Title

CD47 Ligation Repositions the Inhibitory Receptor SIRPA to Suppress Integrin Activation and Phagocytosis

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Summary

CD47 acts as a "Don't eat me signal" that protects cells from phagocytosis by macrophages. Since CD47 upregulation on cancer cells facilitates immune evasion, CD47 is a target for cancer immunotherapy. Here, we utilized a reconstituted system to examine the mechanism by which CD47 binding to its receptor SIRPA prevents phagocytosis. We show that SIRPA is excluded from the phagocytic synapse between a macrophage and its target but is driven into the synapse by CD47 ligation. In the absence of CD47, artificially directing SIRPA to the synapse suppressed engulfment, indicating that receptor localization is critical for inhibition. Downstream of SIRPA engagement, CD47 impeded macrophage spreading and decreased integrin activation. Chemical re-activation of integrins bypassed CD47 inhibition, allowing macrophages to engulf targets including cancer cells. Our results reveal that CD47 suppresses phagocytosis by repositioning SIRPA to inhibit integrin signaling in macrophages and suggest new strategies for cancer immunotherapy.

Introduction

The innate immune system is finely balanced to rapidly activate in response to pathogenic stimuli but remain quiescent in healthy tissue. Macrophages, key effectors of the innate immune system, measure activating and inhibitory signals to set a threshold for engulfment and cytokine secretion. The cell surface protein CD47 is a "Don't Eat Me" signal that protects healthy cells from macrophage engulfment (Oldenborg et al., 2000). Hematopoietic cells lacking CD47 are rapidly engulfed by macrophages and trigger dendritic cell activation (Oldenborg et al., 2000; Yi et al., 2015). CD47 also functions in the nervous system, protecting active synapses from pruning by microglia (Lehrman et al., 2018). CD47 expression is often increased on cancer cells as a mechanism to evade immune detection (Chao et al., 2012; Jaiswal et al., 2009; Majeti et al., 2009; Oldenborg et al., 2001, 2000). CD47 function-blocking antibodies result in decreased cancer growth or tumor elimination (Advani et al., 2018; Chao et al., 2010a; Gholamin et al., 2017; Jaiswal et al., 2009; Willingham et al., 2012). Augmenting macrophage function by CD47 blockade may also be beneficial in other disease contexts, like atherosclerosis or viral infection (Cham et al., 2020; Kojima et al., 2016). Despite the therapeutic promise of manipulating CD47 signaling, the mechanism by which CD47 suppresses macrophage engulfment is unclear.

CD47 on the surface of target cells is recognized by SIRPA (Signal Regulatory Protein α) on macrophages or dendritic cells (Jiang et al., 1999; Liu et al., 2015; Okazawa et al., 2005; Oldenborg et al., 2000; Seiffert et al., 1999; Tseng et al., 2013; Yi et al., 2015). SIRPA is an inhibitory receptor containing multiple intracellular Immune Tyrosine-based Inhibitory Motifs (ITIMs; Kharitonenkov et al., 1997). Macrophages lacking SIRPA do not exhibit reduced phagocytosis of CD47-bearing targets, suggesting that SIRPA is the primary transducer of the CD47 signal (Okazawa et al., 2005; Oldenborg et al., 2000). Activation of the inhibitory receptor SIRPA must be controlled with high fidelity to suppress engulfment of viable cells when CD47 is present while allowing for robust engulfment of targets lacking CD47. CD47 binding triggers SIRPA phosphorylation by Src family kinases (Barclay and Brown, 2006), but how CD47 binding is translated across the cell

48   membrane to drive SIRPA phosphorylation is not known. Phosphorylated SIRPA recruits

49   the phosphatases SHP-1 and SHP-2 (Fujioka et al., 1996; Noguchi et al., 1996; Okazawa

50   et al., 2005; Oldenborg et al., 2001; Veillette et al., 1998), but the downstream targets of

51   these phosphatases and their relationship to the engulfment process are not clear.

