Macrophages show higher levels of engulfment after disruption of cis interactions between CD47 and the checkpoint receptor SIRPα

Brandon H. Hayes1,2,*, Richard K. Tsai1,2,*, Lawrence J. Dooling1, Siddhant Kadu1, Justine Y. Lee1, Diego Pantano1, Pia L. Rodriguez1, Shyamsundar Subramanian1, Jae-Won Shin1,3 and Dennis E. Discher1,2,3

ABSTRACT

The macrophage checkpoint receptor SIRPα signals against phagocytosis by binding CD47 expressed on all cells – including macrophages. Here, we found that inhibiting cis interactions between SIRPα and CD47 on the same macrophage increased engulfment (‘eating’) by approximately the same level as inhibiting trans interactions. Antibody blockade of CD47, as pursued in clinical trials against cancer, was applied separately to human-derived macrophages and to red blood cell (RBC) targets for phagocytosis, and both scenarios produced surprisingly similar increases in RBC engulfment. Blockade of both macrophages and targets resulted in hyper-phagocytosis, and knockdown of macrophage-CD47 likewise increased engulfment of ‘foreign’ cells and particles, decreased the baseline inhibitory signaling of SIRPα, and linearly increased binding of soluble CD47 in trans, consistent with cis-trans competition. Many cell types express both SIRPα and CD47, including mouse melanoma B16 cells, and CRISPR-mediated deletions modulate B16 phagocytosis, consistent with cis-trans competition. Additionally, soluble SIRPα binding to human CD47 displayed on Chinese hamster ovary (CHO) cells was suppressed by SIRPα co-display, and atomistic computations confirm SIRPα bends and binds CD47 in cis. Safety and efficacy profiles for CD47–SIRPα blockade might therefore reflect a disruption of both cis and trans interactions.

KEY WORDS: Phagocytosis, Inhibitory receptor, Erythrocyte, ‘Marker of self’

INTRODUCTION

Immune cells come in frequent contact with cells that either originate from the same organism, and are thus ‘self’, or else are ‘foreign’, such as microbes. ‘Self’ recognition occurs in part through interactions that ultimately inhibit immune activation. An important clinical example in anti-cancer therapy is the inhibitory T-cell receptor PD-1 (also known as PDCD1) that interacts with PD-L1 (also known as CD274) on ‘self’ cells, and injection of anti-mouse CD47 antibody in common macrophages. Here, we found that inhibiting cis interactions between SIRPα and CD47 on the same macrophage increased engulfment (‘eating’) by approximately the same level as inhibiting trans interactions. Antibody blockade of CD47, as pursued in clinical trials against cancer, was applied separately to human-derived macrophages and to red blood cell (RBC) targets for phagocytosis, and both scenarios produced surprisingly similar increases in RBC engulfment. Blockade of both macrophages and targets resulted in hyper-phagocytosis, and knockdown of macrophage-CD47 likewise increased engulfment of ‘foreign’ cells and particles, decreased the baseline inhibitory signaling of SIRPα, and linearly increased binding of soluble CD47 in trans, consistent with cis-trans competition. Many cell types express both SIRPα and CD47, including mouse melanoma B16 cells, and CRISPR-mediated deletions modulate B16 phagocytosis, consistent with cis-trans competition. Additionally, soluble SIRPα binding to human CD47 displayed on Chinese hamster ovary (CHO) cells was suppressed by SIRPα co-display, and atomistic computations confirm SIRPα bends and binds CD47 in cis. Safety and efficacy profiles for CD47–SIRPα blockade might therefore reflect a disruption of both cis and trans interactions.

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*These authors contributed equally to this work

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Anti-CD47 combined with cancer-opsonizing antibody can drive tumor regression, but normal cells are also depleted.

(A) Recent trials of anti-CD47 therapies have shown efficacy only when combined with anti-cancer opsonizing antibodies. In particular, a primary mechanism of anti-CD20-mediated clearance of B-cell lymphoma is via IgG-activated phagocytosis by liver macrophages (i.e. Kupffer cells), requiring B-cells to enter the blood circulation (Montalvao et al., 2013). (ii) Blockade of CD47 often causes loss of blood cells, especially RBCs, presumably through splenic macrophages.

(B) Bar graph, CD47 and SIRPα expression on diverse human-derived cell types, including hematopoietic cells (stem cells, HSCs; PBMC, peripheral blood monocytic cells, a THP-1 monocyte line and RBCs), plus mesenchymal stem cells (MSCs), and A549 lung adenocarcinoma cells. HSCs were differentiated to myeloid cells by treatment with G-CSF according to Shin et al. (2013). Quantification by flow cytometry used primary antibodies (B6H12, SEC72 clones) labeled with fluorescein (mean±s.e.m. for all results; n=4). Table, molecular density estimates for CD47 and SIRPα on several hematopoietic cell types based on Subramanian et al. (2007).

(C) Phagocytosis assays used THP-1 macrophages (Mφ) and human RBC (hRBC) opsonizing antibody with or without anti-CD47 (B6H12) at saturating level. Engulfment of fluorescent hRBCs was confirmed with a secondary antibody against anti-hRBC. Microscopy fields are randomly selected, and >200 macrophages counted (n=3, mean±s.d.). Hyperbolic fits y=A x/(K+x) of each data set [(A,K): (74%, 67 nM) for anti-CD47, (63%, 125 nM) for control; R²>0.97] give the (inset) difference curve with the difference between A values as Δsat. Scale bar: 20 μm. (D) Anti-CD47 pre-incubated for 45 min with THP-1 macrophages (and excess removed) increased phagocytosis of target hRBCs, independently of hRBC opsonization. Results are mean±s.d., n=3. *P<0.03, n.s., not significant.
RESULTS AND DISCUSSION

