Macrophages govern the immune landscape of many cancers and are key proinflammatory and inhibitory signals, the role of macrophage metabolism is unknown. Here, we study the capacity of macrophages to circumvent inhibitory activity mediated by CD47 on cancer cells. We show that stimulation with a CpG oligodeoxynucleotide, a Toll-like receptor 9 agonist, evokes changes in the central carbon metabolism of macrophages that enable antitumor activity, including engulfment of CD47⁺ cancer cells. CpG activation engenders a metabolic state that requires fatty acid oxidation and shunting of tricarboxylic acid cycle intermediates for de novo lipid biosynthesis. This integration of metabolic inputs is underpinned by carnitine palmitoyltransferase 1A and adenosine tri-phosphate citrate lyase, which, together, impart macrophages with antitumor potential capable of overcoming inhibitory CD47 on cancer cells. Our findings identify central carbon metabolism to be a novel determinant and potential therapeutic target for stimulating antitumor activity by macrophages.

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Macrophages enforce antitumor immunity by engulfing and killing tumor cells. Although these functions are determined by a balance of stimulatory and inhibitory signals, the role of macrophage metabolism is unknown. Here, we study the capacity of macrophages to circumvent inhibitory activity mediated by CD47 on cancer cells. We show that stimulation with a CpG oligodeoxynucleotide, a Toll-like receptor 9 agonist, evokes changes in the central carbon metabolism of macrophages that enable antitumor activity, including engulfment of CD47⁺ cancer cells. CpG activation engenders a metabolic state that requires fatty acid oxidation and shunting of tricarboxylic acid cycle intermediates for de novo lipid biosynthesis. This integration of metabolic inputs is underpinned by carnitine palmitoyltransferase 1A and adenosine tri-phosphate citrate lyase, which, together, impart macrophages with antitumor potential capable of overcoming inhibitory CD47 on cancer cells. Our findings identify central carbon metabolism to be a novel determinant and potential therapeutic target for stimulating antitumor activity by macrophages.

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inhibitory signals used by cancer cells to evade elimination by innate immunity.

**Results**

**Macrophage activation, but not loss of inhibitory CD47, is sufficient for antitumor activity in PDAC.** Macrophages can be induced with therapeutic and antitumor functions by activating pro-inflammatory signaling pathways such as CD40 and TLRs\(^ {26} \). However, macrophage biology is ultimately determined by a balance of stimulatory and inhibitory signals that are sensed within the tumor microenvironment (Supplementary Fig. 1a). One major inhibitory signal that is involved in suppressing macrophage antitumor activity is CD47, which is overexpressed on cancer cells across a wide range of solid malignancies\(^ {27} \). Elevated CD47 can be detected in both mouse (Supplementary Fig. 1b,c) and human PDAC\(^ {28} \). Therefore, we initially studied the role of CD47 as a macrophage-inhibitory signal in a murine model of PDAC. To do this, we administered a CD47-blocking antibody via intratumoral delivery to mice implanted with Kras\(^ {12D} \)/Trp53\(^ {R172H} \)/Foxp3\(^ {2L} \) mutant PDAC tumors. In this fully syngeneic and immunocompetent model, antibody blockade of CD47 did not alter tumor growth (Fig. 1a). To address possible limitations in bioavailability or insufficient blockade, expression of Cd47 was ablated in PDAC cells using transient expression of CRISPR/Cas9 (Supplementary Fig. 1d,e). Unlike models of leukemia where CD47 overexpression is a key determinant of immune escape\(^ {29} \), deletion of Cd47 in PDAC cells did not affect tumor engraftment or growth (Fig. 1b). This observation suggested that mechanisms other than CD47 can regulate macrophage-dependent antitumor responses in PDAC, consistent with xenograft models of this disease and other non-hematopoietic malignancies\(^ {30,31} \). In support of this finding, we also found that antibody blockade of CD47 on PDAC cells did not enhance in vitro phagocytosis by murine bone-marrow derived macrophages (BMDMs) (Fig. 1c).

We next considered that PDAC cells might not provide sufficient activating signals to stimulate macrophages with antitumor functions, and that delivery of discrete stimuli may be necessary to induce macrophages with antitumor activity. We tested a panel of TLR pathway agonists for their capacity to stimulate macrophages with antitumor functions, such as phagocytosis. In the absence of TLR stimulation, mock-treated BMDMs lacked the capacity to phagocytose tumor cells on co-culture with PDAC cells (Fig. 1d). Further, the phagocytic capacity of BMDMs increased only modestly with 24-hour pretreatment with Pam3CSK4, Poly(I:C), lipopolysaccharide (LPS), flagellin and imiquimod—which stimulate TLR2 and TLR2, TLR3, TLR4, TLR5 and TLR7, respectively (Fig. 1d). In contrast, ODN1826, a Class B CpG oligonucleotide that preferentially stimulates TLR9 expressed by macrophages and B cells, was found to be a potent activator of macrophage phagocytosis of PDAC cells (Fig. 1d,e). On increasing the duration of CpG pretreatment, CpG-activated macrophages (CpG-BMDM) exhibited enhanced phagocytic capacity relative to mock-stimulated macrophages (mock-BMDM) (Fig. 1f). To ascertain the potential of CpG activation to produce antitumor activity, we also performed an extended co-culture of pretreated macrophages with PDAC cells for 48 hours. We found that CpG activation rendered macrophages with potent antitumor activity leading to a decrease in tumor cell survival in comparison to co-culture with mock-BMDMs (Fig. 1g). Together, these data show loss of inhibitory CD47 alone to be insufficient for unleashing antitumor activity by macrophages in PDAC, and that an activated phenotype is also necessary for macrophages to engage in antitumor functions.

**Tumor-associated macrophages are essential for CpG-activated antitumor responses.** We next evaluated the in vivo antitumor activity of CpG on established murine PDAC tumor growth. Treatment was initiated with intraperitoneal injection of vehicle (PBS) or CpG on day 10 with repeated administration every other day for a total of five doses (Fig. 2a). CpG treatment potently suppressed tumor growth in two independent PDAC tumor models (Fig. 2b,c). Although tumors ultimately relapsed, CpG significantly prolonged overall survival (Fig. 2d) without inducing gross toxicity or lethality. This effect was also independent of any direct cytotoxic activity of CpG on tumor cells, as treatment of PDAC cells in vitro with supratherapeutic concentrations of CpG did not affect tumor cell survival (Supplementary Fig. 2).

