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Self inhibition of phagocytosis: the affinity of ‘Marker of Self’ CD47 for SIRP α dictates potency of inhibition but only at low expression levels

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Abstract

Phagocytes engulf foreign cells but not ‘self’ in part because self cells express CD47 as a ligand for signal regulatory protein SIRP α , which inhibits phagocytosis. Motivated by reports of upregulation of CD47 on both normal and cancerous stem cells [1] and also by polymorphisms in SIRP α [2], we show here that inhibition of engulfment correlates with affinity of CD47 for SIRP α – but only at low levels of CD47. One common human polymorph of SIRP α is studied and binds more strongly to human-CD47 than to mouse-CD47 ($K_d \approx 0.12 \mu\text{M}$ and $6.9 \mu\text{M}$, respectively) and does not bind sheep red blood cells (RBC) – which are well-established targets of human macrophages; in comparison, a common mouse polymorph of SIRP α binds with similar affinity to human and mouse CD47 ($K_d \approx 0.22 \mu\text{M}$). Using immunoglobulin (IgG)-opsonized particles with varying levels of either human- or mouse-CD47, the effective inhibition constants K_i for blocking phagocytosis are then determined with both human- and mouse-derived macrophages. Only human phagocytes show significant differences in man versus mouse K_i 's and only at CD47 levels below normal densities for RBCs. While phosfo-signaling through human-SIRP α shows similar trends, consistent again with the affinity differences, saturating levels of CD47 ($> K_i$) can signal and inhibit phagocytosis regardless of man versus mouse. Quantitative analyses here prompt more complete characterizations of both CD47 levels and SIRP α polymorphisms when attempting to study *in vivo* effects of these key proteins in innate immunity.

Introduction

Critical to innate immunity, macrophages engulf a foreign cell or particle in a coordinated process of adhesion, activation, and internalization, but macrophages come into contact with ‘self’ cells more frequently than foreign. Opsonization of a target by immunoglobulin-G (IgG) facilitates binding to the macrophage's Fc γ receptors (Fc γ Rs), which signal assembly of a focal adhesion-like synaptic structure for firm attachment to the target Error! Reference source not found. Error! Reference source not found.], but self cells are also opsonized by Ig [5]. While an analogy to integrin-based focal adhesions rightly suggests the importance of ligand densities and various phospho-Tyrosine (pTyr) signaling proteins as well as myosin-II to pull on a

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nascent adhesion to promote adhesion maturation [6], the processes by which macrophages distinguish foreign objects from autologous cells of self remain largely unclear. Studies of CD47-knockout mice have shown that red blood cells (RBC) that lack the membrane receptor CD47 are readily taken up by normal mouse macrophages – though not by CD47-null macrophages [6] A.E. Brown and D.E. Discher. Conformational changes and signaling in cell and matrix physics, *Current Biology* 19 (2009) pp.R781–R789.

[3]. Human RBCs with considerably reduced levels of CD47 (10-20% of normal in some Rh variants) show no evidence of enhanced interaction with phagocytes [4][5][6], but hematopoietic stem cells (HSC) in the circulation as well as leukemic cancer stem cells have been reported to overexpress CD47 by 2- to 4-fold (for human and mouse HSC, respectively) relative to marrow resident normal cells [1]. Extra CD47 on cells in both mouse and man is speculated to protect from phagocytic clearance, but quantitative relations to underlying protein-protein interactions and downstream signals have remained obscure.

CD47 binds with high specificity to the inhibitory membrane receptor SIRP α [7], which is expressed at particularly high densities on macrophages [8][9][10][11]. SIRP α belongs to a ‘paired receptors’ class of membrane proteins analogous to Natural Killer (NK) cell receptors involved in MHC recognition [12]. SIRPs have three extracellular immunoglobulin domains, a variable region at the N-terminal and distinct transmembrane and cytoplasmic sequences that function as activating or – as with SIRP α – inhibitory signal motifs [13][14][15]. Macrophages distinguish self from foreign through binding of SIRP α 's N-terminal to the lone Ig domain of ‘marker of self’ CD47 on target cells. The interaction leads to pTyr modifications of SIRP α 's immune-receptor Tyrosine-based inhibitory motif (ITIM) with downstream activation of SHP-1 phosphatase and subsequent deactivation of myosin-II and the contractile cytoskeletal activity involved in pulling a target into a macrophage [16]. Differences in CD47-SIRP α binding presumably affect signaling and inhibitory function, but linearity or not of signal transmission is unclear and has implications for up- or down-regulation of CD47. In fact, saturation in binding and signaling should be anticipated.

