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Review

# Engineering macrophages to eat cancer: from "marker of self" CD47 and phagocytosis to differentiation

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## ABSTRACT

**The ability of a macrophage to engulf and break down invading cells and other targets provides a first line of immune defense in nearly all tissues. This defining ability to "phagos" or devour can subsequently activate the entire immune system against foreign and diseased cells, and progress is now being made on a decades-old idea of directing macrophages to phagocytose specific targets, such as cancer cells. Engineered T cells provide precedence with recent clinical successes against liquid tumors, but solid tumors remain a challenge, and a handful of clinical trials seek to exploit the abundance of tumor-associated macrophages instead. Although macrophage differentiation into such phenotypes with deficiencies in phagocytic ability can raise challenges, newly recognized features of cancer cells that might be manipulated to increase the phagocytosis of those cells include ≥1 membrane protein, CD47, which broadly inhibits phagocytosis and is abundantly expressed on all healthy cells. Physical properties of the target also influence phagocytosis and again relate—via cytoskeleton forces—to differentiation pathways in solid tumors. Such pathways extend to mechanosensing by the nuclear lamina, which is known to influence signaling by soluble retinoids that can regulate the macrophage SIRPα, the receptor for CD47. Here, we highlight some of those past, present, and rapidly emerging efforts to understand and control macrophages for cancer therapy.**
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## Introduction

Phagocytosis is an ancient, cytoskeleton-intensive process of cell-level eating that has continually evolved from amoebae to higher organisms. In humans, phagocytosis is the defining process of the MPS. The two principal cell types of the MPS are Mϕs, which reside in every tissue, and monocytes, which differentiate to Mϕs when exiting circulation to enter tissues [1, 2]. MPS cells, along with highly phagocytic neutrophils, must—for the health of the organism—selectively devour "foreign" targets, such as microbes, rather than phagocytose the human "self" cells or extracellular matrix that typically surrounds our phagocytes. Mϕs have a uniquely efficient capacity to phagocytose multiple targets, digest them, and search for more, including some types of diseased cells among healthy cells [3]. However, Mϕs fail to perceive and attack tumors despite their foreign (i.e. mutated) genomes [4, 5].

Mϕs are abundant and motile in solid tumors [4–6] compared with T cells, which infiltrate minimally [7–9]. The latter observations might help explain the poor clinical trial outcomes for T cell therapy of solid tumors [10, 11]. On the other hand, Mϕs are not only "plastic," in the sense that they exhibit a broad capacity to differentiate in different microenvironments, but also link the density of the "tumor-associated Mϕ" phenotype with promoting tumor growth, inducing angiogenesis, and inhibiting other immune effector cells [5, 12–15]. Clinical data show that a high density of tumor-associated Mϕs is indeed correlated with poor prognosis [16]. *Tumor-associated Mϕ* is perhaps a misnomer in the strict sense of the Mϕ as a giant cell that devours because these cells seem to have lost most or all of their ability to phagocytose, and their low MHC-II expression likely hinders their activation of the adaptive immune system against tumor neoantigens [1, 5, 17]. In efforts to address some of the above challenges, engineering of Mϕs ex vivo for "adoptive transfer" back into patients with cancer has been pursued for many years [18, 19], but some new insights into Mϕ interactions and plasticity—as reviewed here—might prove useful in reinvigorating such approaches (**Fig. 1A**). (The text in this article adheres to nomenclature standards but might sometimes add a species designator. For example, hCD47 and *CD47* symbolize the human protein and gene, whereas mCD47 and *Cd47* symbolize the mouse protein and gene. The designators are added because interactions tend to be species specific,

Abbreviations: MPS = mononuclear phagocyte system, OA3 = ovarian cancer Ag (CD47), SIRPα = signal-regulatory protein α, SHP1 = Src homology region 2 domain-containing phosphatase-1, TAM = tumor associated macrophage

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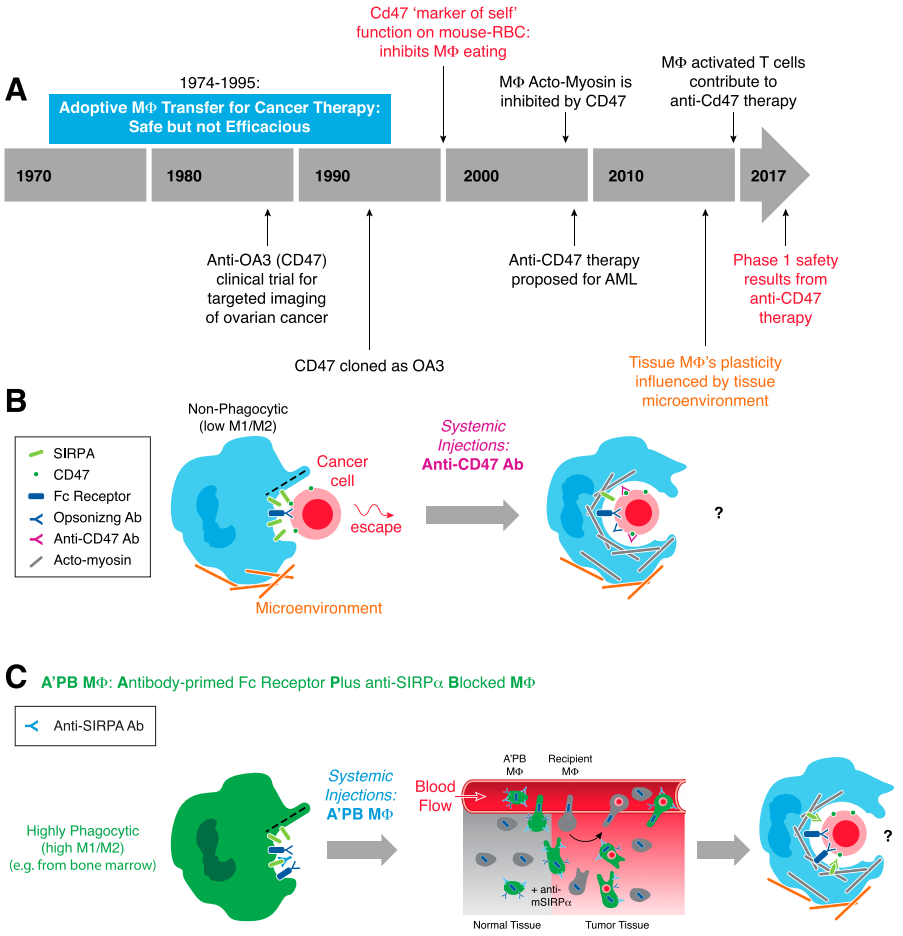