52

53   In vivo, CD47 suppresses multiple different pro-engulfment "Eat Me" signals, including

54   IgG, complement and calreticulin (Chen et al., 2017; Gardai et al., 2005; Oldenborg et al.,

55   2001). This complexity, in addition to substantial variation in target size, shape and

56   concentration of "Eat Me" signals, can make a quantitative, biochemical understanding of

57   receptor activation difficult. To overcome this problem, here we utilized a synthetic target

58   cell-mimic with a defined set of signals to interrogate the mechanism of SIRPA activation

59   and its downstream targets. We found that CD47 ligation altered SIRPA localization,

60   positioning SIRPA for activation at the phagocytic synapse. At the phagocytic synapse,

61   SIRPA inhibited integrin activation to limit macrophage spreading across the surface of

62   the engulfment target. Directly activating integrin eliminated the effect of CD47 and

63   rescued engulfment, similar to the effect of a CD47 function-blocking antibody. Thus, the

64   CD47-SIRPA axis suppresses phagocytosis by inhibiting inside-out activation of integrin

65   signaling in the macrophage, with implications to cancer immunotherapy applications.

Results

CD47 suppresses IgG and phosphatidylserine "Eat Me" signals

To study the mechanism of "Eat Me" and "Don't Eat Me" signal integration during engulfment, we used a reconstituted engulfment target (Figure 1A). Silica beads were coated in a supported lipid bilayer to mimic the surface of a cancer cell. To activate engulfment, we introduced IgG, a well-defined "Eat Me" signal that synergizes with CD47 blockade to promote cancer cell clearance (Chao et al., 2010a; Freeman and Grinstein, 2014). IgG is recognized by the Fcγ Receptor family (FcR), which activates downstream signaling and engulfment (Freeman and Grinstein, 2014). To activate SIRPA, we incorporated the CD47 extracellular domain at a surface density selected to mimic the CD47 density on cancer cells (~600 molecules/μm^2, Figure S1).

Using this system, we tested the effect of CD47 on engulfment across a titration of IgG densities (Figure S1). We mixed beads with the macrophage-like cell line RAW264.7 and measured the number of internalized beads by confocal microscopy. We found that CD47 suppressed engulfment at intermediate IgG densities, but did not appreciably affect engulfment of targets with high densities of bound IgG (Figure 1B-D). The presence of CD47 did not completely eliminate phagocytosis, but rather caused a quantitative decrease in the fraction of cells initiating engulfment and the number of beads engulfed per cell (Figure 1B, C, Figure S1). This suppression was dependent on CD47 binding as a mutated CD47 extracellular domain that is unable to bind to SIRPA (F37D, T115K; Hatherley et al., 2008) was unable to suppress engulfment.

We further examined whether CD47-SIRPA signaling could suppress engulfment of targets mimicking apoptotic corpses. A critical "Eat Me" signal from apoptotic corpses is phosphatidylserine, which becomes exposed on the outer leaflet of the plasma membrane during cell stress, apoptosis (Fadok et al., 1992; Poon et al., 2014), and on some cancer cells (Birge et al., 2016; Utsugi et al., 1991). We found that engulfment of beads containing 10% phosphatidylserine in the supported lipid bilayer was inhibited by the

97   inclusion of CD47 on the bilayer (Figure 1E, Figure S1). Together, these data show that

98   CD47-SIRPA signaling can block engulfment driven by IgG and phosphatidylserine.

99   Moreover, bilayer-coated beads provide a well-defined and tunable platform for studying

100 the integration of "Eat Me" and "Don't Eat Me" signals during engulfment.

101

102 **CD47 ligation relocalizes SIRPA to the phagocytic synapse**

103

104 We next sought to determine the mechanism by which CD47 ligation regulates SIRPA

105 activity. We first examined SIRPA localization during phagocytosis of IgG-coated beads.

106 When not bound to CD47, SIRPA was segregated away from the phagocytic cup that

107 enveloped IgG-coated beads (Figure 2A). Similarly, SIRPA was depleted at the center of

108 the immunological synapse between a macrophage and a supported lipid bilayer

109 containing phosphatidylserine (Figure S2). In contrast, in the presence of CD47, SIRPA

110 remained at the phagocytic cup (Figure 2A). These data demonstrate that unligated

111 SIRPA is excluded from the phagocytic synapse, whereas CD47-bound SIRPA remains

112 at the phagocytic synapse.