Repeated dosing of CpG to non-tumor-bearing mice has been found to stimulate a macrophage-activation syndrome\(^ {32} \). Therefore, we examined the impact of treatment on the systemic release of inflammatory cytokines. We found that in vivo delivery of CpG to tumor-bearing mice increased pro-inflammatory cytokines in the serum, including TNF, IFN-γ and CCL2 (Fig. 2e). Consistent with this increase in inflammatory and chemotactic factors, we observed an increase in tumor-associated macrophages following CpG treatment (Fig. 3a,b). In addition, we detected an increase in F4/80\(^ + \) macrophage phagocytosis of tumor cells in CpG-treated tumors (Supplementary Fig. 3). To ascertain the role of tumor-associated macrophages in the response to CpG treatment, we depleted distinct macrophage populations using GW2580, an inhibitor of colony stimulating factor 1-receptor (CSF1Ri), and CEL, which target phagocytes residing outside of the tumor microenvironment\(^ {33} \). Depletion with either CEL or CSF1Ri alone did not affect tumor outgrowth in the vehicle-treated groups (Fig. 3c). In contrast, CSF1Ri abrogated the antitumor effect of CpG, whereas CEL did not (Fig. 3c). Similar to this, we found that administration of an anti-CSF1R antibody attenuated the CpG-induced antitumor response (Fig. 3d). These findings implicating a CSF1R\(^ + \) population of macrophages, which are not targeted by liposomes, in mediating the antitumor response by CpG. Consistent with this observation, we found that CEL did not alter macrophage presence within tumors (Fig. 3a), whereas both CSF1Ri (Fig. 3a,b) and anti-CSF1R antibody (Fig. 3e) treatment decreased the abundance of tumor-associated macrophages.

To ascertain the possible contribution of other immune effectors, we considered a role for lymphocytes in CpG-induced antitumor activity. We found that CpG did not significantly alter the infiltration of T cell subsets (CD8\(^ + \), CD4\(^ + \) and CD4\(^ + \) Foxp3\(^ + \)) into tumors (data not shown). In addition, we detected no significant change in the expression of immune regulatory markers, including PD-1 and Tim3, by T cells after CpG treatment (data not shown). CpG-induced antitumor activity was also preserved in Rag2-deficient mice bearing PDAC tumors (Fig. 3a), whereas both CSF1Ri (Fig. 3a,b) and anti-CSF1R antibody (Fig. 3e) treatment decreased the abundance of tumor-associated macrophages.

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Fig. 1 | Macrophage activation via TLR agonists, but not disruption of CD47, induces antitumor activity in a model of pancreatic cancer. a, Syngeneic mice with established PDAC tumors were treated with 50 μg isotype or anti-CD47 Ig on days 10 and 14 after tumor implantation (n = 8). Arrows indicate intratumoral delivery of antibody; shown is mean ± s.e.m. b, Syngeneic mice were challenged with PDAC tumors in which CD47 was knocked out (CD47−/−) versus control (CTRL) using CRISPR-Cas9 gene editing (n = 5); shown is mean ± s.e.m. c, In vitro phagocytosis of PDAC cells by BMDMs in the presence of isotype control or anti-CD47 Ig. d, In vitro phagocytosis of PDAC cells by BMDMs stimulated with indicated TLR agonists for 24 h. e, Representative immunofluorescence images showing phagocytosis of PDAC cells (green) by macrophages (BMDM, red) pretreated with vehicle (mock) or CpG for 24 hours. Arrows indicate phagocytic events. Scale bar, 100 μm. f, In vitro phagocytosis of PDAC cells by BMDMs stimulated with CpG for increasing lengths of time (n = 3), error bars represent standard error. g, PDAC cell survival following 48-h co-culture with macrophages pretreated with vehicle (Mock) or CpG for 96 h (n = 3), mean ± s.e.m. Boxplots c,d represent mean ± s.d. Statistical significance determined using two-tailed Student’s t-test with Hochberg correction. NS (not significant). Results are representative of at least two independent experiments (a–g). *P < 0.05; **P < 0.01; ***P < 0.001.

in vivo, blocked both the accumulation of F4/80+ macrophages in tumors (Fig. 3f) and the CpG-induced antitumor response (Fig. 3g). Together, these data implicate myeloid cells marked by expression of Ly6C and CSF1R in the antitumor activity induced by CpG.

CpG activation of macrophages bypasses inhibitory CD47 on PDAC cells. We next sought to understand the mechanism by which CpG stimulates macrophages with enhanced phagocytic and antitumor activity. We considered that CpG might alter the capacity of macrophages to respond to CD47 as a negative regulatory signal. To test this, we assessed the impact of CpG on SIRPα expression. However, we observed no changes in SIRPα expression following CpG-BMDMs (Supplementary Fig. 5). Further, on extended co-culture, CpG-BMDMs effectively eliminated PDAC cells, with loss of CD47 expression in tumor cells providing an additive benefit in antitumor activity (Fig. 4d). However, the ability of CpG to macrophage engulfment of CD47-expressing syngeneic tumor cells derived from breast cancer, melanoma, colorectal cancer, glioblastoma, lung cancer and lymphoma (Fig. 4c). CD47-blockade alone also showed limited capacity to enhance macrophage phagocytosis in these tumors, except for EL4 lymphoma cells. However, despite limited activity by itself, we found that genetic ablation of CD47 in tumor cells (Fig. 4b) as well as CD47-blockade (Fig. 4c) significantly enhanced the capacity of CpG-stimulated macrophages to phagocytose tumor cells in vitro, implying that appropriate macrophage-activation signals are critical for overcoming CD47 in nonhematopoietic tumors.