If the hypothesis of CD47 as a marker of self is valid, then macrophages should be able to discriminate between cells of different species and perhaps between different ‘strains’ or polymorphisms. Earlier studies have indicated limited cross-reactivity across species between SIRP α and CD47 [17]. Initial studies here demonstrate that sheep-RBCs as well as mouse-RBCs bind less soluble human SIRP α when compared to human-RBCs (Figure 1A). Based on this divergence, human macrophages might be expected to engulf opsonized sheep RBCs – an expectation borne out by many prior studies using sheep-RBCs as targets for human macrophages in phagocytosis (e.g. [18][19][20]). In addition to the recognition of SIRP α and CD47 between species, there exists considerable polymorphism in SIRP α between mouse strains [21] and between humans [2]; indeed, from a very limited but worldwide study of 37 human haplotypes, 10 variants of SIRP α 's ~100 amino acid N-terminal domain were reported. Polymorphisms in SIRP α in humans have been suggested, based on xenografts of human-HSC into mice [2], to explain stem cell compatibility differences in humans [22]. SIRP α polymorphisms that alter interactions thus have major implications for human health, contributing perhaps to anemias and autoimmune diseases [23]. Here we exploit man versus mouse differences in quantitation of CD47-SIRP α binding, phagocytosis, and signaling as we seek to define mechanisms by which macrophages discern foreign from self.

RESULTS and DISCUSSION

SIRP α Binding to CD47 is Species-specific for human but not mouse

In order to clarify some of the limits of CD47 as a “self” marker, we assessed the extent to which soluble human SIRP α (hSIRP α) binds to red blood cells (RBC) and purified CD47 from different species. Soluble human SIRP α used in our studies and expressed in the human-derived THP-1 macrophages below express hSIRP α variant 2 – ‘V2’ in the nomenclature of [2] – as assessed by reverse transcriptase PCR (RT-PCR) and sequencing. RBC from mice as well as sheep respectively show weak to no binding to this recombinant hSIRP α when compared to human RBCs (Figure 1A). Based on the lack of sheep RBC interactions with soluble hSIRP α , human macrophages would be expected to phagocytose IgG-opsonized sheep RBC – which is indeed commonly reported in studies of Fc γ receptor mediated phagocytosis [18][19][20]. RBC membranes are nonetheless complex, with many additional differences between species: for example, CD47 on human RBCs is less mobile than on mouse RBCs in part due to human CD47's association with the species-specific Rh complex [24]. To eliminate such differences in measurements of binding, the immunoglobulin-like domain of human CD47 (hCD47) and also of mouse CD47 (mCD47) were recombinantly expressed with a spacer domain plus a C-terminal biotinylation site [25][16]. The attachment of the hCD47 and mCD47 to avidin-coated beads was confirmed using antibodies against the specific species by flow cytometry (Figure S1), and the density of CD47 was adjusted to that of normal RBC [26] with binding affinity then determined by varying the concentration of soluble hSIRP α . Flow cytometry data was fit to saturation binding, giving dissociation constants (K_d) of 0.12 and 6.93 μ M that indicate a ~60-fold weaker binding of mCD47 to hSIRP α (Figure 1B).