113

114 We next sought to address the mechanism of SIRPA segregation away from the

115 phagocytic cup in the presence of IgG and absence of CD47. We hypothesized that

116 exclusion of unligated SIRPA from the synapse could be driven by its heavily glycosylated

117 extracellular domain, either by interactions with the surrounding glycocalyx or steric

118 exclusion from the spatially restricted phagocytic synapse. We therefore created a SIRPA

119 chimeric receptor where the extracellular domain was replaced with a small, inert protein

120 domain (FRB[EXT]-SIRPA; Figure 2B). Unlike full-length SIRPA, FRB[EXT]-SIRPA was not

121 segregated away from the cell-target synapse (Figure 2C). This result demonstrates that

122 the extracellular domain of SIRPA is required for SIRPA exclusion from the phagocytic

123 cup.

124

125 Exclusion of bulky phosphatases, like CD45, is driven by the steric constraints of a small

126 distance between the macrophage and target membrane (Bakalar et al., 2018; Freeman

127 et al., 2016). We hypothesized that the short membrane-membrane distance driven by

128 FcR-IgG ligation (~11.5 nm, Lu et al., 2011) may be sufficient to exclude SIRPA. To test

129 this, we generated a series of synthetic tethers varying in length (Figure 2D). In the

130 macrophage, we expressed synthetic transmembrane proteins containing an intracellular

131 GFP, and an extracellular domain with 0, 1, 3 or 5 repeats of a synthetic FNIII protein,

132 Fibcon (Bakalar et al., 2018; Jacobs et al., 2012), plus half of an inducible dimerization

133 system (Fib0FRB to Fib5FRB). The other half of the inducible dimerization system was

134 attached to bilayer-coated beads (FKBP-His$_{10}$). We then tethered beads to the

135 macrophages in the absence of IgG or CD47 by adding a rapamycin analog to induce

136 dimerization between the synthetic proteins (Spencer et al., 1993). We quantified SIRPA

137 exclusion from the phagocytic synapse. We found that the tethers containing no Fibcon

138 repeats (FRB-FKBP alone, ~6 nm) or one Fibcon repeat (Fib1FRB-FKBP, ~9.5 nm) drove

139 SIRPA exclusion of a similar magnitude to FcR-IgG ligation (Figure 2D and E). The

140 efficiency of SIRPA exclusion decreased with longer tether lengths (Fib3FRB-FKBP, 16.5

141 nm when fully extended; and Fib5FRB-FKBP, 21.5 nm). Together, these data suggest

142 that SIRPA exclusion can be controlled by altering the height of the immunological

143 synapse.

144

145 **Targeting SIRPA to the phagocytic synapse suppresses engulfment**

146

147 Receptor activation by Src family kinases at the phagocytic cup is favored due to

148 exclusion of bulky phosphatases like CD45 (Freeman et al., 2016; Goodridge et al.,

149 2011). SIRPA contains two immune tyrosine inhibitory motifs (ITIMs) that are

150 phosphorylated by Src family kinases and essential for downstream signaling (Fujioka et

151 al., 1996; Tsuda et al., 1998). We therefore hypothesized that positioning SIRPA at the

152 phagocytic cup may drive ITIM phosphorylation and receptor activation. To distinguish

153 between the effects of CD47 binding and synapse localization, we developed a chimeric

154 SIRPA receptor that localized to the phagocytic synapse in the absence of CD47. We

155 replaced the SIRPA extracellular domain with the IgG-binding extracellular domain of the

156 FcγR3 α chain (Figure 3A; termed FcR3^{EXT}-SIRPA^{INT}). This receptor is driven into the

157 synapse by IgG binding instead of CD47 (Figure 3A). Expression of this synapse-

158 localized chimera suppressed engulfment of IgG-coated beads in the absence of CD47