Blocking CD47 on macrophages increases phagocytosis

To first determine relative levels of CD47 and SIRPα on diverse cell types, primary antibodies labeled with fluorescein were used in flow cytometry quantification (Fig. 1B; Fig. S1A). Relative to CD47 levels on human monocyctic THP-1 cells, CD47 levels were within 2–3-fold for most other hematopoietic and non-hematopoietic cell types. RBCs are an exception, but ∼10-fold higher levels are consistent with an 80–90% excess of CD47 relative to levels needed to inhibit engulfment by macrophages (Tsai and Discher, 2008). SIRPα expression was expectedly restricted among hematopoietic cells to phagocytes, including human THP-1 macrophages, cytokine (granulocyte colony stimulating factor; G-CSF)-differentiated human stem cells (HSCs+G-CSF), and peripheral blood (granulocyte colony stimulating factor; G-CSF)-differentiated human stem cells (HSCs−G-CSF), and peripheral blood (granulocyte colony stimulating factor; G-CSF)-differentiated human stem cells (HSCs+G-CSF), and peripheral blood (granulocyte colony stimulating factor; G-CSF)-differentiated human stem cells (HSCs−G-CSF), and peripheral blood (granulocyte colony stimulating factor; G-CSF)-differentiated human stem cells (HSCs−G-CSF), and peripheral blood (granulocyte colony stimulating factor; G-CSF)-differentiated human stem cells (HSCs−G-CSF). Transcriptome analyses further showed that SIRPα was expressed in some mouse cancer cell types as well as primary mouse macrophages, which have an expression profile similar to THP-1 macrophages (Fig. S1B). Nonetheless, the protein analyses showed that CD47 exceeds SIRPα by 3:1 or greater for all cell types, and so all of these cell types should robustly signal ‘self’ to macrophages.

To study anti-CD47 blockade effects on phagocytosis of human RBCs (hRBCs) by human macrophages, fresh hRBCs were pre-incubated with opsonizing anti-hRBC at various levels, with or without saturating levels of anti-CD47, and then added to THP-1 macrophages (Fig. 1C; Fig. S2A–D). Engulfment of opsonized hRBCs increases with anti-CD47 treatment, and saturation binding curves reveal a ∼2-fold higher half-max activity and >10% higher saturation (Δsat) (Fig. 1C). However, for hRBCs without any opsonization (i.e. anti-hRBC=0), anti-CD47 does not promote phagocytosis (Fig. 1C; Fig. S2D). This is surprising because if freshly isolated RBCs display an endogenous opsonizing signal of relevance to the effects of systemic blockade, then anti-CD47 should increase in vitro engulfment. The anemia and loss of other blood cells in clinical blockade of CD47 (Fig. 1Aii) thus motivate a search for other mechanism(s).

Anti-CD47 infused intravenously should bind CD47 on macrophages in the spleen, among other accessible tissues. THP-1 macrophages were therefore pre-incubated with anti-CD47, and excess antibody was removed before assaying phagocytosis. Engagement of hRBCs by THP-1 macrophages increased even with non-opsonized hRBCs (Fig. 1D, left; Fig. S2A,B), and addition of anti-hRBC to hRBCs also increased engulfment, with maximal levels seen for anti-CD47 pre-bound to both THP-1s and hRBCs (Fig. 1D, right; Fig. S2A). The increased percentage of macrophages engulfing hRBCs was also accompanied by more hRBCs being engulfed per macrophage (Fig. S2A, top); THP-1s only phagocytosed ∼1 hRBC in the absence of any added antibodies, whereas ∼2 hRBCs were often engulfed per macrophage under conditions of maximal engulfment. Regardless, pre-binding of anti-CD47 to THP-1 macrophages tended to increase the percentage of engulfment (i.e. percentage of cells with internalized particles) by an amount similar to that achieved with anti-CD47 blockade on fully opsonized hRBCs (Fig. 1D, Fig. S2C). The typical increase (∼Δsat in Fig. 1C,D) underscores the relatively large effect of blocking CD47 on the macrophage. Therefore, hyper-phagocytosis of opsonized targets is a result of blockade of both the target and the macrophage, based potentially on inhibition of the cis CD47-SIRPα interaction.

Knockdown of macrophage CD47 increases engulfment and decreases basal signaling

Anti-CD47 on a macrophage could conceivably dissociate and bind an RBC target and vice versa – although symmetric saturation of both cells will eliminate exchange and was seen to maximize engulfment (Fig. 1D). To achieve a more-stable asymmetric blockade effect, CD47 was suppressed in THP-1 macrophages using shRNA, without affecting SIRPα (Fig. S3A,B). Phagocytosis was assayed (again per macrophage as in Fig. S2A, top) with IgG-opsonized sheep red blood cells (ShRBCs) as targets for knockdown (KD) and wild-type (WT) THP-1 macrophages; it is already known that sheep CD47 does not bind human SIRPα (Tsai and Discher, 2008). ShRBCs were engulfed ~30% more per KD macrophage compared to WT and to KD controls (Fig. 2A; Fig. S3C). Compared to the ShRBCs, opsonized human-RBCs show a decrease in ‘self’-inhibited background engulfment by both WT and KD THP-1 macrophages, following the expected trends (Fig. S3D). IgG-opsonized microbeads were likewise engulfed at higher levels per KD macrophage (Fig. 2A). Macrophages are thus hyper-phagocytic if their CD47 is either blocked with an antibody (Fig. 1D) or knocked down, especially when CD47 on the target is lacking.

Phosphorylation of tyrosine residues in the cytoplasmic immunoreceptor tyrosine-based inhibiting motif (ITIM) of SIRPα increases when CD47 on a phagocytic target binds SIRPα in trans, with phosho-SIRPα activating a downstream phosphatase (Veillette et al., 1998) that turns off actomyosin (Veillette et al., 2005; Ide et al., 2007; Tsai and Discher, 2008). Basal phosphorylation of SIRPα in the absence of phagocytic targets remains unexplained (Oldenborg et al., 2000; Ide et al., 2007; Tsai and Discher, 2008), but we observe lower levels of phospho-SIRPα in CD47 KD THP-1-macrophages than in WT, with a linear increase from zero as a function of CD47 levels (Fig. 2B). Note that the deepest KD essentially inverts the CD47:SIRPα stoichiometry from ∼3:1 excess CD47 to >2:1 excess SIRPα (Fig. 1B; Fig. S3B). Decreased basal signaling with CD47 depletion and the increased phagocytosis led us to hypothesize that CD47 on a WT macrophage binds in cis on the same macrophage membrane to SIRPα.