The SIRP α -V2 used here together with past results for SIRP α -V1 [27] suggest similar binding affinities for human CD47 despite 9 residue differences in the N-terminal domain that binds CD47. With the same types of beads, interactions of soluble mSIRP α were also examined; the N-terminal domain of this mSIRP α – referred to as 129-IgV [2] – differs in only minor ways from mSIRP α on J774A.1 macrophages (used below), which has the sequence of that reported in BalbC mice (see Methods). In contrast, the latter mSIRP α differs from human at 43 residues, including key amino acids at or near hCD47's binding site for hSIRP α [28]. No difference in binding was detected in the moderately high affinity interactions of mSIRP α with both mCD47-coated particles and hCD47-coated particles (K_d = 0.22 μ M; Figure 1C). The lack of species specificity with mouse SIRP α needs to be understood better at a detailed structural level, but phagocytosis results below with macrophages will be seen to be consistent with these binding results.

Species-specific phagocytosis is weakly inhibited by soluble CD47

In a first study of phagocytosis, sheep and human RBC were IgG-opsonized and fed to macrophages. The opsonization level is linear with anti-serum dilution (Figure 2A) while phagocytosis of sheep-RBC by human-derived THP-1 macrophages as well as by mouse-derived J774A.1 macrophages is non-linear, exhibiting a saturable type of response (Figure 2B,C). Addition of soluble human-CD47 at μ M concentrations ($> K_d$) showed at most a slight inhibition of phagocytosis of sh-RBC by THP-1 macrophages (Figure 2C, inset) and by J774A.1 macrophages (not shown). Because phagocytosis is a highly localized process on the macrophage, signaling at the phagocytic synapse would likely be tested better, we thought, if the various recombinant CD47's were attached to otherwise identical target particles.

Inhibition of phagocytosis by CD47 is species-specific for human but not mouse

The same type of avidin-coated particles as used for binding in Figure 1B,C (but made red fluorescent in Figure 3A) were IgG-opsonized with anti-streptavidin for Fc γ R-mediated phagocytosis and then fed to human or mouse macrophages. These particles of course lack

membrane proteins (eg. Rh proteins) and standardize the opsonization as well as the CD47 presentation. After the phagocytosis assay, non-engulfed particles were detected with an anti-Fc (green) against the IgG-opsonin. Phagocytosed particles were protected from the anti-Fc because cells were not permeabilized, thus providing a method to quantify the internalized beads per phagocyte for both human THP-1 and mouse J774A.1 macrophages. With increasing opsonization human macrophages phagocytosed the uncoated “CD47-null” beads significantly more than beads displaying hCD47 (Figure 3B). The two ligands, CD47 and IgG-opsonin, do not compete and do not interfere with SIRP α binding [16], and the phagocytosis results for mCD47 coated beads proved consistent with the hCD47 coated beads (Figure 3C). The dependence on the level of IgG-opsonization fits a saturation binding process, indicative of the specificity of Fc γ -R mediated phagocytosis as seen with RBC (Figure 2C).

Although seminal studies of CD47-knockout mice implicated CD47 as a “marker of self” on mouse RBCs [6] A.E. Brown and D.E. Discher. Conformational changes and signaling in cell and matrix physics, *Current Biology* 19 (2009) pp.R781–R789.

[3], human Rh_{null} genotypes with major co-deficiencies of CD47 on RBCs show no evidence of increased macrophage interactions [5][6]. One could also hypothesize that overexpression of CD47, such as reported for circulating hematopoietic and leukemic stem cells in man or mouse [1], could mediate strong adhesion and thereby promote phagocytosis rather than inhibit it (i.e. adhesion dominates signaling).

Based on results from a 40-fold range of CD47 densities, human-CD47 proved more potent than mouse-CD47 in inhibiting phagocytosis by THP-1 macrophages (Figure 4). The species-specific inhibition constant, K_i differed by 5-fold at high IgG-opsonin and 2.2-fold at low opsonin (Table 1 and Figure S2A). However, maximum inhibition proved to be the same for human- and mouse-CD47 and is achieved in vitro here with particles at CD47 densities at or below those estimated for CD47 on human RBC (~ 250 molecules/ μm^2). Even at several fold higher densities, the same maximum inhibition was found, indicating that CD47-mediated signaling dominates CD47-mediated adhesion. Primary human peripheral blood monocytes incubated with IgG-opsonized particles at high densities of hCD47 or mCD47 (~ 400 molecules/ μm^2) also showed statistically similar reduction in phagocytosis (Figure 4A, inset).