**Figure 1. Anti-cancer Mϕ and CD47.** (A) Timeline of adoptive Mϕ transfer and CD47 studies converging on anti-CD47–focused Mϕ therapies. (B) Inhibition of cancer cell engulfment because of recognition of CD47 by a nonphagocytic phenotype, despite the presence of a pro-phagocytic Ab. Addition of anti-CD47–blocking Ab and a more phagocytic phenotype can drive engulfment. The actomyosin cytoskeleton has a key role in phagocytosis and in linking the microenvironment to influence the phenotype. (C) Ab modification (blocking SIRPα and loading Fc receptor with targeting Ab) of marrow Mϕs, followed by systemic injection, could be an effective method for adoptive Mϕ cancer therapy. In circulation, antibody-primed Fc receptor plus anti-SIRPα blocked Mϕ (A'PB Mϕ) could, in principle, migrate into tumors, phagocytose cancer cells, and then either exit the tumor or continue to destroy tumor cells.

whereas experiments are being conducted with tumor xenografts.)

## RE-ADOPTING Mϕ FOR CELL THERAPY

Adoptive Mϕ transfer was first pursued decades ago in some of the earliest cell therapy efforts against cancer. Monocytes isolated from peripheral blood were cultured in conventional dishes for most preclinical studies and in Teflon bags for clinical trials, and then "engineered" by differentiation into some form of adherent Mϕ such as with IFN-γ and LPS, before ultimately being injected back into patients. Safety was established with injections $<1.5 \times 10^9$ cells [19]. For comparison, roughly $10^5$ WBCs egress from human marrow every second, and only ~5% are monocytes (versus $\sim 10^6$ RBCs egressing per second), so that the Mϕ injections are equivalent to what would be normally produced over a few days as naive cells. However, efficacy assessments in those early clinical trials showed little to no benefit of the in vitro engineered Mϕ [18, 20–22].

It was understood decades ago that, for Mϕs to destroy cancer cells, they needed to be activated, and numerous soluble and/or surface-bound factors could act as molecular cues to stimulate MPS destruction of foreign targets. IgG Abs are among the most modular (and now designable) because they signal via the Mϕ membrane receptor FcγR (involving specific isoforms of FcγR and IgG). IgGs produced by B cells perfuse and diffuse throughout the body and bind to a target surface so that when a Mϕ contacts the target, the constant fragment (Fc) of the IgG binds the FcγR to signal phosphorylation of ITAMs, which then propagate a phosphorylation cascade that regulates adhesion and cytoskeletal remodeling [23]. Phospho-paxillin, F-actin, and myosin-II are just a few among many such proteins that subsequently accumulate within minutes at this dynamic phagocytic synapse [24–26]. Ab-dependent, cell-mediated cytotoxicity and Ab-dependent cellular phagocytosis by Mϕs have indeed been reported to be crucial to anticancer mechanisms in vitro and in vivo [27]. Studies often prove this by depletion of TAMs after systemic injection of clodronate particles, but that approach has shortcomings, as highlighted below. Nonetheless, those pro-phagocytic signals are also balanced by inhibitory signals. Engagement of FcγRIIB (CD32B) causes activation of ITIMs, which promote internalization of pro-phagocytic IgGs, preventing activation of ITAMs. Blocking FcγRIIB can prevent internalization of therapeutic Abs, such as rituximab, and thereby increase cell-surface accessibility of such Abs by Mϕs [28, 29].

Early studies of adoptive Mϕ transfer explored ex vivo incubation of engineered Abs that targeted the Fcγ receptors on Mϕs and specific Ags on tumor cells [30–33]. The approach

failed to control tumor growth [19] with one explanation being a minimal activation of the Mϕ Fcγ receptor because the downstream response varied greatly with engagement, Ab isotype, and species [34]. Unfortunately, the apparent inability to strongly activate and control phagocytosis dampened interest in adoptive-transfer approaches to treat cancer with Mϕs.