Competitive binding between CD47 and SIRPα in cis versus trans

To quantify cis binding in competition with trans binding, binding curves were generated for soluble fluorescent CD47 added to WT and KD THP-1 macrophages. Flow cytometry reveals a saturable, moderate affinity (1.6 μM) interaction for SIRPα on WT cells (Fig. 2C). However, a substantial knockdown of CD47 (to ~13%) strongly increases this apparent binding affinity to 0.26 μM (Fig. 2C), and intermediate knockdown shows that the apparent dissociation constants (Kd) increase linearly with CD47 levels on the macrophage (Fig. 2Ci). Our previous measures of soluble SIRPα binding to CD47 on hRBCs, which lack any cis interactions (Fig. 1Ai), gave Kd=0.2 μM (Sosale et al., 2015); such a value matches the intercept of the THP-1 results. The same studies of hRBCs also showed anti-CD47 (B6H12 clone) binds as expected, with much higher affinity (40 nM).

Mathematically, trans or cis concentrations of CD47 (Ct, Cs, respectively) compete for a single site on macrophage SIRPα (Fig. 2Ci, inset). Given association constants KC and Kc, the fractional occupation of SIRPα by trans-CD47 is:

\[
\theta = \frac{K_{t}C_{t}}{1 + K_{c}C_{c} + K_{t}C_{t}}
\]

(1)
We write $\theta$ as a hyperbolic function of $C_t$ (per Fig. 2Ci) by defining:

$$K_d = \frac{1 + K_c C_t}{K_t}$$

Linearity in $C_c$ fits the CD47-dependence of $K_d$ (Fig. 2Cii) and linear signaling (Fig. 2B). Furthermore, $K_d = (k_{off}/k_{on})$ and agrees with the finding that soluble CD47 binds slower in $trans$ to WT THP-1 cells, with $k_{on}$ of 0.65 min$^{-1}$, compared to 0.36 min$^{-1}$ for KD cells (Fig. S3E).

Fig. 2. Depleting CD47 on macrophages increases engulfment activity, decreases SIRP$\alpha$ signaling and increases SIRP$\alpha$ affinity. (A) Stable knockdown of CD47 on THP-1 macrophages to 48% or 13% of wild-type (WT) levels was used to phenocopy anti-CD47 effects. Phagocytosis assays with WT or knockdown (KD) THP-1 cells used IgG-opsonized sheep RBCs (ShRBCs) or IgG-opsonized streptavidin microbeads. Microscopy fields randomly selected and 200 macrophages counted ($n=3$, mean±s.e.m.). Images, engulfment of ShRBCs. Arrows denote phagocytic events. Scale bar: 10 μm. THP-1 cells with 13% CD47 levels had higher levels of engulfment than WT (∼40%). *P<0.03. (B) SIRP$\alpha$ immunoprecipitation from lysates of THP-1 macrophages under basal conditions using anti-SIRP$\alpha$ (SE7C2 clone) and immunoblotted for phospho-tyrosine (pTyr; C-20 clone) ($n=3$, mean±s.e.m.). The normalized SIRP$\alpha$ pTyr signal increases linearly from zero in relation to the CD47 level. (C) (i) To quantify the effective affinity of CD47 for SIRP$\alpha$ in $trans$ on WT and KD THP-1 macrophages, binding of fluorescent soluble CD47 was measured by flow cytometry and normalized to 8 μM data. All data fits $y=A x/(K_d+x)$ ($R^2>0.93$) for apparent dissociation constants ($K_d$). (ii) $K_d$ increases linearly in relation to the CD47 level, and the non-zero intercept corresponds to the highest affinity for CD47–SIRP$\alpha$ as measured for human RBCs that lack SIRP$\alpha$ (Fig. 1B).

We write $\theta$ as a hyperbolic function of $C_t$ (per Fig. 2Ci) by defining:

$$K_d = \frac{1 + K_c C_t}{K_t}$$

Linearity in $C_c$ fits the CD47-dependence of $K_d$ (Fig. 2Cii) and linear signaling (Fig. 2B). Furthermore, $K_d = (k_{off}/k_{on})$ and agrees with the finding that soluble CD47 binds slower in $trans$ to WT THP-1 cells, with $k_{on}$ of 0.65 min$^{-1}$, compared to 0.36 min$^{-1}$ for KD cells (Fig. S3E).
**SIRPα on cancer cells modulates their engulfment**

Numerous cell types express both CD47 and SIRPα, including human A549 lung cancer cells (Fig. 1B) and mouse B16 melanoma cells (Figs S1B and S4A). We hypothesized that with such cells cis CD47–SIRPα interactions regulate their phagocytosis by modulating trans presentation of ‘self’ (Fig. 3A), as is relevant to cancer therapy (Fig. 1A). B16 cells were studied to generalize species effects and because they are widely used in preclinical immunotherapy, including CD47–SIRPα blockade (Ingram et al., 2017; Chowdhury et al., 2019; Mandal et al., 2019).

CRISPR/Cas9-mediated knockout (KO) of CD47 or SIRPα was performed in B16 cells, alongside a line with a non-targeting guide RNA for control, and all B16 cells were equally opsonized with anti-Tyrp1 antibody after knockout of indicated protein. Results are mean±s.d. (n=3); ***P<0.001 (one-way ANOVA and post-hoc Bonferroni test). (C) Fluorescence microscopy determinations of the phagocytic index, defined as the percentage of BMDMφs that are actively engulfing cells multiplied by the number of target cells engulfed per engulfing BMDMφ. (D) Representative images of BMDMφs engulfing opsonized B16 melanoma cells. Arrows denote phagocytic events. Scale bar: 100 μm.