Mouse J774A.1 macrophages, in contrast to human cells, showed levels of inhibition of phagocytosis IgG-opsonized particles that were similar for both human-CD47 and mouse-CD47 at all CD47 densities (Figure 4B). The lack of difference in phagocytosis by mouse macrophages is fully consistent with the lack of difference in binding affinities of soluble mSIRP α for mCD47 and hCD47 (Figure 1C). More importantly, the results for human and mouse showed that even 10-20% of normal CD47 densities are sufficient to inhibit phagocytosis for CD47-SIRP α within the same species whereas weaker binding can be compensated by increasing CD47 density.

The surface concentrations of CD47 above are not small. Assuming a phagocytic synapse with a gap between macrophage and target of ~ 10 nm [28], the K_i range of 20-200 molecules/ μm^2 corresponds to a gap concentration $K_i^{3D} \approx 3\text{-}30$ μM . Because $K_i^{3D} \gg K_d$, there seem to be impediments to the effectiveness of inhibitory signaling by CD47 – such as overcoming Fc γ receptor activation within the same signaling synapse – which probably explains the relative ineffectiveness of soluble CD47 at ~ 1 μM (Figure 2C, inset). CD47 on the macrophage [26] might also interact in *cis* with SIRP α and thereby compete with CD47 in *trans* (on the target's surface), which would effectively reduce the inhibitory interaction. The fits of phagocytosis inhibition also suggest a Hill coefficient of $m = 2$ which implies cooperative interactions that are characteristic of dimers and might further explain the limited inhibition by soluble CD47. Nano-resolution imaging of the molecular rearrangements within the phagocytic synapse might help to clarify such structure-function issues.

CD47 signaling through SIRP α is species-specific

Based upon the binding and phagocytosis studies above (Figure 1 and 4A), SIRP α binding to CD47 sends species-specific signals primarily through hSIRP α . Recent results have also documented hSIRP α localization to the phagocytic synapse with targets presenting CD47 [16], which is consistent with ligand-receptor interactions that are expected to phospho-activate SIRP α 's immune-tyrosine based inhibitory motif (ITIM) which then activates SHP-1 phosphatase [29][30]. We therefore hypothesized for a last set of experiments that hCD47 would also prove more effective than mCD47 at inducing SIRP α phosphorylation during phagocytosis. Immunoprecipitation of SIRP α followed by Western blot analysis of phospho-Tyrosine (pTyr) showed a clear but saturable difference in signaling by hCD47 versus mCD47 (Figure 5). Normalization of CD47 densities to the phagocytosis inhibition constant for human-CD47 (Figure 4A), denoted as K_{i-h} , and normalization of pTyr levels to SIRP α intensities showed that the effective signaling constant K_s not only approximated the K_i for each species but also differed by ~10-fold between human and mouse.

CONCLUSIONS

Normal macrophages are efficient at removing typical targets such as foreign cells or particles and apoptotic cells [31], but cells or particles that express a recognizable CD47 are engulfed in vitro at a lower frequency. The decision of a macrophage to 'eat' a target is in part made by the extent of target opsonization, and Ig concentrations being very high in bodily fluids seems to lead to absorption or perhaps weak binding at some level to all cells [], especially aged blood cells [36]. Figure 6 summarizes our quantitative studies of IgG-opsonized beads decorated with CD47 and phagocytosed by human or mouse macrophages. A higher CD47-SIRP α binding strength (set by species) results in higher potency inhibition and this increases with increasing opsonin activation of the cell. Such signal amplification through antagonistic interactions might seem counter-intuitive, but enhanced CD47 signaling with increased IgG-opsonization might occur because IgG binding to Fc γ R [33] promotes intimate adhesion between target and macrophage, narrowing the gap between interfaces and thereby promoting CD47 interactions with SIRP α within the phagocytic synapse.

