a total of 7 days, allowing spheres to reach a size of >75µm. For serial passaging, 7-day-old spheres were retained using 40µm cell strainers, dissociated into single cells, and then re-cultured for 7 additional days as previously described (13).

**RNA preparation and quantitative real-time PCR.** Total RNAs from human primary pancreatic cancer cells and spheres were extracted with TRIzol (LifeTechnologies, Norwalk, CT) according to the manufacturer’s instructions. One microgram of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (LifeTechnologies) and random hexamers. Quantitative real-time PCR was performed using SYBR Green PCR master mix (LifeTechnologies), according to the manufacturer’s instructions. Primers sequences used are:

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ACTIN:     Forward - GCGAGCACAGAGCCTCGCCTT
           Reverse – CATCATCCATGGTGAGCTGGCGG

CD47:      Forward - GCGATTGGATTAACCTCCTTCGA
           Reverse - CCATGCATTGGTATACACGCCG
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**Flow cytometry.** Cells were adjusted to a concentration of $10^6$ cells/ml in sorting buffer [1X PBS; 3% FBS (v/v); 3mM EDTA (v/v)] before analysis or sorting with a FACS Canto II or FACS Influx instrument, respectively (BD, Heidelberg, Germany). 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). DAPI was used for exclusion of dead cells. Data were analyzed with FlowJo 9.2 software (Tree Star, Ashland, OR). For the assessment of apoptosis, cells were incubated with DAPI and Annexin V fluorescein isothiocyanate (FITC) staining kit (BD) according to the manufacturer’s instructions.
**Antibody Preparation.** The anti-hCD47 (B6H12) hybridoma was obtained from the ATCC. Hybridoma cells were cultured using previously described conditions (27) and antibodies were purified by Protein G.

**In vitro phagocytosis assay.** For *in vitro* phagocytosis analysis, 5×10^4 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 2h before adding 2×10^5 GFP-labeled live cancer cells. Anti-CD47 (B6H12) antibody (10μg/mL) or IgG1 control antibody was added and incubated for 2h 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 histopathological analysis, FFPE blocks were serially sectioned (3μm thick) and stained with hematoxylin and eosin (H&E). Additional serial sections were used for immunohistochemical (IHC) studies with anti-CD47 antibody (0.2μg/mL, Abcam ab3283). Antigens were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+). Counterstaining was performed with hematoxylin. Histological quantification of digitalized slides was performed using Pannoramic Viewer (3DHistech, Budapest, Hungary).

**Tissue microarrays (TMAs).** Four human TMAs containing quadruplicate 1mm 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 1mm 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) and subcutaneously injected into female 6-8 week old NU-Foxn1nu nude mice (Harlan, Laboratories, UK). In some experiments, macrophages were depleted with the following treatment schedule: 200μl of clodronate was injected intravenously 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 (CIOMS).

In vivo treatment. Primary tumor tissue pieces of ~2mm³ were implanted subcutaneously into the flanks of NU-Foxn1nu nude mice, and once tumors were established mice were randomized to the respective treatment groups. Gemcitabine was administered twice a week (125mg/kg i.p.), Abraxane was administered every 4 days (50mg/kg i.v.) and anti-CD47 was administered daily (500μg/mouse i.p.) (20).

Statistical analyses. Results for continuous variables are presented as means ± standard deviation unless stated otherwise and significance was determined using the Mann-Whitney test. All analyses were performed using SPSS 22.0 (SPSS, Chicago, IL).

RESULTS

CD47 is expressed at higher levels in PDAC compared to normal pancreatic tissue. We evaluated the level of CD47 expression by immunohistochemical analysis of paraffin sections of tissue microarrays containing primary human tissues from “normal” adjacent non-tumor pancreatic tissue, pancreatitis, PDAC, and regional lymph, and liver metastases. CD47 expression was significantly overexpressed in primary PDAC tumors (p<0.001) and metastasis (p<0.05).
versus pancreatitis and normal pancreatic tissue (Fig. 1A). Importantly, while CD47 was still detectable in normal (non-cancer) pancreatic tissue, the level of expression was significantly lower compared with PDAC, patient-derived PDAC xenograft and metastasis samples, where CD47 expression was markedly stronger, but restricted to epithelial cancer cells and absent in the stroma (Fig. 1B and Supplementary Fig. 1A). We next analyzed independent large collections of primary tissues for the expression of CD47. The results indicated that CD47 expression varies considerably within tissues (Supplementary Fig. 1B) and between patients with about 1/3 of the patients bearing low to undetectable levels of CD47. Patients represented on each of the two independent sets of TMAs were dichotomized according to low to undetectable CD47 expression (CD47 negative) and intermediate to high CD47 expression (CD47 positive), however, no association between CD47 expression and outcome could be identified (Supplementary Fig. 1C).