## CD47 SIGNALS "DON'T EAT ME"

In watching a movie of phagocytosis, it is easy to assume that failure of a seemingly activated Mϕ to engulf a target reflects a lack of surface "opsonization" or signaling by molecules, such as IgG. However, it is now clear that, in addition to "foreign" signals, there are also opposing signals for specific recognition of "self." If opsonization is analogous to putting your foot on the gas, the self-signaling is a powerful brake that overrides the phagocytosis process. Indeed, a dominating and passivating interaction occurs between the ubiquitous "marker of self" CD47 membrane protein on a candidate target cell (or particle) and the Mϕ membrane receptor SIRPα [35–37]. Phagocytosis of cancer cells that are targeted by opsonizing IgG might thus benefit by simultaneous blockade of CD47, even given the limited phagocytic capacity of TAMs (Fig. 1B). Alternatively, bone marrow–derived Mϕs are highly phagocytic in studies when SIRPα has been blocked in vitro [35]. Whether systemic injections of such "Ab-primed Fc receptor plus anti-SIRPα blocked Mϕs" can find their way in vivo to a tumor and subsequently phagocytose opsonized cancer cells (Fig. 1C) should be very interesting to assess.

Within a Mϕ that is phospho-activated through engagement of a target via an IgG–FcγR interaction, simultaneous parallel engagement of CD47-SIRPα activates the tyrosine phosphatase SHP1, via SIRPα's ITIMs, which in turn deactivates the myosin-II contractile cytoskeleton to greatly impede phagocytosis [26, 38]. F-actin polymerization is uninhibited, and filopodial protrusions even tend to push a "self-recognized" target away from being engulfed [39]. More studies are needed of such structure–function signaling, in part because a deep understanding of the balance of "eat me" cues (e.g., IgG–FcγR interaction) and "don't eat me" signals (CD47-SIRPα) has implications for therapeutic applications. Initial clinical trials are already focused on anti-cancer therapy [40], but pre-clinical studies also demonstrate CD47 utility in reducing Mϕ uptake of "foreign" nanoparticles and lentiviral vectors for drug and gene delivery [38, 41].

Before the cloning and formal naming of CD47 in the mid-1990s [36, 42], this ubiquitous membrane protein was already referred to as OA3 Ag because of the abundant binding of a monoclonal IgG (OVTL3) to ovarian cancers. Even earlier, bivalent $F(ab')_2$ fragments of this mAb against the single, extracellular, Ig-like domain of CD47/OA3 had already been used for targeted radioimaging. Despite ubiquitous expression of CD47, imaging results were described as showing 80% specificity in 31 patients [43]. Any inhibition of "self-recognition" is unlikely to have affected the growth of the tumors in these studies done decades ago (see below), but through retrospective analyses of the anti-CD47 injection protocols and outcomes might inform current concerns of the safety (or not) of anti-CD47 injections in patients with cancer.

Numerous human cancers have since been reported to display CD47 at levels >3-fold higher than expression on healthy tissues [44, 45]. High levels of CD47 seem to correlate with poor clinical outcomes [44, 46, 47]. CD47 and another immune inhibitor, PD-L1, are either strongly turned on or are simply selected for during early cancer development, and both are transcriptionally controlled by c-Myc as a common oncogene [48, 49]. Low levels of CD47 on various cancerous and noncancerous cells are typical of apoptosis and combine with various opsonizing factors to favor clearance by Mϕs [50–52]. Despite these emerging observations, the processes that occur within the Mϕs during phagocytosis continue to require rigorous study, particularly because most studies of Mϕ involvement in tumor shrinkage have relied on systemic injection of clodronate particles to poison Mϕs even though this approach can cause variability in tumor growth [53, 54] (**Fig. 2A**) and assumes uptake is efficient in its effects on the desired cells (TAMs) with no broader effect on other cells (e.g., other Mϕs or cancer cells). Isolation of Mϕ from tumors for direct assessments of phagocytosis seem essential to advancing the field.