Previous studies have suggested that TLR agonists might promote phagocytic and antitumor activity by stimulating macrophages to produce calreticulin25. To test this possibility, we quantified calreticulin expression—including both membrane-bound and intracellular levels—but detected no differences between mock-BMDMs and CpG-BMDMs (Supplementary Fig. 5). Further, on extended co-culture, CpG-BMDMs effectively eliminated PDAC cells, with loss of CD47 expression in tumor cells providing an additive benefit in antitumor activity (Fig. 4d). However, the ability of CpG to
Fig. 2 | CpG induces antitumor activity in vivo. a, Schematic showing implantation of PDAC tumor cells (day 0) and subsequent treatment schedule (beginning on day 8) for CpG and methods of macrophage depletion using clodronate-encapsulated liposomes (CEL) and a CSF1R-inhibitor (CSF1Ri). b, Two distinct syngeneic KPC-derived PDAC tumors (PDAC.1 and PDAC.2) were treated with vehicle or CpG beginning on day 10. Waterfall plots show percentage change in tumor volume at day 14 relative to baseline before treatment (day 10). c, Longitudinal growth curves shown as mean ± s.e.m. for PDAC tumors treated with vehicle or CpG (n = 6–8); d, Kaplan–Meier curve showing survival of tumor-bearing mice (n = 10) treated with vehicle or CpG (P = 5.3 × 10^{-5}). Treatment was initiated beginning on day 10. Significance was determined using log-rank analysis. e, Syngeneic mice bearing PDAC.1 control (CTRL) or PDAC.1 Cd47^{-/-} tumors were treated with vehicle or CpG beginning on day 10 after tumor implantation. Shown are boxplots displaying mean ± s.d. of cytokine concentration detected in peripheral blood on day 14. Significance determined using two-tailed Student’s t-test with Hochberg correction: NS (not significant). Results are representative of at least two independent experiments (b–e). *P < 0.05; ****P < 0.0001.

Promote antitumor activity by macrophages despite CD47 expression on tumor cells is in contrast with other TLR agonists, for which blockade of CD47 is critical for macrophage activity^{32}.

We then asked whether disruption of CD47 enhances the in vivo antitumor activity of macrophages. Delivery of CpG to mice bearing control or CD47 knockout (Cd47^{-/-}) tumors produced similar
increases in the relative abundance of tumor-associated macrophages (Fig. 4e,f). The impact of Cpg on tumor growth was also similar in tumors that expressed or lacked CD47 (Fig. 4g). These findings underscore the dominant response elicited by Cpg activation of macrophages in vivo that can overcome CD47 expressed by PDAC cells.

**Cpg induces a shift in macrophage metabolism.** Microenvironmental cues can induce distinct phenotypes in macrophages. For example, the combination of LPS and IFN-γ promote an M1 phenotype in macrophages, which is associated with pro-inflammatory and antitumor activity in cancer. In contrast, the combination of IL-4 and IL-13 endows macrophages with M2-polarity, which suppresses inflammation and promotes tumor growth. To study the direct effect of Cpg on macrophage polarity, we evaluated Cpg-BMDMs for markers associated with M1 and M2 macrophages, including arginase 1 (Arg1), CD206, MHC-II, inducible nitric oxide synthase (iNOS), IL-6 and IL-12. We found that Cpg activation increased the expression of both iNOS and Arg1, which are associated with M1 (LPS) and M2 (IL-4) macrophages, respectively (Fig. 5a). However, Cpg did not significantly alter expression of MHC-II or CD206, markers increased by M1 (LPS) and M2 (IL-4) polarization, respectively (Fig. 5b). Cpg also induced production of both pro-inflammatory cytokines (that is, IL-6, IL-12, CCL2 and TNF) and anti-inflammatory cytokines (that is, IL-10) relative to mock-treatment (Fig. 5c). Further examination of additional markers revealed that Cpg did not modulate IL-4R alpha, CD80 or CD86, but upregulated expression of FcyRIII and PD-L1 (data not shown).
Pro-inflammatory stimuli can also alter macrophage metabolism. Therefore, we sought to understand the impact of CpG activation on the metabolic state of macrophages by assessing their ECAR (extracellular acidification rate) and OCR (oxygen consumption rate). We found that CpG and several other TLR agonists increased ECAR relative to mock-BMDMs, indicating increased glycolytic flux (Fig. 5d and Supplementary Fig. 6). This metabolic change seen in CpG-stimulated macrophages is consistent with metabolic activation seen in dendritic cells in response to CpG. Further, we observed that the basal OCR of macrophages increased with prolonged CpG stimulation (Fig. 5e) and corresponded with reduced spare respiratory capacity (Fig. 5h). Collectively, these findings demonstrate that CpG activation confers a unique metabolic shift in macrophages and that CpG-activated macrophages do not fully conform to the classical M1(LPS) or M2(IL-4) classification.

**Lipid metabolism is critical for antitumor functions induced by CpG.** We next investigated the significance of elevated oxygen consumption in response to CpG activation by first assessing mitochondrial abundance in macrophages. CpG-BMDMs exhibited an increase in total mitochondria, in comparison to mock-BMDMs (Supplementary Fig. 7a). To further evaluate changes in mitochondrial function, we tested the contribution of FAO toward elevated metabolic activity. We found that FAO inhibition blocked the CpG-induced increase in macrophage OCR without affecting the maximal respiratory capacity (Fig. 6a,b). Furthermore, FAO inhibition with etomoxir attenuated the capacity of CpG-BMDMs to phagocytose PDAC cells, whether they expressed inhibitory CD47 or not (Fig. 6c,d).