We next determined CD47 mRNA expression in a set of nine primary patient-derived pancreatic cancer cell cultures, two normal pancreas samples, and primary pancreatic stellate cells (PSC). We observed low to undetectable levels of CD47 mRNA expression in normal tissue and in PSCs as compared to PDAC cells. The latter could be subdivided into three groups based on their CD47 mRNA expression: low (JH029 and 247), medium (198, 253, 215, and 354), and high (163, 185, and A6L) (Fig. 1C). In order to confirm the expression of CD47 at the protein level, flow cytometry analysis was performed on both adherent cells and sphere-derived cells, the latter of which are enriched in CSCs (13). We observed relatively homogenous expression of CD47 in differentiated cells (ranging from 40% to 57%) while in sphere culture-enriched CSCs, the surface expression of CD47 was higher although more variable (ranging from 54% to 85%), suggesting an enrichment of CD47 in CSCs (Fig. 1D). We next assessed the percentage of CD47+ cells within the CD133+ [a well-established pancreatic CSC marker (9)] sub-population, and as shown in Fig. 1E, the majority of CD133+ cells expressed CD47 albeit with different percentages (ranging from 77% to 97%).

*Pancreatic cancer stem cells are mostly confined to CD47+ cells.* Since 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 FACSsorted primary pancreatic cancer cells for CD47 (Fig. 2A) and then determined their self-renewal capacity using sphere formation as 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 post-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 expression 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 SIRPα signaling using targeted monoclonal antibodies (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 that in contrast to 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/B) and was independent of the method of macrophage polarization (Supplementary Fig. 2A).

We next attempted to mimic the tumor in vivo microenvironment conditions by polarizing macrophages cultures towards 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, while M-CSF treated and CSC conditioned macrophages both showed a more elongated shape typical of M2-polarized macrophages (Fig. 3C, upper panel). In the absence of anti-CD47 blocking antibodies, CSC-conditioned “M2” macrophages had the lowest phagocytic index levels compared to the other macrophage subtypes (0.9 vs 2.8 for M2-polarized macrophages), consistent with a truly protective and pro-tumorigenic 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, lower panel). 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/non-phagocytosed cells, indicating that anti-hCD47 treatment indeed eliminated the
CSCs pool (Fig. 3F). Using a more stringent ratio of macrophage:cancer 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. 2B-D). In contrast, for non-transformed cells no significant induction of phagocytosis was found (Supplementary Fig. 2B, right panel), which might be attributed to the lack of “eat-me” signals on these cells. Lastly, in order 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 while in the same experiments treatment with anti-CD47 showed strong induction of phagocytosis (Supplementary Fig. 2F). 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 cancer stem cells.** 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 subsequently assessed apoptosis 2 and 12 h post treatment by Annexin V staining. While we observed no induction of apoptosis in non-transformed human cells (Fig. 4A) or in primary murine PDAC tumor cells (Supplementary Fig. 3A), we did detect a significant increase in apoptotic cells across several primary human PDAC cell lines following treatment with the anti-CD47 antibody compared to IgG mAb-treated controls (Fig. 4B). Importantly, no apoptosis was observed in any of the samples tested following two hours of treatment (Supplementary Fig. 3B). Thus, since phagocytosis of CSCs by macrophages was detected as early as 15 minutes post incubation with the anti-CD47 antibody (data not shown), we identify two distinct mechanisms of action, the first being phagocytosis while 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.** In order to test the efficiency of CSCs elimination *in vitro*, we tested the ability of surviving/non-phagocytosed cells after anti-CD47 treatment to form tumors *in vivo*. We observed a significant reduction in the tumorigenicity of anti-CD47-treated cells compared to isotype-treated cells ([Fig. 5A](#)). The few tumors that formed from the anti-CD47 treated cultures were significantly smaller in size compared to isotype control tumors ([Fig. 5B](#)). In addition, while 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. 2E](#)).

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 (~100mm$^3$), mice were randomized to one of the following six treatment groups: Diluent control; Gemcitabine (biweekly 125mg/kg i.p.) from day 14 to 56; Abraxane (every 4 days 50mg/kg i.v.) from day 14 to 28; Anti-CD47 (daily 500μg/mouse i.p.) from day 14 to 35; Gemcitabine + anti-CD47; and Abraxane + anti-CD47. Interestingly, for both utilized PDX models no significant differences were observed for chemotherapy and anti-CD47 single treatment, however, tumors treated with a combination of chemotherapy + anti-CD47 were significantly reduced compared to control tumors and single treatment tumors. Specifically, for PDAC-185, treatment with Abraxane plus anti-CD47 significantly stalled tumor growth. While 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. 4A). 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. 4B/C).