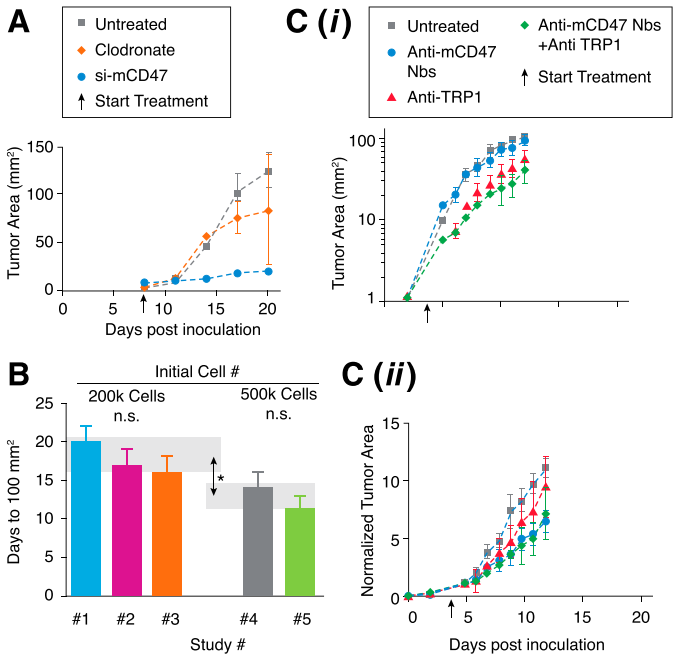


**Figure 2. Tool kit for studying the effect of CD47 inhibition on tumor growth.** (A) Growth curve of syngeneic, orthotopic B16F10 tumors in C57 mice show the effects that si-mCD47 and clodronate liposomes have on tumor growth. Data adapted from Wang et al. [53]. (B) Analysis of how long untreated orthotopic B16F10 tumors in C57 mice take to reach 100 $mm^2$ when challenged with either 200,000 or 500,000 cells. Data are adapted from Alvey [unpublished results] and studies, Wang et al. [53], Bencherif et al. [63], Ali et al. [110], and Sockolosky et al. [57]; $^*P \leq 0.05$. (C) Growth curves of orthotopic B16F10 tumors in C57 mice treated with a combination of anti-CD47 nanobodies and an Ab that binds tyrosinase-related protein 1 (Trp1). Data adapted from Sockolosky et al. [57]. *i*) Log-scale growth highlights differences in tumor sizes between treatment conditions near the start of the treatment. *ii*) Normalizing growth data to d 5 gives a different interpretation from the reported conclusions in (*i*): anti-TRP1 has only a small effect, but a combination with the anti-CD47 nanobody can significantly reduce tumor growth.

## ANTI-CD47 THERAPY AND SAFETY

Clinical trials of CD47 blockade for therapy are rapidly emerging with anti-human CD47 Abs (**Table 1**). These trials rely on TAMs and, perhaps, on infiltrating monocytes that are partially or fully inhibited from recognizing tumors as self [44, 46]. A few preclinical models with syngeneic tumors have shown partial inhibition of tumor growth when using either anti-mCD47 or small interfering RNA knockdown of *Cd47* with nanoparticles [53, 55]. Success against human-derived xenografts has extended to human cancer stem cells in mice [56]. However, most preclinical models show CD47 disruption alone is insufficient [46, 55, 57]. Strongly pro-phagocytic signals (like a heavy foot on the gas pedal) combined with effective inhibition of CD47 (to eliminate any braking) seem necessary to drive phagocytosis of cancer cells by TAMs [46, 55, 57] (Fig. 1B). Anti-CD47 Abs had been thought sufficient to inhibit CD47 and activate phagocytosis through Fc engagement, but results have been mixed at best, even when combined with tumor pro-phagocytic signals, such as calreticulin and phosphatidylserine [58–61].

One recent study [62] failed to replicate any efficacy with anti-mCD47 inhibition and questioned the statistical significance of past data [44]. An additional concern arose with the syngeneic orthotopic breast tumor model used in both studies because the tumors were reported to spontaneously regress during the replication studies [62]. An alternative, syngeneic, orthotopic tumor model with well-documented robustness in reproducibility is the melanoma model B16F10, derived from, and engrafted in, C57 mice. This model shows consistent tumor growth rates between different laboratories and over time, which suggests it is a very predictable and useful model [53, 63] (Fig. 2B); although the B16F10 cells can reportedly change phenotype with loss of their dark melanin pigmentation after extended passage in standard culture. Even with a reliable tumor model, another cause of uncertainty in the field seems to arise from the numerous ways tumor growth data are reported: publications commonly show either tumor volume (often assuming shape or height), tumor cross-sectional area (measured or estimated from shape) or normalized tumor area, but each tumor metric can yield a different conclusion. In one recent study of B16F10 tumors, for example, the authors reported that injection of an Ab against the melanin-pathway factor tyrosinase-related protein 1 (TRP1) was sufficient to significantly reduce tumor growth in mice [57]. However, anti-TRP1–treated tumors were 2–3-fold smaller than control tumors within 1 d after treatment started (Fig. 2C[*i*]). When the data are normalized to that d 5 time point, anti-TRP1 shows no effect, whereas the combination of anti-TRP1 and anti-CD47 nanobody does seem to inhibit tumor growth when normalized (Fig. 2C[*ii*]). Such reanalyzed findings again suggest that shrinkage of tumors with anti-CD47 requires at least a combination with another tumor-opsonizing Ab, such as rituximab or trastuzumab used in other studies [46, 55].

Safety of anti-CD47 Ab injections remains a concern. Injections of anti-mCD47 in mice and anti-hCD47 monkeys led to a 30% decrease in RBC counts within days after a single injection [55, 64]. Abs and other serum proteins bind both specifically and nonspecifically to RBCs [65, 66], to viruses [67], and even to particles coated with polyethylene glycol [68], and so "eat me" signals are always present. Perhaps related, one strain of CD47 knockout mouse survived for only 6 mo and had detectable IgG against mouse RBCs, as well as anemia and organ failure [69]. Inhibiting the receptor for CD47 on Mϕ, SIRPα, also enhances phagocytosis in vitro [35, 70] and in vivo [38], and the latter studies showed that systemic injection of anti-SIRPα Abs led to rapid clearance of circulating components [38]. Despite the caution required from these data, a growing number of clinical trials are using anti-CD47 Abs in patients with diverse liquid and solid tumors that range from leukemia to colorectal cancer [71–80] (Table 1).