To test the role of FAO for the in vivo antitumor activity induced by CpG, etomoxir was administered daily to mice beginning on...
**Fig. 5 | CpG evokes metabolic changes in macrophages without polarization to M1 or M2.**

**a,** Normalized mean fluorescence intensity of iNOS (left) and Arg1 (right) in mock, M1 or M2 as indicated and CpG-activated BMDMs (n = 3). **b,** Mean fluorescence intensity for BMDMs pretreated with vehicle (mock) or CpG for 96 h, compared with M1 and M2 BMDMs (n = 3). **c,** Heatmap of cytokine concentrations produced in vitro by BMDMs treated with vehicle (mock), LPS or CpG for 48 h (n = 3), scaled to \( \log_{10}(\text{Concentration in pg ml}^{-1}) \). **d,** Extracellular acidification rate (ECAR) of BMDMs after 24-h activation with indicated TLR agonists (n = 8), relative to mock-treated baseline. **e,** Basal oxygen consumption rate (OCR) of BMDMs after 24-h activation with indicated TLR agonists (n = 8), relative to mock-treated baseline. **f,** Profile of OCR in BMDMs pretreated for 24 h with vehicle (mock) or CpG, in comparison to M1 and M2 BMDMs (n = 8). OCR was measured as picomoles of \( \text{O}_2 \) per minute on sequential administration with oligomycin, FCCP and Rotenone + Antimycin A. **g,** Basal OCR of BMDMs following activation with CpG for increasing durations (n = 8), relative to mock-treated baseline. **h,** Spare respiratory capacity of BMDMs treated with vehicle (mock) or CpG for 96 h (n = 8), shown as the difference between basal OCR and maximal OCR (after addition of FCCP). Significance determined using two-tailed Student’s t-test with Hochberg correction: NS (not significant). Plots **a–b, d–f, h** show mean ± s.d. Plots **e** show mean ± s.e.m. Results are representative of at least two independent experiments (**a–h**). *P < 0.05; ****P < 0.0001.
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day 9 before initiating CpG treatment on day 10. We found that etomoxir treatment blocked the antitumor effect of CpG, whereas treatment with etomoxir alone had no significant effect on tumor outgrowth (Fig. 6e). Importantly, etomoxir treatment also did not alter the abundance of F4/80+ macrophages in the tumor microenvironment, indicating that the inhibitory effect observed was not a result of depletion or exclusion of macrophages from the tumor microenvironment (Fig. 6d). Rather, the inhibitory effect of etomoxir was associated with decreased engulfment of tumor cells by F4/80+ macrophages (Supplementary Fig. 3). Together, these data demonstrate a key role for FAO in macrophage-dependent antitumor activity induced with CpG.

De novo lipogenesis, but not exogenous fatty acids, are critical for CpG-activated antitumor activity. To define the metabolic changes induced by CpG, we assessed the abundance of short chain coenzyme A (CoA) species in mock- and CpG-treated macrophages. We considered that an increase in FAO in CpG-BMDMs would require breakdown and incorporation of exogenous fatty acids into acetyl-CoA and tricarboxylic acid (TCA) substrates. Surprisingly, we found that the incorporation of labeling from 13C-palmitate into acetyl-CoA and TCA substrates. Surprisingly, we found that substrates other than exogenous palmitate probably serve as precursors for generating acetyl-CoA and succinyl-CoA in response to CpG stimulation (Fig. 7a,b). We next investigated the impact of CpG on increased fatty acid uptake, which is a key feature of M2 macrophages. We found that CpG-BMDMs did not upregulate fatty acid transporters such as CD36 that instead was significantly downregulated in response to CpG stimulation (Supplementary Fig. 7b). In addition, CpG-BMDMs showed a limited increase in the uptake of boron-dipyrromethene- (BODIPY-)labeled palmitate, indicating that utilization of exogenous fatty acids was not significantly altered in macrophages by CpG stimulation (Supplementary Fig. 7c). Together, these findings illustrate that while CpG-macrophages share several metabolic features with M2 macrophages, such as their oxidative phenotype, they differ significantly in their use of exogenous fatty acids.

We proceeded to examine alternative pathways that may support metabolic activation in CpG-BMDMs. On substitution of glucose or glutamine with 13C-glucose or 13C-glutamine in the media, the relative total pool sizes for acetyl-CoA, succinyl-CoA and HMG (hydroxymethylglutaryl)-CoA increased in CpG-BMDMs compared to mock-BMDMs (Fig. 7c). The percentage of acetyl-CoA derived from glucose (M+2 isotopepeak) increased
in CpG-BMDMs relative to mock-BMDMs (Fig. 7d). This shift was accompanied by a decreased percentage of acetyl-CoA derived from glucose (M+2 isotopologue) in CpG-BMDMs (Fig. 7d). Conversely, incorporation of \(^{13}\)C-glucose into succinyl-CoA (M+4 isotopologue) decreased on CpG stimulation, and was accompanied by an increase in labeled, glutamine-derived succinyl-CoA (M+4 isotopologue) in CpG-BMDMs compared to mock-BMDMs (Fig. 7e). Together, these data show that CpG activation of macrophages promotes a shift away from complete utilization of carbon from glucose and toward glutamine anaplerosis for generating TCA cycle intermediates such as succinyl-CoA.

The incorporation of glucose into acetyl-CoA and HMG-CoA (a precursor for the cholesterol synthesis pathway) seen in CpG-stimulated BMDMs was consistent with a shunting by adenosine tri-phosphate citrate lyase (ACLY) and de novo biosynthesis of cholesterol and/or lipids\(^{16,37}\). Lipid metabolism can impact multiple biological processes in macrophages, including cellular organelle production, cytokine secretion and lipid membrane properties\(^{36,39}\). Therefore, we examined a role for secretory capacity in CpG-induced macrophage phagocytosis. We found that blocking endoplasmic reticulum secretion using brefeldin A impaired CpG-induced phagocytosis of tumor cells (Supplementary Fig. 7d). This finding was consistent with previous work showing a role for the endoplasmic reticulum as a source of membrane for phagocytosis\(^{34}\). Therefore, we next hypothesized that disruption of ACLY would alter membrane properties and directly impede the antitumor activity of CpG-activated macrophages. We found that inhibition of ACLY during CpG-induced macrophage activation attenuated the oxidative phenotype (Fig. 7f) and reversed membrane fluidity (Supplementary Fig. 7e), an established regulator of macrophage phagocytosis. Consistent with this, we also found that inhibition of ACYL abrogated the antitumor phagocytic activity of CpG-stimulated macrophages even with combined CD47 ablation (Fig. 7g). Together, our findings demonstrate that CpG imparts macrophages with a unique metabolic state, defined by enhanced oxidative phosphorylation and a shunting of TCA cycle intermediates, which determines their antitumor potential and capacity to circumvent inhibitory signals received by CD47 expressed on malignant cells.