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): (1) the classically activated (M1) macrophage that plays an important pro-inflammatory effector role in TH1 cellular immune responses, including the secretion of cytokines and phagocytosis of target cells or (2) the alternatively activated (M2) macrophage that is involved in type II helper T-cell processes, such as wound healing and humoral immunity. In cancer, pro-tumorigenic M2 TAMs enhance neoplasia via matrix remodeling, angiogenesis and the secretion of pro-tumor growth factors, such as TGF-β (34). In contrast, M1 macrophages are believed to inhibit tumor growth via anti-tumor-adaptive immunity mechanisms that include phagocytosis. The latter, however, mainly depends on macrophage recognition of pro-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 monoclonal antibodies 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). While CD47 was significantly overexpressed in about 2/3 of neoplastic tissues, we did not observe a correlation between high CD47 protein expression and poor clinical outcome (Supplementary Fig. 1C), which is in contrast to 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. 1A-B). Thus, analysis of complete sections of primary patient PDAC samples is likely needed before a correlative connection can be definitively determined for PDAC. Notably, while 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 to 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 phagocytose CSCs 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 PDAC cells treated with anti-CD47 mAbs compared to 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, IL-4, IL-13, and IL-10) (33). Therefore, our finding that MCSF-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 biological 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 monoclonal antibodies 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 phagocytic 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 to mice treated with Abraxane alone where relapse was evident in all mice by day 77. Regarding the tumor CSC content, we observed that only the anti-CD47 therapy was able to reduce the
percentage of CD133+ cells in the tumor, which confirms our in vitro results and indicate 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 anti-cancer therapeutic agents, such as Abraxane, is needed.

While 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 (PCD) in normal and leukemic cells (31, 32, 37), thus in addition to blocking the anti-phagocytic 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, pro-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 pro-phagocytic (“eat me”) signal calreticulin. Therefore, anti-CD47 therapy may have multiple mechanisms of action, each of which likely facilitate macrophage phagocytosis of CSCs.

Is CD47 targeting in PDAC suitable and ready for further clinical exploration? While our in vivo 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 SIRPa monomers are available in larger amounts (39), it should be determined if the abundant stroma in PDAC tumors represents a relevant physical barrier for the antibodies to reach the cancer cells. Therefore, it should be tested if co-administration 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 de-differentiation of cancer cells rendering them more aggressive (40, 41). In addition, a recent clinical trial on hedgehog pathway inhibition was prematurely stopped based on 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. Secondly, 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). Thirdly, 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 towards 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 monoclonal antibodies 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 pre-clinical studies, which may eventually lead to first trials in human patients with PDAC. While 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 by the authors.

AUTHOR CONTRIBUTIONS

Michele Cioffi developed the study concept, acquired, analyzed, interpreted the data, and wrote the manuscript; Michele Cioffi and Sara M. Trabulo designed and performed the in vivo experiments. Bruno Sainz, Jr. helped in the study design, interpretation of the data, and wrote the manuscript; Manuel Hidalgo provided extensively characterized PDAC samples; Eithne Costello, William Greenhalf, Mert Erkan, and Joerg Kleeff provided PDAC samples and/or tissue microarrays. Christopher Heeschen developed the study concept, obtained funding, interpreted the data, and wrote the manuscript.

GRANT SUPPORT

The research was supported by the ERC Advanced Investigator Grant (Pa-CSC 233460), European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n°256974, the Subdirección General de Evaluación y Fomento de la Investigación, Fondo de Investigación Sanitaria (PS09/02129 & PI12/02643) and the Programa Nacional de Internacionalización de la I+D, Subprogramma: FCCI 2009 (PLE2009-0105; both Ministerio de Ciencia e Innovación, Spain), all to C.H.; and the Spanish Ministry of Economy and Competitiveness (SAF2011-30173 to M.B.). The authors declare no conflict of interest. M.C. is supported by the La Caixa Predoctoral Fellowship Program.