**TABLE 1. CD47-SIRPα clinical trial data**

| Compound | Company | Target | Treated disease | Start date | Estimated completion date | Phase |
|---|---|---|---|---|---|---|
| Hu5F9-G4 | Forty Seven Inc., Menlo Park, CA, USA | CD47 | Colorectal neoplasms/ solid tumors | November 1, 2016 | March 1, 2023 | Phase I/phase II (cetuximab) |
| Hu5F9-G4 | Forty Seven Inc. | CD47 | Non-Hodgkin/large B cell lymphoma | November 1, 2016 | January 1, 2023 | Phase I/phase II (rituximab) |
| TTI-621 | Trillium Therapeutics Inc., Mississauga, ON, Canada | CD47 | Melanoma/breast carcinoma/solid tumors | September 1, 2016 | December 1, 2019 | Phase I |
| CC-90002 | Celgene, Summit, NJ, USA | CD47 | Acute myeloid leukemia | July 27, 2016 | July 1, 2019 | Phase I |
| SIRPα Ab | Nantes University Hospital, Nantes, France | SIRPα | Hepatocellular carcinoma | June 16, 2016 | May 1, 2019 | Investigation |
| Hu5F9-G4 | Forty Seven Inc. | CD47 | Acute myeloid leukemia | January 27, 2016 | January 1, 2018 | Phase I |
| TTI-621 | Trillium Therapeutics Inc. | CD47 | Hematologic malignancies | January 19, 2016 | June 1, 2019 | Phase I |
| CC-90002 | Celgene | CD47 | Hematologic cancers/ solid tumors | February 13, 2015 | January 1, 2018 | Phase I |
| Hu5F9-G4 | Forty Seven Inc. | CD47 | Solid tumors | August 12, 2014 | August 1, 2017 | Phase I |
| None | Medical University South Carolina, Charleston, SC, USA | CD47 | Multiple myeloma | July 13, 2011 | July 1, 2014 | Prognostic potential for chemotherapy |

Chronological order of anti-CD47 antibody clinical trials on a variety of human cancers. Most of these trials were started in 2016 and include phase II studies in combination with an additional opsonizing antibody. Data adapted from references 63–72.

Blood analyses will likely provide the first evidence of safety in such human trials with the therapeutically relevant, high doses of anti-hCD47 injected intravenously. A short-term, mild anemia is expected and might even be evident in a retrospective analysis of patient data from the early radioimaging trial that used anti-CD47 targeting of ovarian cancer [81]. Leukocytes and platelets in human circulation are all likely affected by systemic anti-CD47 because CD47 is displayed on all cells and is known to prevent phagocytosis for most cell types. Nonetheless, the ease of a blood draw and the relatively tight control of hematocrit makes changes in RBCs easiest to quantify, and the youngest blood cells are routinely quantified only for RBCs (i.e., reticulocytes) in providing the clearest measure of an ongoing perturbation. RBC-clearing of Mϕs in the human spleen will likely initially phagocytose older RBCs, which are the most IgG opsonized and the stiffest [39]. However, a new steady state for the RBC life span is difficult to predict, given that CD47-null cells exist only in mice and not in humans.

Further background on blood production can be informative given the above. In the normal steady state, every second, ~1-million reticulocytes emerge from marrow to mature in about 1 d to discocyte RBCs, which replace old, opsonized, stiff RBCs that are cleared at ~100 d (reticulocytes are thus ~1% of RBCs). Within days of injecting anti-CD47 systemically, the oldest RBCs should decrease in age to about 70 d, based on noted mouse and primate studies (~30% loss in RBCs) [55, 64]. This will likely saturate engorgement of splenic Mϕs (splenomegaly when chronic), which may limit clearance of even younger, CD47-blocked RBCs. Enhanced production of reticulocytes (perhaps increasing to ~10%) will compensate for the rapid loss of RBCs. This degree of compensation is also observed in humans, who, secondarily to other genetic defects, lack ~90% of CD47 on their RBCs [82, 83]. Within weeks of continued anti-CD47 injections, the anemia is likely to become better compensated, and reticulocyte production should gradually decrease with the oldest RBC age remaining low at ~90+ d. An overabundance of CD47 on RBCs allows for a half-max effectiveness in 'self'-signaling with just ~10% of normal levels (i.e. 10% of ~250 molecules per sq. micron on RBC [38]). It will be important, therefore, to determine whether systemic anti-CD47 binds and blocks up to ~90% of CD47 and thereby mimics tolerable human deficiencies of CD47 or greatly exceeds such conditions. These projected estimations illustrate the careful consideration of CD47 quantities on various cells; determining how much anti-CD47 binds and blocks can thus make sense of past studies and new clinical results with humanized anti-CD47 IgG isotypes. Whether some patients develop anti-RBC antibodies as occurs in *Cd47*-null non-obese diabetic (NOD) mice will be a crucial safety issue to address.

## Mϕ BRIDGE TO ACQUIRED IMMUNITY

Although Mϕ engulfment of cancer cells can contribute to tumor reduction, phagocytic cells can also present neoantigens to T cells. Early hints of this have included the noted presence of IgG against RBCs in some strains of *Cd47* knockout mice (i.e. NOD strain), and differences in the effects of mCD47 blockade between syngeneic and immune-deficient tumor models [45, 53, 57, 69]. Absent any targeting of mCD47, vaccination studies have certainly documented T cell activation by Mϕ and phagocytic dendritic cells in cancer therapies: for example, implanted scaffolds that contain tumor lysates and cytokines lead to acquired immunity—probably after being phagocytosed—in syngeneic models such as the B16 melanoma model [63, 84]. With mCD47 blockade, T cells are recruited to tumors by phagocytic Mϕs, even though tumor clearance seems dominated by Mϕs in some studies [57, 64]. Surprisingly, even though PDL1 on cancer cells is primarily considered to inhibit T cell interactions and thereby enhance T cell responses, anti-PDL1 IgG can also engage Mϕ Fc receptor and indeed has a major role in driving phagocytosis [55]. With a standard melanoma model (in which initial treatments were begun before tumors became palpable), blockade of PDL1 also required blockade of mCD47 for long-term mouse survival and rechallenge with cancer cells [55].