159 (Figure 3B, Figure S3). As a control, we expressed a chimeric construct with the four
160 tyrosines of the ITIM domains mutated to phenylalanines, prohibiting phosphorylation and
161 activation of SIRPA (FcR3^{ext}-SIRPA 4F^{int}). This construct did not affect engulfment,
162 suggesting that the inhibitory effect of the SIRPA chimera is signaling dependent (Figure
163 3B). Thus, targeting SIRPA to the phagocytic cup is sufficient to inhibit engulfment, even
164 in the absence of its natural ligand CD47.
165
166 As an alternative strategy to control the localization of SIRPA activity, we fused one half
167 of the chemically inducible dimer to FcR (FcR γ chain-FKBP) and the second to a soluble
168 SIRPA intracellular domain (FRB-SIRPA^{INT}, Figure 3C). We then recruited the SIRPA
169 intracellular domain to FcR by adding a rapamyacin analog to induce dimerization of FRB
170 and FKBP. In the absence of rapamycin, cells efficiently engulfed IgG-coated beads
171 (Figure 3C). In contrast, rapamycin-induced recruitment of the SIRPA intracellular domain
172 to the FcR γ chain significantly suppressed engulfment (Figure 3C). When the ITIM
173 domain of SIRPA was mutated, this construct no longer affected engulfment (Figure 3C).
174
175 We next returned to the extracellular domain truncation of SIRPA (FRB^{EXT}-SIRPA), which
176 was not excluded from the phagocytic synapse (Figure 2B,C), to determine if eliminating
177 SIRPA exclusion is sufficient to suppress engulfment. FRB^{EXT}-SIRPA constitutively
178 suppressed engulfment (Figure 3D), demonstrating that exclusion of SIRPA is essential
179 for efficient engulfment of targets presenting "Eat Me" signals. Taken together, these
180 experiments show that exclusion of unligated SIRPA is essential for efficient phagocytosis
181 and that CD47 activates SIRPA by positioning SIRPA at the phagocytic synapse.
182
183 **CD47 does not suppress engulfment by altering Syk recruitment to IgG**
184 **microclusters**
185
186 We next sought to determine how activated SIRPA inhibits engulfment. Phosphorylated
187 SIRPA recruits the phosphatases SHP-1 and SHP-2 via their phosphobinding SH2
188 domains but the downstream targets of SHP-1 and SHP-2 are not known (Fujioka et al.,
189 1996; Noguchi et al., 1996; Okazawa et al., 2005; Oldenborg et al., 2001; Veillette et al.,

190 1998). One potential target of SIRPA-bound SHP phosphatases is FcR itself. We used
191 TIRF microscopy to examine the initial steps in the engulfment signaling cascade with
192 high temporal and spatial resolution. When macrophages interacted with an IgG-bound
193 supported lipid bilayer, the cells formed IgG microclusters that recruited Syk (Figure 4A;
194 Lin et al., 2016). When we compared static images of macrophages that had landed on
195 a bilayer containing IgG and CD47, or IgG and the inactive CD47$^{F37D, T115K}$, we did not
196 detect a significant difference in the fraction of cells forming IgG microclusters or the total
197 area of the IgG microclusters under the cells. There was also no significant difference in
198 the fraction of cells containing Syk microclusters or the amount of Syk-mCherry recruited
199 to these clusters (Figure 4B,C). Further, we found that SIRPA did not co-localize with IgG
200 clusters when macrophages landed on a bilayer containing IgG and CD47, suggesting
201 that SIRPA is not positioned to dampen receptor activation (Figure 4D). Overall, this
202 suggests that changes to FcR activation and Syk recruitment are unlikely to account for
203 the effect of SIRPA, consistent with previous biochemical observations (Okazawa et al.,
204 2005; Tsai and Discher, 2008).
205

206 **CD47 prevents integrin activation**
207

208 We next assessed the dynamics of cells landing on functionalized supported lipid bilayers.
209 We found that cells on IgG-coated bilayers spread across the bilayer surface (Figure 5A,
210 Movie S1). In contrast, macrophages encountering an IgG and CD47-containing bilayer
211 exhibited reduced cell spreading (Figure 5A, Movie S2). TIRF imaging at a static timepoint
212 revealed that fewer macrophages were interacting with the bilayer, and those interacting
213 had a smaller footprint (Figure 5B). These data show that CD47 inhibits cell spreading
214 across a target substrate.
215

216 Cell spreading is thought to involve activation of integrins and the actin cytoskeleton
217 (Springer and Dustin, 2011). Inactive integrins exist in a low affinity, bent confirmation
218 (Springer and Dustin, 2011). Upon activation, the extracellular domain extends into an
219 open conformation that can bind many ligands with high affinity (Freeman and Grinstein,
220 2014; Springer and Dustin, 2011). FcR activation stimulates inside-out activation of