Based on the various man versus mouse results here, a 60-fold higher binding affinity of CD47 for SIRP α (Figure 1B) produces a 10-fold more specific pTyr signal in SIRP α (Figure 5), which leads to a 5-fold more specific inhibition of phagocytosis at high opsonin (Table 1). Biochemical differences propagate but seem blunted rather than amplified in this process of signaling self. Specificity is also lost altogether at high CD47 density, consistent with a single saturable process of binding $\rightarrow$ signaling $\rightarrow$ local inhibition. While the interactions might be crucial to some species-specific recognition, polymorphisms in SIRP α within a given species have been reported for human [34][35][2] and mouse [21] and raise important questions about altered interactions. Structural analysis of the human SIRP α -CD47 complex has suggested that SIRP α polymorphisms occur outside the binding site and therefore seem unlikely to directly affect CD47 binding [28]; polymorphisms have been speculated to frustrate pathogens that seek to exploit the SIRP α inhibitory signal [28]. Other paired receptors seem to use this strategy including Ly49 which interacts with viral particles [36] and the paired immunoglobulin-like receptor (PIR) that binds bacteria [37]. However, human hematopoietic stem cells (HSCs) have been shown to engraft efficiently only into the NOD strain of mouse because human-CD47 binds sufficiently strongly to SIRP α^{NOD} but not other mouse SIRP α 's [2]. Our binding, signaling, and phagocytosis results motivate additional characterization of CD47 expression levels on all cells involved, especially in light of changes in CD47 expression levels on HSCs [1]. The results here nonetheless help to make it clear that in man, and probably in mouse, innate immune cells distinguish cells or other targets for clearance through specific CD47-initiated "self" signals that ultimately inhibit engulfment.

Materials and Methods

Chemicals

Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} or Mg^{2+} (Invitrogen) was supplemented with either 1% BSA or 1% BSA + 0.05% Tween 20 (Sigma-Aldrich). PKH26 (Sigma-Aldrich) hydrophobic dye was used for red cell labeling. Tris-buffered saline (TBS) and TBS with Tween 20 (TTBS) were used in Western blotting.

Antibodies

The fluorescein-labeled antibody B6H12-FITC was used against human CD47 (BD Biosciences). Opsonizing antibodies against human and sheep RBCs included rabbit anti-human RBC, and rabbit anti-sheep RBC (Sigma-Aldrich); these were used as IgG opsonin in phagocytosis assays. Antibodies against 1.1 μm radius streptavidin-coated polystyrene beads (Spherotech) included rabbit anti-streptavidin (Sigma-Aldrich); and rabbit anti-streptavidin conjugated with FITC (Rockland Immunochemicals) used also as IgG opsonin in phagocytosis assays. Secondary antibodies used for detecting opsonin levels and uningested beads included goat anti-rabbit FITC or goat anti-rabbit F(ab')₂ R-PE (Sigma-Aldrich). Secondary antibodies used for in conjunction with soluble SIRP α or CD47 binding included anti-GST Alexa 488 (Invitrogen) and anti-biotin Cy5 (Sigma-Aldrich) respectively.

SIRP α reverse transcriptase PCR and sequence

The SIRP α variant expressed in THP-1 macrophages was confirmed by RNA extraction (Qiagen) using a one step reverse transcriptase PCR (RT-PCR) amplification (Invitrogen). Samples of the PCR product were run on 1% agarose gel and gel purified for sequencing. Primers used for both RT-PCR sequencing include the following 5'-GGGTGAGGAGGAGCTGCAGGTGATT-3' and 5'-GCGCTCGAGCCGTTTCATTAGATCC-3'. In the case of mSIRP α expressed in J774A.1 macrophages the primers used were 5'-CGTCCTGTTTCTGTACAGGA-3' and 5'-AGTTCACCTTCTGGTCAGGT-3'. J774A.1 cells are derived from Balb/C mice, and compared to our soluble mSIRP α , the mSIRP α in the J774A.1 cells differs in the N-terminal domain by 8 residues, with 6 conservative changes and the other 2 being far from the site of CD47 interaction [28].