Discussion

Macrophages dominate the microenvironment of many cancers, wherein they are directed toward a pro-tumor role and restricted in their antitumor potential. The capacity of macrophages to engulf and kill tumor cells is counterbalanced by their phenotype as well as inhibitory signals present within their surrounding microenvironment, including the anti-phagocytic molecule CD47, which is overexpressed by malignant cells\(^{34}\). Our study identifies the central
carbon metabolism of macrophages as a regulator of their antitumor functions, including the capacity to phagocytose tumor cells. We found that redirection of macrophages toward an oxidative phenotype is essential for antitumor activity induced by CpG stimulation in vitro and in vivo. CpG-stimulated alterations in macrophage metabolism were necessary for circumventing inhibitory signals mediated by CD47. Together, our findings highlight rewiring macrophage metabolism—by promoting FAO and redirecting the use of TCA intermediates—as a mechanism to circumvent negative regulatory signals present on malignant cells and to endow macrophages with antitumor potential.

Inhibitory signals expressed by tumor cells may not always be dominant in regulating antitumor activity by macrophages. Unlike findings in other cancers, we found that disruption of CD47 activity via antibody blockade was not sufficient for inciting antitumor responses in an immunocompetent tumor model of PDAC. Deletion of tumor-expressed CD47 was also not sufficient for inducing in vitro phagocytosis of tumor cells or in vivo antitumor responses. The absence of macrophage activity in this setting was not due to a lack of pro-phagocytic signals as treatment with CD47-blocking antibodies, which contain Fc-domains for driving Fc receptor dependent pro-phagocytic signals in macrophages, also failed to elicit macrophage antitumor activity in vitro and in vivo.

Further, in the absence of macrophage activation via CpG stimulation, we found that CD47-blocking antibodies were insufficient for stimulating macrophage phagocytosis of multiple solid tumor types including colorectal, glioblastoma, melanoma, breast cancer and lung cancer. Our data support a conceptual model in which inhibitory signals, such as CD47, can be overcome by the activation state of macrophages, which is defined by core metabolic pathways that govern their antitumor potential. Consistent with this, we observed a notable role for CD47 in regulating macrophage antitumor activity in vitro, but only when macrophages were activated with CpG, which provides rationale for combining CD47 antagonists with myeloid agonists. However, this finding was not appreciated in vivo where CpG-induced antitumor activity was indistinguishable between tumors that expressed or lacked CD47. This may reflect the emergence of additional anti-phagocytic signals that are redundant with CD47 expressed by tumor cells or even the capacity of CpG to more effectively activate macrophages in vivo to circumvent inhibitory signals mediated by CD47.

The functional consequences of metabolic changes in macrophages has predominately been defined in cancer, despite findings to suggest that lipid metabolism has a pivotal role in governing the antitumor functions of several other immune cell types (for example, myeloid-derived suppressor cells, dendritic cells and T cells)\(^\text{13,22,40}\). We showed that, similar to M1 macrophages, CpG-activated macrophages redirected exogenous fatty acids and glucose toward acetyl-CoA generation and de novo lipid biosynthesis. We also found, as seen in M2 macrophages, that FAO and glutaminoysis were essential for the oxidative phenotype of CpG-activated macrophages\(^\text{17,14}\). Notably, M2 macrophages are recognized to possess superior abilities than M1 macrophages in phagocytosing antibody-opsonized tumor cells\(^\text{41}\), suggesting an association between FAO and phagocytic capacity. Functionally, FAO in antitumor macrophages may enable the breakdown of large lipid loads after engulfment of target cells or fulfill the metabolic demands for phagocytosis\(^\text{42}\). Together, our findings substantiate the importance of lipid metabolism as a dominant regulator of antitumor activity by macrophages.

Changes in lipid metabolism can alter plasma membrane properties in macrophages and in doing so, confer them with an ability to phagocytose more effectively\(^\text{43}\). Our data show enhancement of membrane fluidity induced by CpG that is dependent on acetyl-CoA shunting for de novo lipogenesis. In turn, membrane fluidity can modulate CD47-SIRP\(\alpha\) signaling and receptor clustering at phagocytic synapses\(^\text{43,44}\). Macrophage activation by TLRs has been shown to promote lipid-modifying programs that alter ceramide and sphingolipid species, which may influence the membrane properties of macrophages\(^\text{45}\). Analogously, CpG activation enhances lipogenesis in macrophages, which consequently modulates secretory function\(^\text{13,46}\). Consistent with this, we identified secretory pathways between the endoplasmic reticulum and Golgi to be critical for CpG-induced phagocytosis. In non-cancerous settings, lipogenesis supports macrophage engulfment of microparticles, due to its role in phospholipid synthesis and expansion of cellular organelles (for example, endoplasmic reticulum and Golgi)\(^\text{44}\). However, the relevance of de novo lipogenesis for macrophage activity in cancer and for circumventing inhibitory signals of phagocytosis (that is, CD47) has not been previously reported. We find that CpG increases mitochondrial abundance and enhances FAO, along with shunting of acetyl-CoA toward cholesterol biosynthesis. This mechanism is necessary for CpG to endow macrophages with the capacity to phagocytose tumor cells, without need for engaging Fc-receptors or blocking CD47 activity.

Most clinical studies evaluating CpG oligonucleotides have investigated subcutaneous delivery and weekly administration with the goal of stimulating dendritic cells and mobilizing T cell immunity. However, thus far, CpG has demonstrated limited efficacy\(^\text{47–49}\). In contrast to these studies, we administered CpG systemically and repeatedly to incite robust and sustained macrophage activation. Our data suggest that activation of adaptive immunity may not always be required for CpG-induced antitumor activity and support evaluating the potential of CpG to stimulate macrophage antitumor activity in the clinical setting.