221   integrins (Dupuy and Caron, 2008; Jones et al., 1998). Activated integrins can then

222   promote engulfment, either by increasing adhesion to the target particle or by providing a

223   platform for intracellular signaling and actin assembly (Dupuy and Caron, 2008; Wong et

224   al., 2016). We found that inhibiting integrin with a β2 integrin function-blocking antibody

225   (2E6) or Fab dramatically decreased the efficiency of IgG-mediated engulfment (Figure

226   5C and S4). We could also detect a role for αM integrin in engulfment, but not for β3 or

227   αL (Figure S4). Thus, blocking αMβ2 integrin is sufficient to suppress engulfment.

228

229   Because integrin is required for cell spreading and engulfment (Springer and Dustin,

230   2011), we hypothesized that CD47-SIRPA signaling may inhibit engulfment by preventing

231   inside-out activation of integrin. Supporting this hypothesis, a previous study identified

232   phosphopaxillin, which is specifically recruited to sites of integrin activation, as one of a

233   number of phosphoproteins affected by CD47 (Geiger et al., 2009; Tsai and Discher,

234   2008). Consistent with this, we found that the enrichment of phospho-paxillin at the

235   interface of the macrophage with an IgG-coated bead was substantially diminished by the

236   simultaneous presence of CD47 on the bead (Figure 5D). Together, these data indicate

237   that CD47-SIRPA prevents integrin activation.

238

239   **Activating integrin bypasses CD47-SIRPA inhibitory signaling**

240

241   CD47-SIRPA has previously been reported to affect phosphorylation of multiple proteins,

242   including paxillin and myosin (Tsai and Discher, 2008). We also found that SIRPA

243   inhibited F-actin accumulation at the phagocytic cup (Figure 5D). It is not clear which of

244   these pathways is a direct target of CD47 signaling and which is a secondary effect of

245   altered upstream signaling. We hypothesized that if SIRPA signaling suppresses

246   engulfment primarily by inhibiting integrin inside-out activation, then directly activating

247   integrin might bypass SIRPA-mediated inhibition and permit bead engulfment (Figure 6A).

248   Alternatively, if the target of CD47-SIRPA signaling is in a parallel pathway or downstream

249   of integrin activation, then activating integrin should not rescue engulfment following

250   SIRPA activation. To activate integrin, we treated macrophages with manganese, which

251   locks integrin into a high-affinity open conformation (Dransfield et al., 1992). We found

252   that macrophages treated with 1 mM manganese engulfed beads with a similar efficiency

253   whether or not CD47 was conjugated to the supported lipid bilayer (Figure 6B).

254   Importantly, manganese did not trigger bead engulfment on its own or dramatically

255   enhance engulfment of IgG-coated beads in the absence of CD47 (Figure 6B,C),

256   establishing that increasing integrin activation is not sufficient to trigger engulfment. Thus,

257   a manganese-induced increase in engulfment was specific to beads coated with CD47

258   and IgG.

259

260   As an alternative strategy to activate integrins, we incubated macrophages with beads

261   containing a surplus of high affinity integrin ligand, ICAM-1 (Springer and Dustin, 2011).

262   ICAM-1 was sufficient to activate integrin and recruit phophopaxillin even in the presence

263   of CD47 (Figure 6D, 5D). Inclusion of high concentrations of ICAM-1 abrogated the

264   inhibitory effect of CD47 on phagocytosis, but did not dramatically alter the engulfment

265   efficiency of IgG coated beads in the absence of CD47 (Figure 6E). Despite the presence

266   of CD47, ICAM-1-bound beads had similar levels of actin accumulation as beads lacking

267   CD47 (Figure 6D, 5D). This demonstrates that activating integrins restores the ability of

268   a macrophage to engulf targets in the presence of CD47. Together, these data suggest

269   that inside-out activation of integrins may be a primary target for repression following

270   CD47-SIRPA engagement.

271

272   CD47 has also been shown to suppress complement-mediated phagocytosis (Oldenborg

273   et al., 2001). Because complement directly activates αMβ2 integrin (Freeman and

274   Grinstein, 2014), we sought to determine if preventing integrin activation could account

275   for the suppressive effect of CD47 in complement-mediated phagocytosis. To address

276   this, we examined whether manganese treatment could increase macrophage

277   engulfment of complement-opsonized mouse red blood cells (RBCs), which present

278   CD47 on their surface (Figure 6F; Oldenborg et al., 2000; Yi et al., 2015). We found that

279   activating integrin with 1 mM manganese dramatically increased engulfment of

280   complement-opsonized RBCs but not control IgM-treated RBCs. This demonstrates that

281   activating integrin enhances complement mediated engulfment, and is consistent with

282 integrin activation bypassing the suppressive CD47 signal on complement-opsonized red

283 blood cells.