Cell culture

COS-1, CHO-K1, J774A.1, and THP-1 cells (American Type Culture Collection) were respectively maintained in DMEM, MEM α , and RPMI 1640 media (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich). Differentiation of THP-1 cells was achieved in 100 ng/mL phorbol myristate acetate (PMA) (Sigma-Aldrich) for 2 days and confirmed by attachment of these cells to tissue-culture plastic. Peripheral blood monocytes from human donors were obtained through the Human Immunology Core at Penn (University of Pennsylvania). Human blood was obtained from finger pricks of healthy donors. Blood from other species was obtained from Covance and washed 3x in PBS plus 0.4% BSA.

Soluble human and mouse SIRP α production

COS-1 cells were transfected with pcDNA3-based vector [10] encoding a human SIRP α extracellular domain (variant: NA18949_V2) fused to GST using Lipofectamine 2000 (Invitrogen). Secreted SIRP α 1-GST (referred as hSIRP α) was affinity-purified using Glutathione Sepharose 4B (Amersham Biosciences) and dialyzed against PBS (Invitrogen). The protein was stored at -20°C with or without addition of 10% v/v glycerol (Fisher Scientific). The extracellular domain of mouse SIRP α (variant: 129_IgV) was also prepared as a GST fusion and will be referred as mSIRP α .

Production of recombinant human and mouse CD47

Plasmid encoding the extracellular domain of human and mouse CD47 were PCR amplified, digested with XbaI and SalI (New England Biolabs, Inc.), and ligated to similarly digested vector, pEF-BOS-XB [15], which results in an in-frame fusion of CD4d3+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). Secreted CD47-CD4d3+4 was concentrated using a 10K MWCO Amicon filter (Millipore) and biotinylated at the C-terminus using a biotin-protein ligase (Avidity, LLC) and dialyzed against PBS (Invitrogen). The protein was affinity-purified using monomeric avidin (Promega) and dialyzed against PBS (Invitrogen).

Preparation and quantification of CD47 density on polystyrene beads

Streptavidin-coated polystyrene beads of 2.1 μm diameter (Spherotech) were washed and blocked 3x in PBS plus 0.4% BSA. Biotinylated human CD47 was attached to streptavidin-coated beads at room temperature for 30 min, washed 3x and resuspended in PBS plus 0.4% BSA. The attachment of mouse CD47 to streptavidin coated beads was performed in the same way.

The density of human and mouse CD47 present on the beads was assessed by labeling with saturating levels of B6H12-FITC and mIAP301-FITC (BD Biosciences) respectively for 30 min at room temperature. Beads were washed and resuspended in PBS/0.4% BSA and flow cytometry measurements were performed using BD Calibur (BD Immunocytometry Systems). Mean fluorescence intensities were calibrated against uncoated streptavidin beads labeled with saturating B6H12-FITC/ mIAP301-FITC levels (Figure S1). The fluorescent intensities were standardized using Quantum FITC Molecule of Equivalent Soluble Fluorochrome (MESF) units (Bangs Laboratories). The MESF value for the human and mouse CD47 beads were then divided by the number of fluorophore molecules per antibody to obtain the number of molecules per bead. The density of CD47 molecules on the streptavidin beads was determined by dividing the surface area (SA) of the 2.1 μm bead ($\text{SA} = 13.9 \mu\text{m}^2$). The density of CD47 molecules on human RBC ($\text{SA} = 128 \mu\text{m}^2$) and mouse RBC ($\text{SA} = 85 \mu\text{m}^2$) was also determined as described for the CD47 beads.

Binding isotherm for soluble hSIRP α^{ex} and mSIRP α^{ex} for CD47 coated beads

Human and mouse CD47 were attached at the identical density as described above. The binding isotherm of soluble hSIRP α^{ex} and mSIRP α^{ex} were performed for the different species 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 10^4 events using a FACScan or FACSCalibur (BD Immunocytometry Systems). Data points from flow cytometry was plotted and fitted.