The mechanisms involved in shifting the role of macrophages from pro- to antitumor have remained poorly defined. We found that CSF1R\(^+\) macrophages were necessary for mounting an antitumor response on CpG activation, despite this subset of macrophages being associated with immunosuppression\(^\text{10}\). Our findings show a role for core metabolic processes, which act together as a key regulator of macrophage antitumor activity. Our findings also highlight the importance of the metabolic state of macrophages for circumventing immune checkpoint signals with implications for broadening the impact of immunotherapy and identify macrophage metabolism as a novel therapeutic target that must be appropriately wired to enable macrophages to carry out antitumor functions.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at [https://doi.org/10.1038/s41590-018-0292-y](https://doi.org/10.1038/s41590-018-0292-y).

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Mice. C57BL/6J, Rag2−/− (B6Cg-Rag2−/−J), CD11c-DTR/εGFP (B6. FVB-Tg(creERTM1(Asc))1Mcl mice were obtained from Jackson Laboratories. CD11c-DTR/εGFP heterozygotes were enrolled in tumor studies. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania and conducted in compliance with the guidelines for animal research by the National Institutes of Health.

**Cell lines.** PDAC.1 (152 PDA) and PDAC.2 (69 PDA) cell lines were derived from PDAC tumors, as previously described, which arose spontaneously in KrasG12D−/−, Trp53R16H−/−, Pdx-1Cre mice backcrossed into the C57BL/6 background (Jackson Labs). Cell line authentication was performed as previously described. Isogenic PDAC lines were established by cloning single cells for in vitro and in vivo experiments. PDAC knockout lines were generated as previously described (Supplementary Table 3). Tumor lines from American Type Culture Collection (ATCC) were reviewed and approved by the Institute of Animal Care and Use Committee (IACUC) of the University of Pennsylvania and conducted in compliance with the guidelines for animal research by the National Institutes of Health.

**Tissue culture.** Tumor cells were cultured in DMEM (Dulbecco’s Modified Eagle’s Media, Thermo Scientific Fisher) supplemented with 10% v/v FCS (fetal bovine serum, Thermo Scientific) and 1% antibiotic-antimycotic (Sigma). Tumor cells were maintained at 37 °C and 5% CO2. Cell counts were determined using a CytoFLEX S flow cytometer (Beckman Coulter). PDAC cells were gathered from culture, washed and used as controls when appropriate.

**Generation of BMDMs.** BMDMs were derived by isolating bone marrow from mice euthanized with CO2 overexposure. Following treatment with ACK lysis (10 ng ml−1) and Eagle’s Media, Thermo Scientific Fisher) supplemented with 10% v/v FCS (fetal bovine serum, Thermo Scientific渔父) or DMSO vehicles were used as controls when appropriate. Macrophages were applied to activated macrophages for 12–24 h before assays. For mock-treatment, PBS or DMSO were used as controls.

**Flow cytometry.** Tumor cell cultures were treated with trypsin (Thermo Scientific Fisher) and prepared as a single-cell suspension for flow cytometric analysis. BMDMs in culture were incubated in cell dissociation buffer (Thermo Scientific Fisher) before mechanical detachment and resuspension. For intraacellular stains, cells were permeabilized in 0.1% Triton-X (Sigma) for 15 min, washed and stained with 5 µM DiO (Life Technologies) and washed before being seeded at 5 × 104 cells per well. Following 4 h co-culture, samples were washed and fixed in 4% PFA (paraformaldehyde, Sigma) for 15 min before image collection. For antibody blockade, tumor cell suspensions were incubated with 10 µg ml−1 isotype (2A3, BioXcell) or rat-anti-mouse anti-CD47 (Mipat301, BioXcell) antibodies for 20 min, before being washed and co-incubated with macrophages. Macrophages random fields were captured using a CellHarvest microscope, and the number of phagocytic events were scored and averaged for each replicate.

**Immunohistochemistry.** Frozen sections of PDAC tumors were fixed for 20 min in 4% PFA, followed by permeabilization in ice-cold methanol for 20 min. Samples were fixed for 1 h in 10% goat serum and stained for 4 h in primary antibodies (rabbit-anti-mouse cytokeratin 19, rat-anti-mouse F4/80). After washing, secondary antibody (488 goat-anti-rabbit, 568 goat-anti-rat) was applied for 1 h. Samples were washed, and images were captured using an Olympus IX83 microscope. Background reduction was performed in CellSens software (Olympus) and images were subsequently processed using EBImage software analysis in R to perform color deconvolution, adaptive thresholding of F4/80 signal, Gaussian blur and binarization of signal. Size exclusion was applied to remove particles with a radius less than 10 pixels or greater than 200 pixels. The green (CK) signal was normalized intrinsically for each image to minimize variability in staining intensity. The phagocytic index was determined by calculating the normalized green (CK) intensity within each F4/80+ cells.

**Immunofluorescence microscopy.** Treated BMDMs were collected and fixed using 4% PFA and treated with 1% hydrogen peroxide (Sigma). Samples were fixed for 10 min in 10% goat serum (Vector Laboratories) in PBS and treated overnight with primary antibodies (Supplementary Table 2). Secondary antibody was applied for 1 h, and samples were stained with ABC HRP kit ( Vectastain) per manufacturer instructions. Samples were washed with DAB (3, 3-diaminobenzidine) HRP substrate (Vector Labs) before counterstaining with hematoxylin (Sigma) and dehydration. Images were captured using the Olympus BX-43 microscope.

**Membrane fluidity.** BMDMs were seeded at 2.5 × 104 cells per well in 24-well plates, and stimulated under mock, CpG, CpG + ALCY inhibitor (BMS 303141) or CpG + e-toxinrix conditions for 72 h. Samples were then fixed in 4% PFA, permeabilized in 0.1% Triton-X for 15 min, and stained with 5 µg ml−1 BODIPY-C16 (Thermo Scientific) for 1 h. After labeling, cells were washed, detached and analyzed by flow cytometry on a FACScanto Canto.

**Antitumor activity assay.** Luciferase-labeled PDAC cells were collected as a single-cell suspension and seeded into a 96-well tissue culture plate at 5 × 104 cells per well. Pretreated BMDMs were collected by mechanical detachment and seeded at a 0.1, 1:1, 2:1 and 5:1 macrophage-to-tumor ratio. For 48 h co-culture, tumor cell survival was determined using Luciferase assay system (Promega). Luminescence measurements were performed using a SpectraMax M3 reader ( Molecular Devices). Tumor cell survival was determined by normalizing luminescencce to tumor-only controls.