**Fig. 3. Knockout of either CD47 or SIRPα on mouse B16 melanoma cells affect CD47 availability to signal ‘self’ to primary mouse macrophages.** (A) A diagram of the hypothesis. Knockout of SIRPα on antibody-opsonized B16 shifts CD47 interactions from cis to trans interactions, inhibiting phagocytosis of the tumor cell. Knockout of CD47 abolishes ‘self’ signaling and increases phagocytosis. (B) Primary mouse bone marrow-derived macrophages (BMDMφs) phagocytose mouse B16 melanoma cells opsonized with anti-Tyrp1 antibody after knockout of indicated protein. Results are mean±s.d. (n=3); ***P<0.001 (one-way ANOVA and post-hoc Bonferroni test). (C) Fluorescence microscopy determinations of the phagocytic index, defined as the percentage of BMDMφs that are actively engulfing cells multiplied by the number of target cells engulfed per engulfing BMDMφ. (D) Representative images of BMDMφs engulfing opsonized B16 melanoma cells. Arrows denote phagocytic events. Scale bar: 100 μm.
marrow derived macrophages (BMDMs). Flow cytometry and imaging both show that engulfment of SIRPα-KO cells was reduced by 30–40% relative to control B16s, compared to the 2–3-fold increase for CD47-KO cells (Fig. 3B–D; Fig. S4B); also 1–2 cells were again engulfed per BMDMφ (Fig. 3C,D). These results support the general hypothesis that CD47–SIRPα cis interactions modulate trans interactions.

**Co-display of CD47–GFP and SIRPα suppresses binding of soluble SIRPα**

Heterologous display of human CD47–GFP on Chinese hamster ovary (CHO) cells varies broadly, but the variation includes physiological levels of CD47 (Subramanian et al., 2007). Co-expression (or not) of human SIRPα (unlabeled but expressed similarly) with CD47–GFP, allowed us to assess trans binding of anti-CD47 or soluble SIRPα. Co-expressing cells show a ~90% reduction of the red fluorescence from anti-CD47 binding compared to cells expressing CD47–GFP alone (Fig. 4A,B). Such a reduction is consistent with cis-trans competition. For red-fluorescent soluble SIRPα, co-expressing cells showed almost no trans-SIRPα binding relative to cells expressing CD47–GFP alone (Fig. 4B). The larger effect on trans-SIRPα binding relative to anti-CD47 is consistent with weaker association of SIRPα with CD47.

**Simulated SIRPα can bend and bind CD47 in cis**

Among its three Ig domains, the N-terminal domain of SIRPα binds the single Ig domain of CD47 in a crystallizable interaction (Hatherley et al., 2008). The Ig domains of SIRPα move as near-rigid bodies linked by hinges in computations of normal mode motions (Fig. 4C). Insertion of SIRPα with its transmembrane domain into a simulated lipid bilayer while in crystallographic association with CD47 in the same membrane (Fig. 4D) provides a molecularly detailed view of SIRPα bending over and binding to CD47 in cis. Bending energy could destabilize binding energy, and a first step toward determining this might be to relate SIRPα docking probabilities for CD47-derived ‘self’ peptides to measured binding affinities (Rodriguez et al., 2013).

**Conclusion**

Safety of CD47 blockade is a concern across multiple clinical trials, with loss of various blood cells (RBCs, platelets, etc.; see Andrechak et al., 2019). Clearance by macrophages has been claimed to result from opsonizing signals on aged cells (Advani et al., 2018), such as opsonizing IgG (against oxidation-generated epitopes) or membrane rigidity (Sosale et al., 2015). Paradoxically, fresh hRBCs are minimally eaten by macrophages and are not engulfed more when treated with anti-CD47 (Fig. 1C,D). This paradox is resolved by disruption of the inhibitory CD47–SIRPα cis interaction on the macrophage. Engulfment clearly increases, even though blocking CD47 on only the macrophage should release SIRPα molecules to conceivably interact in trans and increase inhibition. More study is clearly needed, but this is the first study to illustrate the importance of CD47 on the macrophage, with clear implications for clinical blockade of CD47.

The presence of cis interacting CD47–SIRPα is surmised from our four experimental approaches, including anti-CD47 on human-derived THP-1 macrophages (Fig. 1C,D; Fig. S2). These approaches are shRNA-mediated knockdown of CD47 on THP-1 macrophages (Fig. 2; Fig. S3), phagocytosis by primary mouse macrophages of mouse melanoma B16 cells with SIRPα or CD47 deleted (Fig. 3; Fig. S4), and heterologous co-display of SIRPα and CD47 (Fig. 4A,B). Simulations further indicate a cis interaction is physically reasonable. The potency of the phospho-signaling of SIRPα in cis versus that seen in trans remains unclear, but phospho-signaling in trans saturates with increased CD47 on a target (Tsai and Discher, 2008), which differs from the linearity seen upon knockdown (Fig. 2B) and perhaps reflects a limit to SIRPα accumulation at the phagocyte synapse.

The efficacy of anti-CD47 in human cancer trials depends on macrophage activation, such as with tumor-opsonizing antibodies (Fig. 1A1) (Advani et al., 2018; Andrechak et al., 2019). Expression of anti-CD47 from bacteria within a mouse tumor might activate macrophages via bacterial pathways (Chowdhury et al., 2019), but the same anti-CD47 is also likely to disrupt CD47–SIRPα cis interactions on tumor-associated macrophages and thereby drive hyper-phagocytosis. SIRPα blockade should lead to similarly high activation and hyper-phagocytosis, which is interesting to consider in light of anti-tumor efficacy with systemically injected anti-SIRPα blocked macrophages (Alvey et al., 2017).