Phagocytosis assay

For phagocytosis assays, macrophages were plated in 4-cm² Lab-Tek II chambered coverglasses (Nalge Nunc International) at $10^5/\text{cm}^2$. Streptavidin polystyrene beads or RBCs were added to macrophages at a ratio of 20:1 and allowed to incubate at 37°C for 45 min. Non-phagocytosed beads was washed with PBS. Cells were fixed with 5% formaldehyde (Fischer Scientific) for 5 min, followed by immediate replacement with PBS. For differentiation of non-internalized beads, beads were labeled with a primary antibody, rabbit anti-streptavidin (Sigma-Aldrich) at 1:1,000, 1:2,000, or 1:8,000 in PBS for 20 min at 25°C. A second antibody, anti-rabbit R-PE (Sigma-Aldrich) was added at 1:1,000 in PBS to the cells and incubated for an additional 20 min at 25°C. Cells were then washed with PBS/ 0.4% BSA and then quantified

by light and fluorescent microscopy. At least 200 cells were scored per well and experiments were repeated at least three times.

For stimulated phagocytosis assays, beads with or without CD47 were incubated with rabbit anti-streptavidin serum (Sigma-Aldrich) as the opsonin. Beads were opsonized at the respective concentrations for 30 min at RT. In some experiments, human CD47 was blocked with a F(ab')₂ monoclonal antibody against CD47. The Fc fragment of mAb B6H12 were removed by enzymatic digestion with immobilized Ficin (Pierce) and separation by protein A chromatography after 10 min of the addition of opsonin. Opsonized beads were washed 2x and resuspended in 50µl of PBS/0.4% BSA.

Immunofluorescence microscopy

Immunostaining was performed after cells were fixed and blocked for 1 h with 5% BSA in PBS. Staining with primary antibody anti-rabbit PE-conjugated (1:200) was used for detection of non-phagocytosed beads for 1 h at room temperature in PBS. After washing, samples were fixed with 5% formaldehyde and imaged. In order to ensure that the cells were not permeable to labeling an antibody against myosin IIA was used to confirm not labeling occurred (Sigma-Aldrich).

Images were acquired on an inverted microscope (IX71; Olympus) with a 40x or a 60x (oil, 1.4 NA) objective using a Cascade CCD camera (Photometrics). Image acquisition was performed with Image Pro software (Media Cybernetics, Inc.). All subsequent image analysis was done using ImageJ.

Immunoprecipitation and Western blotting

Human phagocytes, THP-1 cells (2×10^6) were cultured and differentiated in 6-well plates for 48 hours after PMA differentiation. Human and mouse CD47 was attached to 2.1µm beads at specific densities as described in the text and added at a bead to cell ratio of 20:1 for 2, 5, 10, or 30 minutes. Following the incubation time, the cells were washed with ice-cold PBS and then lysed on ice in 400 µl of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM EDTA, 1% NP-40, 1% protease inhibitor cocktail [Sigma-aldrich] and 2mM activated sodium orthovanadate). For immunoprecipitation, whole lysate was mixed with 2µg of anti-SIRPα antibody (SE7C2 clone)(Santa Cruz Biotechnology, Inc.) and protein G (Fischer Scientific) at 4°C overnight. Precipitated proteins were washed with lysis buffer 3x and resuspended in 2x LDS buffer (Invitrogen) and boiled at 90°C. The supernatant was collected and run on a 10% SDS-PAGE gel (Invitrogen), transferred to PVDF membrane for Western blotting, where anti-phosphotyrosine HRP-conjugated antibodies and anti-SIRPα (clone c-20) followed by anti-goat HRP-conjugated (Santa Cruz Biotechnology, Inc.) as a primary antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Species-specific binding of soluble human-SIRPα to various species RBCs and CD47-coated beads

(A) Fresh human, sheep, and mouse RBC binding to soluble hSIRPα (4 μM of GST conjugate), as detected by FITC-anti-GST. 'Bkgd' is obtained with RBC plus anti-GST. Species-specific affinities of hCD47- and mCD47-coated beads binding to soluble (B) hSIRPα or (C) mSIRPα based on mean fluorescence in flow cytometry. Saturation binding fits gave the indicated dissociation constants, K_d .