284

Adhesion alone is not sufficient not bypass CD47

286

287 We next sought to clarify whether integrin bypassed the CD47 signal by acting simply as

288 a physical tether, or if intracellular integrin signaling was required. To distinguish between

289 a tethering role and a signaling role for integrins, we created a DNA-based synthetic

290 receptor that tethered the bilayer-coated beads to the macrophage but contained no

291 intracellular signaling domains (Figure 6G). Unlike integrin activation, this inert tether was

292 unable to bypass the suppressive CD47 signal (Figure 6H). This suggests that adhesion

293 alone cannot overcome the effect of CD47 and that the intracellular signaling capabilities

294 of integrin are essential.

295

Integrin activation drives cancer cell engulfment

297

298 Many cancer cells overexpress CD47 to evade the innate immune system despite

299 increased expression of "Eat Me" signals such as calreticulin or phosphatidylserine (Birge

300 et al., 2016; Chao et al., 2010b; Gardai et al., 2005; Utsugi et al., 1991). Blocking CD47

301 with a therapeutic antibody allows "Eat Me" signals to dominate, resulting in engulfment

302 of whole cancer cells (Jaiswal et al., 2009; Majeti et al., 2009). We hypothesized that

303 exogenous activation of integrin would bypass the CD47 signal on the surface of cancer

304 cells, allowing for engulfment. To test this, we incubated bone marrow derived mouse

305 macrophages expressing a membrane tethered GFP (GFP-CAAX) with a CD47-positive

306 murine leukemia line, L1210, expressing nuclear H2B-mCherry (Chen et al., 2017). We

307 then imaged macrophage-cancer cell interactions for 8 hours and found that activating

308 integrins with 100 µM manganese increased the ability of macrophages to engulf cancer

309 cells, reaching a similar efficiency as treatment with a CD47 function-blocking antibody

310 (Figure 6I; Movie S3). Manganese did not directly affect cancer cell viability over the time

311 course of this experiment (Figure S5). To confirm this result, we dyed L1210 cancer cells

312 with CFSE. We then incubated these dyed cancer cells with primary bone marrow derived

313   macrophages for 2 hours at a 2:1 cancer cell:macrophage ratio. We found that

314   manganese increased whole cell engulfment in this assay as well (Figure S5). These data

315   suggest that activating integrins bypasses the suppressive CD47 signal on the surface of

316   cancer cells.

Discussion

CD47-SIRPA signaling suppresses engulfment, protecting viable cells and allowing cancer cells to evade the innate immune system (Jaiswal et al., 2009; Majeti et al., 2009; Oldenborg et al., 2000). Although CD47 blockade is a promising new target for cancer therapies (Advani et al., 2018; Gholamin et al., 2017; Willingham et al., 2012), there are many unresolved questions concerning CD47-SIRPA mechanism and potency. Using a reconstituted engulfment target, we quantitatively probed CD47-SIRPA signaling. By titrating CD47 and IgG, we found that CD47 dampened IgG-mediated phagocytosis but this suppressive effect could be overcome by a surplus of IgG. Mechanistically, we demonstrated that localizing SIRPA to the phagocytic synapse was sufficient to activate this inhibitory receptor. Once active, SIRPA suppressed engulfment by preventing integrin activation.

Our results demonstrate that SIRPA localization is a key determinant of its activity. In the absence of CD47, SIRPA is relegated to the phosphatase-rich zone outside the cell bead interface (Freeman et al., 2016; Goodridge et al., 2011). This localization prevents SIRPA activation. Conversely, CD47 binding retained SIRPA at the Src-kinase rich phagocytic cup, where it is activated and suppresses engulfment. Spatial segregation of Src-family kinase activity at the central phagocytic synapse and CD45 phosphatase activity at the periphery underlies the activation of many activating receptors (TCR, Fc Receptor, (Freeman et al., 2016; James and Vale, 2012). Our work expands this model, suggesting that exclusion of inhibitory receptors like SIRPA may be a pre-requisite for efficient engulfment. Further, these data suggest a new paradigm for regulating inhibitory receptors based on conditional recruitment to the immunological synapse.