Other syngeneic tumor models using mCD47 blockades have relied on endogenous opsonization (e.g., calreticulin [57]) and showed shrinkage in days, but injection of anti-CD8—which should deplete T cells—removes any therapeutic effect [85]. This suggested to the authors that the primary effector cell was the T cell. Alternatively, T cells displaying an intact anti-CD8 IgG (typically, IgG1, which strongly engages Fc receptor) could be the most opsonized cell in or near a tumor (assuming T cell infiltration), and thus, mCD47-blocked TAMs phagocytosing such T cells would distract from phagocytosis of weakly opsonized tumor cells. The process which dominates in the imbalance is sometimes unclear, but the various reports do seem to question whether TAMs are effective phagocytic cells and Ag presenters. TAMs certainly promote tumor growth and are weakly phagocytic, at least when compared with peritoneal Mϕs [12–14, 17]. TAMs also have low MHC-II, which is required for activation of T cells [1, 2, 86]. Regardless of the extent to which T cells contribute, the ability of Mϕs to activate T cells should be considered when evaluating efficacy as well as safety. The ubiquitous and abundant expression of CD47 on all cells has already given cause for concern over anti-CD47 therapy, first in terms of the massive amount of Ab that needs to be injected and secondly in terms of the possible autoimmune response against healthy cells, such as the rapidly cleared RBCs.

Concerns over TAMs could perhaps be addressed by adoptive Mϕ therapy in combination with CD47–SIRPα blockade (Fig. 1C). Mϕs and dendritic cells would be isolated and/or differentiated, as done in early adoptive-transfer studies, but they would be first engineered with Abs and/or SIRPα knockdown or CRISPR knockout [87, 88]. When SIRPα depletion is combined with transfection of Mϕs with presentable cancer Ags, implantation of both the Mϕs and melanoma cells are found to prevent tumor growth [88]. Safety becomes a major concern, however, because SIRPα knockdown in Mϕs, followed by systemic injection, enhances growth of liver cancers [87]. SIRPα activates the Tyr-phosphatase SHP1, which has a multitude of targets and is, therefore, likely involved in multiple signaling pathways that affect phenotype [26].

## Mϕ PLASTICITY AND MECHANOSENSING

Phenotypes of Mϕ have classically been divided into 2 or 3 states: a proinflammatory state (M1); an immune inhibitory, angiogenetic

state (M2) [89–93]; and a more passive M0 state. Mϕs are instead far more diverse and plastic: Mϕs from different tissues indeed exhibit distinct expression profiles [1]. Studies of Mϕ diversity use a variety of surface markers that should be factored into the interpretation of any study for humans or mice [1, 15, 47, 48, 57, 63, 93–96] (**Table 2**). In the mouse, the most common Mϕ Ag is F4/80, and the $CD11b^+$ subset is only used occasionally, which might explain some differences in phenotype. Importantly, Mϕs taken from donor tissue and transplanted into a different tissue partially convert over days or weeks to be increasingly like Mϕs in the new host tissue [1]. Mϕ phenotype is thus plastic and controlled by the local microenvironment, with potential effects of both biochemical and biophysical cues. Any adoptive Mϕ approach used to treat solid tumors will, therefore, contend with their differentiation to TAMs. Broadly understanding and controlling such differentiation is thus key to Mϕ-based therapies.

Differentiation of cultured Mϕs into the classic M1 phenotype was done biochemically in early trials of adoptive Mϕ therapy before transfer into the host [19, 89]. Plastic culture dishes are rigid and are now known to affect differentiation, with stem cell phenotypes in culture affected by the softness of the underlying matrix in a mechanosensing process that depends on Myosin-II contractions of the substrate [97] (Fig. 1B). Mϕ plated on soft gels exhibit high M1/M2 ratios, whereas stiff gels lead to low M1/M2 ratios [98]. Stiffening of tissues, such as breast and liver, is often associated with cancer [99–101] and might even contribute to genomic heterogeneity of cancer [102], which complicates therapies with a single molecular target. For Mϕs, premalignant stiffening of tissue could favor conversion to a nonphagocytic phenotype with a reduced capacity to clear damaged cells, which again favors cancer.