**Antibody activity assay.** Luciferase-labeled PDAC cells were collected as a single-cell suspension and seeded into a 96-well tissue culture plate at 5 × 104 cells per well. Pretreated BMDMs were collected by mechanical detachment and seeded at a 0.1, 1:1, 2:1 and 5:1 macrophage-to-tumor ratio. For 48 h co-culture, tumor cell survival was determined using Luciferase assay system (Promega). Luminescence measurements were performed using a SpectraMax M3 reader ( Molecular Devices). Tumor cell survival was determined by normalizing luminescencce to tumor-only controls.

**Tumor growth studies.** PDAC cells were gathered from culture, washed and pretreated as a single-cell suspension for implantation into the subcutis of mice. Rat-anti-mouse anti-CD47 (Mipat301, BioXcell) and isotype control antibodies (2A3, BioXcell) were diluted in saline, and 50 µg of each antibody was delivered intratumorally on days 10 and 14 after tumor implantation, as previously described (Supplementary Table 3). Tumor-bearing mice were treated with vehicle (PBS) or 50 µg CpG by intraperitoneal injection on every other day, beginning on day 10. For mouse survival (depletion), 200 µl of CEI (clodronate liposomes) was delivered by retroorbital injection into mice. 400 µg of anti-CSFIR Ig (AF598, BioXcell) diluted in saline was delivered by intraperitoneal injection on days 8, 10, 13, 16 or GW5280
(AdooQ) was delivered daily at 160 mg kg⁻¹ by oralavage. For natural killer cell depletion, 200 µg anti-NK.1 Ig (PK136, BioXcell) diluted in saline was delivered by intraperitoneal injection on days 8, 10, 13 and 16. For dendritic cell depletion in CD11c-DTR/eGFP, diphtheria toxin (Sigma) was administered by intraperitoneal injection at 8 ng g⁻¹ body weight. Etomoxir (Sigma) was resuspended in PBS and delivered daily at 40 mg kg⁻¹ by intraperitoneal injection as previously described (Supplementary Table 3). Tumor volume was monitored by caliper measurement and calculated using a formula for an ellipsoid: volume = 1/2 × length × width × length. For survival studies, mice were euthanized when tumors reached 1,000 mm³.

**ECAR and OCR measurements.** BMDMs were pretreated with TLR agonists or inhibitors and seeded into XF96-well plates (Agilent) at 1 × 10⁶ cells per well. Before measurements, samples were washed and incubated in Seahorse media (Agilent) supplemented with 0.5 mM D-glucose (Sigma). The mitotrigger kit (Agilent) was prepared per manufacturer instructions by loading 1.5 µM oligomycin 1.5µM FCCP and 1 µM rotenone/antimycin A into injection ports. Measurements were made using an XF96 Extracellular Flux Analyzer (Agilent) and results processed with Wave v.2.2.0 software.

**Cytotoxicity studies.** BMDMs and tumor cells were seeded at 1 × 10⁶ cells per well into a 96-well plate and treated with inhibitors diluted in Dulbecco’s Modified Eagle’s Media supplemented with 10% v/v FCS, 2 mM glutamax and 10 ng ml⁻¹ gentamicin. After 4h, the media was removed and samples were stained with 0.05% w/v crystal violet (Sigma) in 20% ethanol/80% H₂O. Samples were then washed and optical density measured at 570 nm using a Spectramax M3 spectrophotometer. Cell viability was determined by normalizing optical density of treated conditions to mock-treated controls.

**¹³C metabolite tracing.** After pretreatment with CpG or vehicle for 96 h, macrophages were counted and seeded at 4 × 10⁶ cells per sample. For glucose and glutamine labeling, U⁻¹³C-glucose and U⁻¹³C-glutamine (Cambridge Isotope Laboratories) were substituted into DMEM supplemented with 100 µM U⁻¹³C-palmitate (Cambridge Isotope Laboratories) were substituted into DMEM supplemented with 10% v/v dialyzed FCS (Life Technologies), 4 mM L-glutamine (Sigma) and 25 mM glucose (Sigma). Cells were incubated at 37 °C in labeled media for 2 h before collection. For palmitate labeling, cells were cultured in DMEM supplemented with 100 µM U⁻¹³C-palmitate (Cambridge Isotope Laboratories), 10% v/v charcoal-stripped FCS (Life Technologies), 4 mM L-glutamine (Sigma) and 25 mM glucose (Sigma) for 4h. Both tracer experiments included unlabeled control samples not exposed to ¹³C-labeled substrates. After incubation, cells were gathered and placed on ice. Samples were then pelleted, washed in cold PBS and collected in 750 µl of 5% 5-sulfosalicylic acid and 10 µl of 15N1-labeled internal standard. Samples were centrifuged at 1,200g for 10 min at 4 °C and pulse-sonicated with a probe sonicator (Fisher Scientific). Lysates were centrifuged at 15,000g for 15 min, and the supernatants were further purified by solid-phase extraction using (OASIS HLB) columns. Supernatants were then applied, and columns were washed with 1 ml H₂O. Analytes were eluted in 25 mM ammonium acetate in methanol and evaporated to dryness overnight by N₂ gas. Samples were resuspended in 50 µl of 5% 5-sulfosalicylic acid and 10 µl injections were applied in liquid chromatography/electrospray ionization/tandem mass spectrometry analysis. Isotopologues were designated as unlabeled (M + 0), containing one ¹³C isotope (M + 1), two ¹³C isotopes (M + 2) and so on, following tracer labeling.

**Cytokine analysis.** Peripheral blood was collected by tail vein bleed on day 14, and the serum fraction was isolated following centrifugation at 10,000g for 15 min. For in vitro experiments, 1 × 10⁴ macrophages were cultured in 1 ml of media. Supernatant was collected following 48 h activation with cytokines and TLR agonists. Serum and supernatants were analyzed per manufacturer instructions using cytokine bead array kits (BD Biosciences) for IFN-γ, TNF and CCL2. Beads were analyzed using a FACS Canto II.