SIRPA exclusion from the phagocytic synapse in the absence of CD47 prevents basal inhibition of engulfment and allows positive signaling to dominate. SIRPA may be sterically excluded from the phagocytic synapse based on the size of its bulky extracellular domain, as replacing the extracellular domain with a small, inert protein (FRB) allowed SIRPA to enter the phagocytic synapse. Could the bulky SIRPA

14

348   extracellular domain be sterically excluded from the phagocytic synapse based on height

349   alone? The FcR-IgG complex is ~11.5 nm tall (Lu et al., 2011), and our data demonstrate

350   that both unligated SIRPA and CD47-bound SIRPA are excluded from these receptor-

351   ligand clusters. Between IgG clusters, integrin forms a diffusion barrier in the phagocytic

352   synapse that prevents bulky proteins from entering (Freeman et al., 2016). While

353   extended integrin is quite tall, engaged integrin is tilted and has been shown to drive

354   exclusion of the bulky transmembrane phosphatase CD45 (Freeman et al., 2016;

355   Swaminathan et al., 2017). Although aglycosylated CD45 is larger than SIRPA (17 nm

356   and 12 nm respectively), the size of both extracellular domains is increased by extensive

357   glycosylation (Chang et al., 2016; Hatherley et al., 2008). Thus, steric exclusion may be

358   sufficient to explain the depletion of SIRPA at the immunological synapse. To support

359   this, we demonstrated that shortening the distance between the macrophage and its

360   target increased SIRPA exclusion. How does CD47 binding alter SIRPA localization?

361   Biophysical studies show that unligated proteins that are the same size or even slightly

362   smaller than the height of a cell-cell synapse are excluded from the synapse (Schmid et

363   al., 2016). Ligand binding is sufficient to drive synapse localization (Schmid et al., 2016).

364   Thus, SIRPA may be sterically excluded unless CD47 ligation overcomes the energetic

365   barrier preventing SIRPA from entering the immunological synapse. While our data

366   demonstrates that SIRPA exclusion can be driven by altering the height of the

367   immunological synapse, our studies do not rule out possible contribution of other

368   exclusion mechanisms, such as lateral crowding or interactions with the surrounding

369   glycocalyx.

370

371   After addressing the mechanism of SIRPA activation, we sought to identify the targets of

372   CD47-SIRPA signaling. Previous work shows that SIRPA activation dramatically reduces

373   global phosphotyrosine, including phosphorylation of mDia, paxillin, talin, alpha-actinin

374   and non-muscle myosin IIA (Okazawa et al., 2005; Tsai and Discher, 2008). However,

375   discerning between direct targets of SIRPA-bound phosphatases and indirect targets

376   resulting from an upstream block in the engulfment signaling cascade has been

377   challenging. Because blocking non-muscle myosin II decreases phagocytosis to a similar

378   extent as CD47, myosin has been presumed to be the primary target of SIRPA,