**MATERIALS AND METHODS**

**Chemicals**

Dulbecco’s phosphate-buffered saline (DPBS) without Ca2+ or Mg2+ (Invitrogen, Carlsbad, CA) was supplemented with either 1% bovine serum albumin (BSA) or 0.05% Tween 20 (Millipore Sigma, Darmstadt, Germany). Tris-buffered saline (TBS) and TBS with 0.5% Tween 20 (TTBS) were used in western blotting. Hoechst 33342 (Invitrogen) was used for DNA stains.

**Antibodies**

The anti-human CD47 antibody clones B6H12 (catalog no. 556044, BD Biosciences, San Jose, CA), 2D3 (catalog no. 14-0478-82, Thermo Fisher Scientific, Waltham, MA), and 6H9 (a gift from Marilyn Telen at Duke University, Durham, NC) were used for detection of human CD47 on macrophages or CHO cells expressing full-length human CD47. Quantification of CD47 and SIRPα was performed using B6H12–FITC (catalog no. 556045, BD Biosciences, San Jose, CA) and anti-SIRPα clone SE7C2 (catalog no. sc-23863, Santa Cruz Biotechnology, Dallas, TX), respectively. Human SIRPαex [produced as a secreted and purified SIRPα1-GST as per Subramanian et al. (2007), see below] was used for experiments comparing binding affinities between species. The following antibodies were used as IgG opsonin in our phagocytosis assays: polyclonal rabbit anti-human NBC (refer to as anti-hRBC in the text) (catalog no. 109-4139, Rockland Immunochemicals), polyclonal rabbit anti-sheep NBC (catalog no. 113-4139, Rockland Immunochemicals, Limerick, PA) to target sheep red blood cells (anti-sRBC), rabbit anti-strepavidin (catalog no. S6390-1ML, Sigma-Aldrich), and rabbit anti-strepavidin conjugated to FITC (catalog no. 200-042-0955, Rockland Immunochemicals) to target strepavidin coated polystyrene beads (catalog no. SVP-50-5, Spherotech, Lake Forest, IL), Anti-Cas9 (7A9-3A3 clone) antibody (catalog no. ab191468, Abcam, Cambridge, MA) was used to detect Cas9 expression in transduced cell lines in western blot analysis. Secondary antibodies used for detecting opsonin levels and uningested beads included goat anti-rabbit FITC or goat anti-rabbit F(ab’2) R-PE (Millipore Sigma). Secondary antibodies used for detecting SIRPαex binding included anti-GST Alexa Fluor 488 (Invitrogen). Secondaries antibodies used for confirming complete RBC engulfment included donkey anti-rabbit Alexa Fluor 647 (Invitrogen). Cytokine granulocyte colony stimulating factor (G-CSF) was purchased from R&D Systems (Minneapolis, MN).

**Cell cultures and THP-1 differentiation**

COS-1, CHO-K1, A549, THP-1 and B16F10 cells (American Type Culture Collection, Manassas, VA) were maintained in culture with DMEM, MEM, F-12, RPMI 1640 and RPMI 1640 (Invitrogen), respectively. All cell culture media, unless otherwise specified, was supplemented with 10% (v/v) fetal bovine serum (FBS; F2442, Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Gaithersburg, MD). Human mesenchymal stem cells (MSCs; Osiris Therapeutics, Columbia, MD) were maintained in
Co-expression of CD47 and SIRPα on CHO cells reveals less ligand is available for binding in trans.

(A) Images, CHO cells expressing human CD47–GFP were labeled with anti-CD47 as detected with Alexa Fluor 647 secondary antibody. Cells co-displaying full-length human SIRPα suppress anti-CD47 binding. Scale bar: 10 μm. Diagram, anti-CD47 and soluble SIRPα bind membrane-associated CD47 in trans while competing with membrane SIRPα. (B) Quantification of anti-CD47 binding to CD47–GFP, and soluble SIRPα (labeled with red fluorophore) binding to CD47–GFP. Flow cytometry shows CD47–GFP levels vary between cells, and bound ligand signal increases proportionately. Co-displayed SIRPα reduces ligand binding in trans by >90%, consistent with cis-trans competition. Data points were generated by averaging 10,000 events from flow cytometry. (C) Top, the extracellular region of SIRPα in ribbon representation with hinge regions as yellow Cα carbons from Arg-114 to Lys-116, and Arg-220 to Phe-222. The covariance matrix of all Cα carbons is based on the first ten normal modes in a Gaussian network model; bottom, snapshots show the first three modes, highlighting rigid body domains bending and rotating around hinges. (D) Coarse-grained atomistic computation of lipid bilayer with SIRPα binding in cis to CD47 (PDB: 2JJS).