**Statistics and software analysis.** P values were calculated using a two-tailed unpaired Student’s t-test and Hochberg correction for multiple comparison testing, unless stated otherwise. Linear mixed effects models were built to include random effects for each cell and image fields; statistical differences between treatment conditions were determined by ANOVA comparison of linear mixed effects models. For survival analysis, significance was determined using the Kaplan–Meier log-rank test. P values of 0.05 or less were considered significant. Error bars indicate standard deviation unless stated otherwise. Data analysis and graphical design was performed using R software (v.3.4.3), R-studio and additional R-packages: ggplot2, dplyr, EBimages, lme4, kmsurv and heatmap.2

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data and code that support the findings of this study are available within the paper and its Supplementary Information files and are available from the corresponding author upon reasonable request.

**References**

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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- [ ] n/a
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- [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- [ ] A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- [ ] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- [ ] Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- [ ] Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection N/A

Data analysis

Data analysis was performed using R software (v3.4.3), R-studio, and additional R-packages: ggplot2, dplyr, reshape2, EBimages, Ime4, and heatmap.2 (CRAN). Flow cytometric analysis was completed using FlowJo (v10.3). Figure development utilized Adobe Illustrator CS6.

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were estimated based on pilot experiments, in which control tumors reached an average volume of 300 mm³ at day 18 post-implantation, with a sigma of 50 mm³. Assuming a medium effect size with treatment (35% tumor size reduction), sample sizes were determined for a two-sided statistical test, with the potential to reject the null hypothesis with a power (1-β) of 80%, subject to α=5. |
| --- | --- |
| Data exclusions | Tumor-bearing mice were enrolled for study if tumors reached 5 mm diameter at day 10 post-implantation. Tumors that implanted intraperitoneally prior to commencing treatment on day 10 were censored. Tumors demonstrating severe ulceration were censored from flow cytometric endpoint analysis. |
| Replication | All attempts at replication were successful. Experiments were reproduced in at least two independent experiments as indicated. |
| Randomization | For in vivo studies, mice were stratified by gender and block randomized for tumor implantation and treatment. |
| Blinding | Investigators were not blinded to most studies as interventions required knowledge of the individual experimental groups. To establish objective measurements from studies incorporating immunohistochemistry and immunofluorescent microscopy, multiple random fields were collected per replicate, and cell counts in each field were averaged for every biological replicate. |

Reporting for specific materials, systems and methods

Materials & experimental systems

- n/a Involved in the study
- ✗ Unique biological materials
- ✗ Antibodies
- ✗ Eukaryotic cell lines
- ✗ Palaeontology
- ✗ Animals and other organisms
- ✗ Human research participants

Methods

- n/a Involved in the study
- ✗ ChIP-seq
- ✗ Flow cytometry
- ✗ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

| Obtaining unique materials | All unique materials used are readily available from the authors |

Antibodies

| Antibodies used | Rat-anti-mouse anti-CD47 (clone MIAP301, BioXcell, BE0270) and isotype control antibodies (clone 2A3, BioXcell, BE0089) were administered intratumorally as previously described in (Liu X et al, Nature Medicine; PMCID: PMC4598283). Details for commercial antibodies used in flow cytometry and immunofluorescence/immunohistochemistry microscopy are described in supplementary tables 1 and 2. |
| Validation | Antibodies were validated by flow cytometry with corresponding data including in figures presented in the manuscript. |

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | PDAC.1 (152 PDA) and PDAC.2 (69 PDA) cell lines were isolated from pancreatic tumors, as previously described (Long et al, |
Cell line source(s) | Cancer Discovery 2016), which arose spontaneously in KrasLSL.G12D/+; Trp53R172H/+; Pdx-Cre mice were backcrossed >10 generations onto the C57Bl/6 background. For genetic studies, isogenic clones from these lines were used to minimize intratumoral heterogeneity.

Authentication | Tumor cell lines were authenticated to be syngeneic with the C57BL/6 background using DartMouse sequencing.

Mycoplasma contamination | Tumor cell lines were routinely tested for mycoplasma using MycoAlert (Cambrex).

Commonly misidentified lines (See ICLAC register) | No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals | C57BL/6 mice were obtained from Jackson Laboratories and were bred at the University of Pennsylvania. Male and female C57BL/6 mice aged between 9-16 weeks were enrolled in studies or used for derivation of bone marrow macrophages. Tumor bearing male and female KPC mice (KrasLSL.G12D/+; Trp53R172H/+; Pdx-Cre mice backcrossed onto the C57Bl/6 background) were age and gender matched with Pdx-Cre mice for analysis.

Wild animals | N/A

Field-collected samples | N/A

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation | Details regarding the biological source of cells and tissue processing steps are describes in the Methods. Specifically, pancreatic tumor cell cultures were treated with trypsin and prepared as a single-cell suspension for flow cytometric analysis; murine macrophages in culture were incubated in cell dissociation buffer prior to mechanical detachment and resuspension. For intracellular stains, cells were permeabilized in 0.1% Triton-X for 15 min. For tumors, samples were harvested upon necropsy, mechanically separated, and digested in 1 mg/ml collagenase and 100 μUnits/ml DNase I. Tumor digests were filtered and resuspended as single cell suspensions. Cells were treated with Fc-block, then stained with Amcyan live/dead dye.

Instrument | FACS Canto (BD Biosciences)

Software | Samples were collected using FACSDiva software (v8.0.1) and analysis was performed using FlowJo (v10.3)

Cell population abundance | Following CRISPR-deletion in an isogenic line, CD47+ and CD47- cells sorted from populations treated with control or CD47-targeting guide RNA resulted in post-sort fractions that were respectively 95-100% CD47+ and CD47-. Following GFP-labeling, sorting for GFP+ cells in CD47+ and CD47- populations resulted in comparable 95+% GFP+ populations.

Gating strategy | Gating strategies are described in supplementary figures 4 and 5. Specifically, singlet cells were gated on using FSC-H versus FSC-A, and live cells were identified by exclusion of AmCyan Live/Dead stain. Additional gating was based on thresholds from staining of FMO and isotype antibody when possible.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.