Figure 2. Phagocytosis of Ig-opsonized sheep-RBC and human-RBC by human-THP-1 macrophages

(A) Fresh human and sheep RBC were incubated with antiserum at different dilution ratios and detected by fluorescent secondary antibodies, exhibiting a linear opsonization based on mean fluorescence in flow cytometry. (B) Phagocytosis of shRBC by human-derived THP-1 macrophages at different opsonization level observed by DIC microscopy, which showed a saturable type of response (C). Addition of soluble human-CD47 at μM concentrations showed at most a slight inhibition of phagocytosis of sh-RBC by THP-1 macrophages but not enough to reach hRBC phagocytosis levels at 1/900 antiserum dilution ratio.

Figure 3. CD47 is sufficient to inhibit phagocytosis

(A) Microbeads with streptavidin were coated with anti-streptavidin IgG as the opsonin ± biotinylated human-CD47 or mouse-CD47. Phagocytosis of beads (red) by THP-1 or J774A.1 cells was assessed in DIC and fluorescence with non-ingested beads (green) visible with rabbit anti-streptavidin plus a second, fluorescent goat anti-rabbit antibody. White arrow indicates non-phagocytosed beads. Scale bar, 10μm. Streptavidin beads coated with both anti-streptavidin IgG as the opsonin and ± biotinylated (B) human CD47 particle targets with THP-1 macrophages or (C) mouse CD47 particle targets with J774A.1 in a phagocytosis assay.

Figure 4. Inhibition of phagocytosis by CD47 is density dependent

IgG-opsonized particles with human (solid line) and mouse CD47 (dashed line) were used as targets for (A) human THP-1 macrophages or (B) mouse J774A.1 macrophages. Grey shaded vertical bar indicates normal RBC density (~ 250 CD47/ μm^2). Species-specific differences are quantitated by fits to $y = a - b x^m / (K_i^m + x^m)$ with effective K_i [molecules/ mm^2] that differ by 5-fold at high opsonin, although the maximum inhibition is similar (see Table 1). A Hill coefficient of $m = 2$ suggests cooperativity.

Figure 5. Species-specific signaling through SIRPα

hCD47 or mCD47 were bound at varying densities to opsonized beads and phagocytosed by THP-1 macrophages. From macrophage lysates, SIRPα was immunoprecipitated and immunoblotted (inset) for quantitation of phospho-Tyr and total SIRPα for normalization. Fits of the data gave effective signaling constant K_s for each species that depends on the CD47 density; all densities are scaled by hCD47's inhibitory constant ($K_{i,h}$) as determined in Figure 4.3A at the same opsonization.

Figure 6. Potency and binding strength depend on species-specific CD47-SIRP α interactions
 IgG-opsonization modulates the density-dependent activity of hCD47 or mCD47 with either human THP-1 macrophages (hSIRP α) or mouse J774A.1 macrophages (mSIRP α). Each point on the plot represents a K_d obtained from binding studies per Figure 4.1B and a K_i obtained per Figure 4.3A as fitted by a Hill-type model. Larger symbols indicate results with higher opsonin, which promotes synapse formation and thereby more potent inhibition than at lower opsonin.

Table 1
Human THP-1 phagocytosis of beads

Summary of conditions used to fit the data points for both hCD47 and mCD47 coated beads at high and low opsonization. The K_i for each of conditions are summarized with low K_i having a higher inhibitory effect.

	<u>a</u>	<u>b</u>	<u>m</u>	<u>K_i</u>
hCD47 (High Opson.)	1.07	0.53	2	21
mCD47 (High Opson.)	1.07	0.53	2	110
hCD47 (Low Opson.)	0.46	0.25	2	87
mCD47 (Low Opson.)	0.46	0.25	2	190