Fig. 4. In heterologous display, CD47–SIRPα trans interactions are out-competed by cis, and molecular modeling shows SIRPα bends and binds CD47 in cis. (A) Images, CHO cells expressing human CD47–GFP were labeled with anti-CD47 as detected with Alexa Fluor 647 secondary antibody. Cells co-displaying full-length human SIRPα suppress anti-CD47 binding. Scale bar: 10 μm. Diagram, anti-CD47 and soluble SIRPα bind membrane-associated CD47 in trans while competing with membrane SIRPα. (B) Quantification of anti-CD47 binding to CD47–GFP, and soluble SIRPα (labeled with red fluorophore) binding to CD47–GFP. Flow cytometry shows CD47–GFP levels vary between cells, and bound ligand signal increases proportionately. Co-displayed SIRPα reduces ligand binding in trans by >90%, consistent with cis-trans competition. Data points were generated by averaging 10,000 events from flow cytometry. (C) Top, the extracellular region of SIRPα in ribbon representation with hinge regions as yellow Cα carbons from Arg-114 to Lys-116, and Arg-220 to Phe-222. The covariance matrix of all Cα carbons is based on the first ten normal modes in a Gaussian network model; bottom, snapshots show the first three modes, highlighting rigid body domains bending and rotating around hinges. (D) Coarse-grained atomistic computation of lipid bilayer with SIRPα binding in cis to CD47 (PDB: 2JJS).
low-glucose DMEM, supplemented with 20% (v/v) FBS with the previously mentioned penicillin-streptomycin concentrations. All cell lines were regularly tested for mycoplasma contamination. Cells were detached using 0.05% trypsin and 0.5 mM EDTA (Invitrogen) for passaging. Human blood was obtained from finger pricks of healthy donors. Blood from other species was obtained from Covance (Princeton, NJ) and washed three times in 0.4% BSA. Human hematopoietic stem cells (HSCs) were obtained from fresh purified bone marrow (BM)-derived human CD34+ cells from Lonza (Basel, Switzerland). All HSC experiments were performed in hematopoietic stem cell expansion medium (StemLine-H; Millipore Sigma) and supplemented with 1× antibiotics and G-CSF (100 ng/ml) for 7 days for cultured cells. Peripheral blood monocytes from human donors were obtained through the Human Immunology Core (University of Pennsylvania, Philadelphia, PA). Differentiation of THP-1 cells followed a protocol based on several recent publications that all refer to the differentiated cells as THP-1 macrophages (Genin et al., 2015; Starr et al., 2018; Sedlyarov et al., 2018). Differentiation was achieved in 100 ng/ml phorbol myristate acetate (PMA) (Millipore Sigma) for 2–3 days, which leads to these cells switching from growth in suspension (as monocytic cells) to attaching to tissue-culture plastic. Gene expression data shown in Fig. S1B confirms that PMA-differentiated THP-1 cells express key macrophage markers, while differing greatly from two epithelial cell types.

Expression of human CD47–GFP and SIRPα

Human CD47 (hCD47; isoform 2) was PCR amplified, digested with XhoI and BamHI (New England Biolabs, Ipswich, MA) and ligated to a similarly digested vector pEGFP-N3 (Takara Bio USA, Inc., Mountain View, CA). CHO cells were plated at 1×10^5 cells/cm² at 1 day prior to transfection. On the day of transfection, medium was replaced with 2 ml Opti-MEM I (Invitrogen) per 25 cm² surface area and 10–15 μl Lipofectamine 2000 and 5 μg plasmid DNA were diluted in 0.25 ml Opti-MEM I separately and, 5 min later, mixed and incubated for a minimum of 20 min at 25°C. Lipid–DNA complexes in a total volume of 0.5 ml Opti-MEM I were added to the flasks and incubated for 4–6 h. The flasks were then replenished with fresh growth medium. Transfected cells were harvested using DPBS supplemented with 2 mM EDTA (Invitrogen) at 1–2 days post transfection for analysis. Full-length human SIRPα was expressed in CHO cells in a similar manner to human CD47–GFP.

Soluble human SIRPα production

COS-1 cells were transfected with pcDNA3-based vector encoding a human SIRPα extracellular domain fused to GST using Lipofectamine 2000 (Invitrogen). Secreted SIRPα-GST (referred as hSIRPαex) was affinity-purified using glutathione–Sepharose 4B (Amersham Biosciences, Little Chalfont, UK) and dialyzed against DPBS (Invitrogen). The protein was stored at -20°C with or without addition of 10% (v/v) glycerol (Thermo Fisher Scientific).

Production of recombinant human CD47

Plasmid encoding the extracellular domain of human CD47 or mouse CD47 were PCR amplified, digested with XbaI and Sall (New England Biolabs) and ligated to a similarly digested vector, pEF-BOS-XB, which results in an in-frame fusion of CD43+4-biotin at the C-terminus of the extracellular domain of CD47. The above vector containing the extracellular domain of CD47 was transfected into CHO-K1 cells using Lipofectamine 2000 (Invitrogen). Secretd CD47-CD43+4 was concentrated using a 10 kDa molecular mass cut-off (MWCO) Amicon filter (Millipore Sigma) and biotinylated at the C-terminus using a biotin-protein ligase (Avidity, LLC) and dialyzed against DPBS (Invitrogen). The protein was affinity-purified using a monoclonal Avidin (Promega) and dialyzed against DPBS (Invitrogen).

Measurement of human SIRPα and CD47 on cells

Human RBCs, THP-1 cells, AS549 cells, peripheral blood monocytes and human MScs were labeled with 15 μl of B6H12–FITC at saturating levels (BD Biosciences) against human CD47 for 30 min at room temperature. Cells were washed and resuspended in DPBS supplemented with 5% FBS for flow cytometry analysis. For measurement of human SIRPα expression on CHO-K1 cells anti-SIRPα (clone P3C4) (catalog no. LS-C179629, MBL International, Woburn, MA) was used. Anti-human CD47 measurements and detections were performed using different antibodies above recognizing different epitopes of CD47. Saturating concentration of anti-SIRPα or anti-human CD47 antibodies were used as similarly described for the above cells.

Lentiviral knockdown of CD47 in THP-1 cells

shRNA lentiviral supernatants to CD47 were purchased from Millipore Sigma (TRC#: TRCN000007836, TRCN000007837) to target CD47 and resulted in 52 and 87% knockdown, respectively. Further details of these clones are available from the Millipore Sigma website. Target THP-1 cells were infected with lentiviral supernatants at a multiplicity of infection (MOI) of 10 in the presence of 80 μg/ml polybrene (hexadimethrine bromide) (Millipore Sigma) and remained in culture for 24 h at 37°C and replaced with fresh RPMI 1640 supplemented with 10% FBS. Cells with integrated viral sequence were selected using puromycin (Millipore Sigma) at 2 μg/ml and then passaged with continuous puromycin selection. The degree of CD47 silencing was regularly monitored by flow cytometry and western blotting with 1:200 anti-SIRPα (clone SE7C2, Santa Cruz Biotechnology). Control cell cultures were generated with control lentivirus in parallel.