ACKNOWLEDGEMENTS

We are indebted to Magdalena Choda for excellent technical assistance. We would also like to thank Maria Lozano for assistance with the PDAC TMA analyses.
FIGURE LEGENDS

**Figure 1: CD47 is expressed in pancreatic cancer (stem) cells.** (A) Quantification of CD47 expression in a 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. (B) Representative pictures of CD47 stained TMA cores including FFPE section of human-derived xenografts. (C) RTqPCR analysis of CD47 in normal pancreas samples, PSC cells, and several primary human pancreatic cancer cultures. β-actin was used as a normalization control. (D) Flow cytometry analysis of CD47 cell surface expression comparing adherent cells and sphere-derived cells. (E) Flow cytometry analysis of CD47 and CD133 expression on sphere-derived cells (left panel) and quantification of CD133+ cells also expressing CD47 (right panel).

**Figure 2: Pancreatic cancer stem cells 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 panel) and quantification of spheres (right panel) 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 cancer stem cells.** (A) Representative confocal images (upper panel) and phagocytic index (lower panel) 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. (B) 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) 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. (E) Sphere formation quantification of cells after treatment with anti-CD47 mAbs, compared to IgG1 control treated cells.

**Figure 4: Anti-CD47 treatment directly induces apoptosis of pancreatic cancer stem cells.** (A) Flow cytometry analysis of apoptosis as determined by Annexin V/DAPI staining, in non-transformed human cells and (B) several primary human pancreatic sphere-derived cell cultures after treatment for 12 h with anti-CD47 mAb (B6H12) or IgG1 isotype control mAb.

**Figure 5: Anti-CD47 treatment inhibits in vivo tumorigenicity and tumor progression, preventing relapse.** (A) In vivo tumorigenicity, (B) tumor weight and (C) flow cytometry analysis for EPCAM, CD133 and SSEA1 for primary pancreatic sphere-derived cells after treatment with anti-CD47 mAb (B6H12) or IgG1 isotype control mAb. (D) Experimental setup for in vivo treatment (upper left panel) and effects of allocated treatment regiments in 185 tissue xenografts transplanted in immunocompromised mice (lower left panel). The mean tumor volume is given; n=6 tumors per group. Representative images of tumors extracted from mice at the end of the respective observation period (right panel). (E) H&E staining and IHC analysis of CK19 expression in paraffin section from the tumors. (F) Flow cytometry analysis of CD133 cell surface expression in cells isolated from tumors of mice treated as indicated.
References


Figure 1 – CD47 is variably expressed in pancreatic cancer (stem) cells

A

Mean Relative Intensity

Normal Pancreatitis PDAC Metastasis

0 10 20 30 40

B

C

D

E

CD47 mRNA expression (absolute values)

CD47 surface expression (% of positive cells)

CD133

CD47 expression in CD133+ cells

Normal Pancreatitis PDAC Metastasis

PSC

JH029

247 198 253 215 354 163 185 A6L

Adherent Spheres

* p<0.05

Author Manuscript Published OnlineFirst on February 23, 2015; DOI: 10.1158/1078-0432.CCR-14-1399

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 2 – Pancreatic cancer stem cells are mostly confined to CD47+ cells

**A**

Pancreatic cancer stem cells are mostly confined to CD47+ cells.

**B**

CD47− and CD47+ cells.

**C**

Tumorigenicity

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<tr>
<th>(# tumors / # injections)</th>
<th>10^5</th>
<th>10^6</th>
<th>10^7</th>
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<th>P value</th>
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<td>215 CD47−</td>
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<tr>
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<td>3/4</td>
<td>3/4</td>
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**D**

Number of spheres

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**E**

Tumorigenicity

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**Figure 3** – Anti-CD47 treatment enables phagocytosis of pancreatic cancer stem cells

**A**  
IgG1 Isotype  
Anti-CD47  
Phagocytic Index

**B**  
CD47+CD133-  
CD47+CD133+  
Phagocytic Index

**C**  
Human Macrophages  
Phagocytic Index

**D**  
Mouse Macrophages  
Phagocytic Index

**E**  
Fold Change of CD133 expression

**F**  
Number of spheres
Figure 4 – Anti-CD47 treatment directly induces apoptosis of pancreatic cancer stem cells

A

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B

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<td>31%</td>
<td>45%</td>
<td>35%</td>
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</table>
Figure 5 – Anti-CD47 treatment inhibits in vivo tumorigenicity and tumor progression

A

B

C

D

E

F

Experimental setup:

Day 0 14 28 35 56 70 100

Experimental setup:

Day 0 14 28 35 56 70 100

Tumor volume (mm^3)

Time (days)

CD133 surface expression (%) * p<0.05

CD133 expression (%) * p<0.05
Inhibition of CD47 effectively targets pancreatic cancer stem cells via dual mechanism

Michele Cioffi, Sara Maria Trabulo, Manuel Hidalgo, et al.

Clin Cancer Res Published OnlineFirst February 23, 2015.

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-1399

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