Mechanistically, transcriptional control is provided by the nuclear envelope protein, lamin-A, which regulates the nuclear localization of retinoic acid receptor transcription factors; the latter is interesting because epigenetic analyses have implicated retinoic acid in microenvironment regulation of the Mϕ phenotype [1]. Different cell types exhibit different expression changes in response to tissue stiffness, but at least one common factor—*lamin-A*—appears mechanosensitive in most (perhaps all) cell types in tissues [103]. Stiff tissues tend to be under high mechanical stress, and that stress is transmitted from the cell surface through the actin-myosin cytoskeleton and to the nuclear envelope, with *lamin-A* adjusting its level to sustain the stress (dissipate is more accurate) [104] and ultimately protect chromatin from damage [102]. Average levels of *lamin-A* protein and transcript increase systematically from soft marrow and soft brain to stiffer muscle and rigid bone whereas the levels of *lamin-B* isoforms remain relatively constant. Mϕ can of course be isolated from any tissue or disease site and provide an in vivo test of the broader nuclear mechanosensing hypothesis. Meta-analysis of RNA-seq results for monocytes or Mϕ isolated from different tissues show *lamin-A* increasing with tissue stiffness and *lamin-B* remaining nearly constant (**Fig. 3A**).

Solid tumors are typically high in collagen, which generally determines tissue stiffness and has already been shown for numerous human cancer types xenografted into mice [103]. TAMs that are isolated from such tumors using standard markers (F4/80, CD11b) have recently been subject to RNA-seq analysis, which shows that the ratio of *lamin-A* reads to *lamin-B* reads is similar in TAMs to the same ratio in stiff, normal tissues (Fig. 3B). Such results are thus consistent with mechanosensing of matrix microenvironments by Mϕs, and such physical effects on the expression of other genes require careful study. SIRPα is especially interesting because it was recently shown to be strongly regulated by retinoic acid [105], which is mechanosensitive in its downstream effects according to the studies above. If the sensing of microenvironment and the affected gene circuits do drive an increase in SIRPα on TAMs within stiff solid tumors, then TAMs could recognize "self" cancer cells more readily and thus be passivated. Knockdown of SIRPα would seem logical to counter such protumorigenic effects, but systemic injections of such engineered Mϕs are found to enhance the growth of liver tumors in the absence of added tumor opsonization [87].

## TARGET RIGIDITY AND SHAPE OVERRIDE SELF SIGNALING

Mϕs not only respond to physical cues, such as the stiffness of their microenvironments, but also to the targets that they engulf. With spherical microparticles made of hydrogels and opsonized

**TABLE 2. Commonly used markers to identify mouse phagocytes**

| Cell type | Tissue | F4/80 | CD11b | CD11c | CD45 | CD86 | Ly6G | CD45 | MHC II | CCR7 | CD206 | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phagocyte | Tumor | + | | | | | | | | | | Majeti et al. [47] |
| Mϕ | Tumor | + | | | | | | | | | | Casey et al. [48] |
| Mϕ | Pan tissue | + | + | | | | | | | | | Lavin et al.[a] [1] |
| Mϕ | Cultured | + | | | | | | | | | | Sockolosky et al. [57] |
| Dendritic | Cryogel implant | | | + | | + | | | | | | Bencherif et al. [63] |
| Monocyte | Blood, lung tumor | | + | + | + | | | | | | | Hann et al. [94] |
| Neutrophil | Bone marrow | | + | | | | + | | | | | Dorward et al. [95] |
| Neutrophil | Bone marrow | | + | | | | + | + | | | | Swamydas et al. [96] |
| MO | Cultured | − | + | | | | | | | | | Jablonski et al. [93] |
| M1 | Cultured | + | | | | | | | + | + | − | Jablonski et al. [93] |
| M2 | Tumor | + | | | | | | | − | | + | Colegio et al. [15] |

Markers frequently used to identify phagocytic cells (macrophages, neutrophils, monocytes) and different macrophage polarizations organized by publication. [a]The Lavin et al. [1] study of Mϕs in multiple tissues used the indicated markers, sometimes supplemented with additional surface markers.

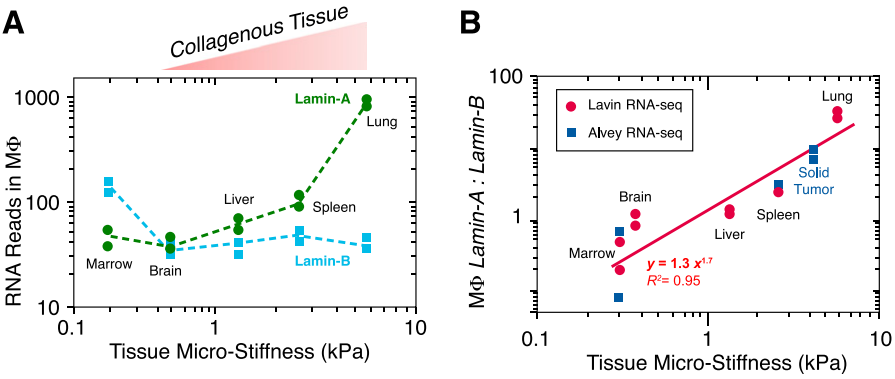


**Figure 3. Stiff matrix regulation of Lamin-A.** (A) RNA-seq reads per million for Lamin-A and Lamin-B in tissue Mϕ from [1], plotted as a function of tissue stiffness measurements in Swift et al. [103]. (B) Ratio of RNA reads for lamin-A: lamin-B in Mϕs, including tumor-associated Mϕs isolated from human tumor xenografts per Lavin et al. [1] and Swift et al. [103]. Subcutaneous A549 tumors were engrafted in NSG mice and allowed to grow to 80 $mm^2$ before tumor stiffness was measured and Mϕ were profiled.

by IgG, engulfment is proportional to stiffness, which was also shown to drive focal adhesion protein assembly at the phagocytic synapse [106]. Stiffness changes occur with cancer cells and with chemotherapy [107, 108]; soft cancer cells might thus escape anticancer efforts aimed at inhibiting CD47-SIRPα interactions [55]. To test the relevance of cell stiffness and any modulation by CD47 signaling of "self," human RBCs were controllably stiffened with a dialdehyde cross-linker, and both IgG opsonization and CD47 blockade were separately controlled [39]. Phagocytosis of rigidified, discocyte-shaped, human RBCs exceeded that of flexible RBCs and proved almost independent of CD47 (**Fig. 4**). Myosin-II contractile forces are again key in responding to target rigidity.