CRISPR/Cas9-mediated knockout of CD47 and SIRPα in B16F10 cells

Lentiviral shRNA plasmids (Addgene #108100 and 65656, respectively) were gifts from Christopher Vakoc. The single guide RNA (sgRNA) oligonucleotides (CD47; 5′-TCCCGTCTAGA-GATTACAATG-3′; SIRPα; 5′-TAAATCTAAAGGTCTAGCAGC-3′) were designed using the Broad CRISPR algorithm. sgRNAs were cloned into the sgRNA vector using a BamBI restriction digest.

HEK293T cells were used for lentiviral delivery of Cas9 and sgRNA and cultured in high-glucose DMEM supplemented with 10% (v/v) FBS (F2422, Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). To generate lentivirus, transfer plasmid, pVSVG and psPAX2 were co-transfected into HEK293T cells at a 2:2:1 ratio using Mirus TransIT (MIR6603) transfection reagent (Mirus, Madison, WI) following the manufacturer’s protocol. Viral supernatants were collected 48 h after transfection and added to target cells at a 1:1 ratio of culture medium to viral supernatant. Successfully transduced cells were selected using puromycin at 2 μg/ml. Cas9 expression was confirmed using western blot analysis. Clonal knockout cells lines were developed and confirmed using both flow cytometry and western blot analysis using the previously described antibodies.

Binding isotherm for soluble CD47 for wild-type and knockdown THP-1 cells

The binding isotherm of soluble CD47 was performed for THP-1 wild-type and CD47 knockdowns, as noted, over a range of concentration using flow cytometry. Forward scatter, side scatter and fluorescence (FL1, FL2, FL3, FL4 channels in logarithmic mode) were acquired for at least 10⁹ events using a FACSScan, FACS Calibur or LSR II (BD Biosciences, San Jose, CA). Data points from flow cytometry were plotted and analyzed using a hyperbolic fit of the form y=A/(K+x) to obtain the Ka values shown.

Kinetics measurements

The rates of association (k on) of SIRPα with soluble CD47 were measured at room temperature. Mixtures of 8, 4 and 2 μM soluble CD47 with 15 μl of B6H12–FITC against human CD47 at saturating levels (BD Biosciences) were pre-equilibrated for 3 h to produce the primary complex. After mixing, aliquots were periodically withdrawn and added to the human THP-1 cells, then washed and diluted in PBS (1:30) to measure the CD47 and SIRPα binding using flow cytometry. Forward scatter, side scatter and fluorescence (FL1, FL2, FL3, FL4 channels in logarithmic mode) were acquired for at least 10⁶ events using a FACSScan, FACS Calibur or LSR II (BD Biosciences) Data points from flow cytometry were plotted and fitted to obtain the k off values shown.
Phagocytosis assay of human RBCs by PMA-differentiated THP-1 macrophages

For the phagocytosis assays, macrophages were plated in 4 cm² Lab-Tek II Chambered Coverglass (Nalge Nunc International, Rochester, NY) or 12-well tissue culture plates (Corning, Corning, NY) at 1×10⁵ cells per 4 cm². Streptavidin polystyrene beads or RBCs were added to macrophages at a ratio of 20:1 and allowed to incubate at 37°C for 60 min. Non-phagocytosed beads or RBCs were washed with DPBS. For assays using RBCs as targets, lysis of non-ingested RBCs was performed by adding deionized H₂O for 30 s, followed by immediate replacement with 0.4% BSA and fixing with 5% formaldehyde for 5 min.

For stimulated phagocytosis assays, beads (with or without CD47) were incubated with rabbit anti-streptavidin serum, sheep RBCs with rabbit anti-sheep RBC antibody, and human RBCs with rabbit anti-human RBC antibody, respectively, as the opsonin. Beads or RBCs were opsonized at their respective titrated concentrations for 45 min at room temperature. Opsonized beads and RBCs were washed twice and resuspended in 50 µl of PBS containing 0.4% BSA. If the opsonized target required an additional CD47 block, they were treated with anti-CD47 (B6H12 clone) diluted in DPBS to a final concentration of 0.5 µg/ml for 45 min at room temperature.

For phagocytosis assays in which the interactions in cis on THP-1 cells was disrupted, we added 1.5 µM anti-CD47 clone B6H12 antibody to the THP-1 cells prior to the addition of RBCs. The antibody was allowed to bind for 1 h prior to the assay and then washed out with DPBS. Finally, if RBCs were used, they were stained with PKH26 membrane dye (Sigma-Aldrich) for 45 min at room temperature prior to their addition to THP-1 cultures.

To identify non-internalized beads, beads were labeled with a primary antibody, rabbit anti-streptavidin (catalog no. S6390-1ML, Millipore Sigma) at 1:1000 in DPBS for 20 min at 25°C. A second antibody, anti-rabbit IgG conjugated to R-phycocerythrin (R-PE) (catalog no. S2412-1MG-F, Millipore Sigma), was added at 1:1000 in PBS to the cells and incubated for an additional 20 min at 25°C. Cells were then washed with 0.4% BSA and then quantified by light and fluorescence microscopy. For differentiation of non-internalized RBCs, fixed THP-1 cells were blocked with 5% BSA for 30 min followed by addition of donkey anti-rabbit IgG (to target rabbit anti-human RBC) conjugated to Alexa Fluor 647 at 1:500 dilution in DPBS for 45 min at 25°C. Secondary antibody would not be able to bind to completely engulfed RBCs but would stain RBCs that simply adhered to the THP-1 surface. For quantification purposes, we define true phagocytic events as those in which RBCs are completely internalized and do not stain for secondary antibody. At least 200 macrophages were scored per well and experiments were repeated at least three times.

Primary mouse BMDMφs and phagocytosis of B16 cell lines

Bone marrow cells were isolated from femurs and tibias of healthy male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) and cultured in 10-cm petri dishes containing Iscove’s modified Dulbecco’s medium (IMDM, Gibco 12440-053) supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 20 ng/ml M-CSF (Biologend 576404) for 7 days at 37°C, 5% CO₂. All animal experiments were performed according to protocols approved by the University of Pennsylvania’s IACUC (protocol #805977 and #804455). The resulting BMDMφs were detached with 0.05% trypsin-EDTA (TRED, Gibco) and re-plated in 6- or 24-well tissue culture plates at a density of 2×10⁵ cells/cm².