Rigid, spherical CD47 beads signal self and thereby impede engulfment both in vitro and in vivo [38], and sphered RBCs also recovered some "self" signaling, probably because the discocyte's rigid concavities could not contact and signal "self" [39]. Target shape is, therefore, an additional factor in phagocytosis. Indeed, polystyrene microbeads melted and distorted into diverse shapes, for example, are engulfed by Mϕ more readily as spheres than as nonspheres when IgG opsonized [109]. Such findings seem relevant to the diverse shapes of bacteria and fungi, which invariably have rigid cell walls. With cancer cells that are soft but CD47-blocked and IgG-opsonized for targeted engulfment by Mϕ, phagocytosis could distort and elongate the cells—as seen for RBCs [39], and this would also tend to weakly oppose successful phagocytosis. Understanding the details of the various physical and chemical cues to Mϕ, therefore, remains an important endeavor.

## CONCLUDING REMARKS

During the past 4–5 decades, Mϕs have been found safe, albeit ineffective, in anticancer therapy, but the general approach is perhaps reemerging based on the discovery of "marker of self" CD47 signaling to Mϕs. That signaling ultimately turns off cytoskeletal myosin-II, which otherwise makes the very active process of engulfing a foreign cell or particle efficient, and so, inhibiting this signaling at various upstream or downstream points in the CD47-SIRPα pathway can likewise make engulfment of "self" cells more efficient. Considerable progress during the past decade has separately been made toward understanding the broad plasticity of Mϕs and their responses to microenvironments. Initial analyses of ≥1 mechanosensitive nuclear proteins suggest that such responsiveness includes the stiffness of the microenvironment. Phagocytosis is also favored by the stiffness of a cell or particle, and myosin-II has again been shown to be key. Myosin-II thus has a vital role in multiple, cytoskeletal-intensive activities of Mϕs.

Complementary to these basic insights into pathways is a current focus on blockade of CD47-SIRPα to engineer Mϕs in situ for therapy against cancer. The various clinical trials are

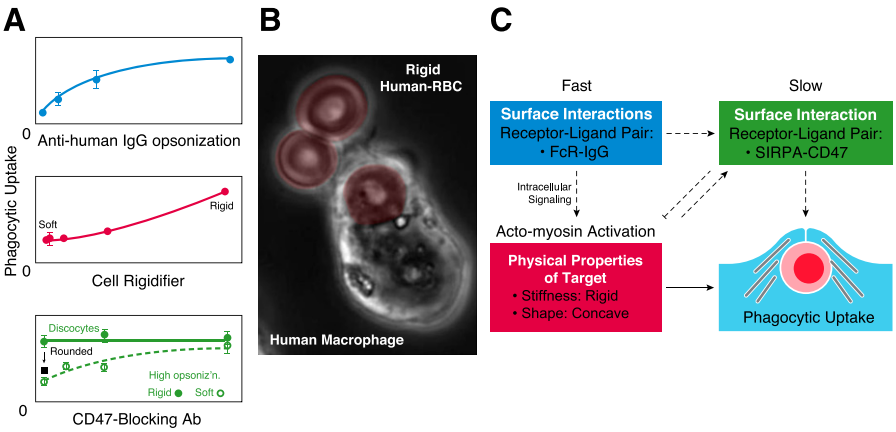


**Figure 4. Targeting the physical properties and molecular interactions at the cell surface determines the efficiency of human RBC engulfment by human Mϕ.** (Adapted from Sosale et al. [39]). (A) Phagocytosis increases with IgG opsonization and with cross-linker–based rigidification of hRBCs. Phagocytosis of rigid, opsonized RBCs is independent of hCD47 inhibition in contrast to "soft," native RBCs whose uptake is enhanced by an hCD47-blocking Ab. A "sphering" treatment, which gives a rounded and rigid hRBC, shows reduced uptake relative to the discocytes. (B) Time-lapse images of rigidified hRBC discocytes show rapid engulfment and lack of deformation by the Mϕ. (C) Surface interactions combine kinetically with physical properties of a candidate target in the calculus that determines phagocytic uptake.

likely to encounter some challenges in safety and efficacy, but injection of anti-hCD47 in patients with cancer was conducted decades ago for imaging of tumors. Regardless of the success in Mϕ engineering in situ or ex vivo for specific applications, the ability of these fascinating and ubiquitous cells to migrate, engulf, digest, and perhaps activate the broader immune system against foreign and diseased cells merits the heightened interest in understanding basic functions of macrophages.

## AUTHORSHIP

C.A. and D.E.D wrote text. C.A. and D.E.D made all figures.

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### DISCLOSURES

The authors declare no conflicts of interest.

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