The following day, macrophages were stained with 0.5 µM CellTracker Deep Red (C34565, Thermo Fisher Scientific) for 10 min at 37°C. The labeled BMDMφs were washed twice with serum-free IMDM. B16 cell lines were labeled with 1 µM CFDA-SE (V12883, Thermo Fisher Scientific) for 10 min at 37°C. The labeled BMDMφs were washed twice with serum-free IMDM. B16 cell lines were opsonized in suspension with 5 µg/ml anti-Tyrp1 (clone TA99, Bio X Cell BE0151, Lot #715419A1) or mlgG2a isotype control (clone C1.18.4, Bio X Cell BE0085, Lot#6924181S) on 30 min ice.

For analysis of phagocytosis by flow cytometry, 2 ml (∼4×10⁵) of the CFDA-labeled, opsonized B16 suspension was added to each well of a six-well plate containing Deep Red-labeled BMDMφs and incubated at 37°C, 5% CO₂ for 2 h. All cells were detached with 0.25% TRED and resuspended in FACS buffer [PBS plus 1% (v/v) BSA and 0.1% sodium azide] and analyzed on an LSRRI flow cytometer (BD Biosciences). For analysis of phagocytosis by fluorescence microscopy, 0.5 ml containing opsonized B16 cells was added to each well of a 24-well plate containing Deep Red-labeled BMDMφs and incubated at 37°C, 5% CO₂ for 2 h. For the final 15 min of the incubation period, cells were stained with 1 µg/ml Hoechst 33342 (Thermo Fisher Scientific) before fixation with 4% formaldehyde. Epifluorescence images were acquired on an EVOS XL auto imaging system with either a 20× objective or on an Olympus IX inverted microscope with a 40× objective. For imaging, we quantified the phagocytic index, which is defined as the product of the percentage of the total BMDMφs population phagocytosing and the number of target cells engulfed per BMDMφ.

Immunoprecipitation and western blotting

Wild-type THP-1 phagocytes and CD47 knockdowns (2×10⁵) were cultured and differentiated in six-well plates for 48 h after PMA differentiation. Human CD47 was attached to 2.1 µm diameter beads at specific densities as described above and added at a bead-to-cell ratio of 20:1 for 10 min. Following the incubation time, the cells were washed with ice-cold PBS and then lysed on ice in 300 µl of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% protease inhibitor cocktail (Millipore Sigma) and 2 mM activated sodium orthovanadate]. For immunoprecipitation, whole-cell lysate was mixed with 1:200 anti-SIRPα (SET2C2 clone) antibody (Santa Cruz Biotechnology, Inc.) with Pierce Protein G agarose (Santa Cruz Biotechnology) primary antibodies and anti-goat-HRP (Amersham Biosciences). All western blots were run in duplicate, along with an additional blot for actin to ensure constant protein load among samples.

Fluorescent labeling of transfected CHO with soluble SIRPα and CD47 antibodies

A mix of 5 µl of soluble SIRPα (final concentration ~1 µM), 5 µl of 2 mg/ml Alexa Fluor 647-conjugated rabbit anti-GST, 40 µl DPBS, 1% BSA and 2.5×10⁶ CHO cells was prepared, and incubated at room temperature for at least 30 min. Cells were pelleted (500 g for 5 min) and resuspended in 1 ml cold DPBS and analyzed immediately. For antibody labeling, saturating levels of anti-CD47 antibody in 50 µl of DPBS, 1% BSA and 2.5×10⁶ CHO cells were mixed together and incubated as above. Cells were washed in 0.5 ml DPBS with 1% BSA and then resuspended in 50 µl of DPBS with 1% BSA containing 5 µl of secondary antibody (2 mg/ml). After incubation for 30 min at room temperature, cells were washed once in 0.5 ml DPBS and resuspended in 1 ml of DPBS with 1% BSA and imaged immediately.

Quantification of fluorescent intensity

Images were acquired with an inverted microscope (Olympus, IX71) with a 60× (oil, 1.4 NA) objective using a Cascade CCD camera (Photometrics, Tuscon, AZ). Image acquisition was performed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). All subsequent image analysis was done using ImageJ.

GEO microarray data analysis

Data from the GEO database was used to obtain gene expression data for key genes associated with macrophage identity (Fig. S1B). The cell types included in this analysis were human HEK 293T (GEO accession GSE28715), human PMA-differentiated THP-1 macrophages (GEO accession GDS4258), primary mouse macrophages (GEO accession GDS2454) and mouse B16F10 melanoma (GEO accession GSE33607).

Molecular modeling

To gain insight about the degree of flexibility and mobility of SIRPα used the Normal Mode Analysis technique. For this purpose, we used the α-carbon backbone of the published crystal structure for SIRPα as model (PDB: 2WNG). Our first goal was to detect the presence of hinges. This problem is challenging when only one structure is known, and several algorithms and techniques have been developed to tackle such a problem...
we needed to make use of the Anisotropic Network Model (ANM) (Hinsen et al., 1999). We were also interested in having a spatial description of the normal modes, the regions delimited by residues 113–117 and 220–222 act as hinges. Since we were also interested in having a spatial description of the normal modes, we needed to make use of the Anisotropic Network Model (ANM) (Hinsen et al., 1999). Despite being less realistic than GNM, we used ANM since it is better suited for assessing the directions of motions (Cui and Bahr, 2007).

**Quantitative and statistical analysis**

All statistical analyses were performed using GraphPad Prism 7. Unless otherwise noted, all statistical comparisons were made by unpaired two-tailed Student’s t-test and were considered significant if \( p < 0.05 \). Unless otherwise stated, all plots show mean±s.e.m. or mean±s.d. Figure legends specify the exact meaning of ‘n’ for each figure.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

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**References**