379 suggesting a model where CD47 inhibits "pulling" of the phagocytic target into the
380 macrophage (Chao et al., 2012; Tsai and Discher, 2008). However, we demonstrate that
381 the inhibitory effect of CD47-SIRPA can be eliminated by re-activating integrin,
382 suggesting that the direct targets of SIRPA-bound SHP phosphatases are upstream of
383 integrin activation. Instead of a pulling model, we propose that CD47 inhibits spreading
384 of the macrophage around the phagocytic target. In our system, we found that blocking
385 αMβ2 integrin had the largest effect on engulfment. However, instead of targeting a
386 specific integrin subset directly, we hypothesize that SIRPA bound phosphatases
387 deactivate an upstream step in the inside-out activation signaling pathway or an integrin
388 regulator. SHP-2 has previously been shown to directly dephosphorylate Fak (Yu et al.,
389 1998) and vinculin (Campbell et al., 2018), thus SHP-2 may act upon these key integrin
390 regulators. However, given the broad specificity of SHP-1 and SHP-2, these
391 phosphatases may dephosphorylate several targets at the phagocytic cup to suppress
392 signaling.
393
394 We show that CD47-SIRPA prevents integrin activation, allowing macrophages to quickly
395 discriminate between targets based on the presence of CD47. In addition to immediately
396 inactivating integrin to prevent engulfment of a CD47-positive cell, SIRPA may also
397 contribute to a long-term transcriptional down regulation of integrins (Liu et al., 2008).
398 While this decrease in integrin expression does not explain how SIRPA prevents
399 phagocytosis specifically of CD47-bound targets, it suggests that long-term exposure to
400 activated SIRPA may decrease overall phagocytic capacity. Paradoxically, SIRPA may
401 also be required for integrin-dependent cell migration, as fibroblasts lacking SIRPA have
402 impaired motility (Alenghat et al., 2012; Inagaki et al., 2000; Motegi et al., 2003). In this
403 context, SIRPA may promote integrin turnover to provide the dynamic interactions
404 necessary for motility.
405
406 By suppressing integrin activation, CD47-SIRPA signaling may be able to suppress many
407 different signaling pathways. CD47 has been reported to affect dendritic cell activation,
408 cancer cell killing via a nibbling behavior (called trogocytosis), and complement-mediated
409 engulfment (Caron et al., 2000; Matlung et al., 2018; Oldenborg et al., 2001; Tamada et

410 al., 2004; Wu et al., 2018; Yi et al., 2015). These processes are triggered by diverse
411 positive signaling receptors, but all require inside-out activation of integrin (Caron et al.,
412 2000; Matlung et al., 2018; Oldenborg et al., 2001; Tamada et al., 2004; Wu et al., 2018;
413 Yi et al., 2015). Targeting integrin, a common co-receptor, may explain how CD47-SIRPA
414 signaling can regulate these diverse processes.

415

416 Finally, we found that integrin activation by manganese can drive engulfment of whole
417 cancer cells by bone marrow-derived macrophages. As a cancer treatment, CD47
418 blockade synergizes with therapeutic antibodies, like rituximab (Advani et al., 2018; Chao
419 et al., 2010a). Activating integrins with a small molecule agonist in combination with
420 antibody therapeutics may have a similar synergistic effect as CD47 blockade. Small
421 molecule agonists of the αM integrin subunit drive tumor regression in a macrophage-
422 dependent manner (Panni et al., 2019; Schmid et al., 2018). Our data suggest that these
423 small molecules may promote tumor regression partially by allowing macrophages to
424 bypass the CD47 inhibitory signal.

425

426 **Limitations of Study**
427 To allow for high resolution imaging, this study was conducted *ex vivo* using mouse
428 macrophage cell lines or bone marrow derived macrophages. Validating these findings in
429 a model that more closely recapitulates the human tumor microenvironment would be an
430 additional step forward. While we demonstrate that CD47-SIRPA prevents integrin
431 activation, we have not shown whether this is through direct dephosphorylation of an
432 integrin regulator by SIRPA-bound phosphatases, or through a less direct mechanism.

433

Author contributions

Conceptualization, MAM, NK, RDV; Methodology, MAM, NK; Validation, MAM, NK; Formal Analysis, MAM, NK; Investigation, MAM, NK; Writing – Original Draft Preparation, MAM; Writing – Review & Editing Preparation, MAM, NK, RDV; Visualization Preparation, MAM, NK; Supervision, MAM, RDV; Funding Acquisition, MAM, RDV.

Acknowledgments

Declaration of Interests

Ron Vale is a founder of Myeloid Therapeutics. Meghan Morrissey and Ron Vale have submitted a patent application related to this work.

Figure Legends

Figure 1: CD47-SIRPA suppresses IgG and PS dependent engulfment

(A) Schematic shows the supported lipid bilayer system used in this study. Anti-biotin IgG is bound to biotinylated lipids. IgG is recognized by Fc Receptor on the macrophage. The extracellular domain of CD47-His$_{10}$ is bound to Ni-NTA-conjugated lipids and recognized by SIRPA expressed by the macrophage. (B) Silica beads are coated with a supported lipid bilayer and incubated with the indicated concentration of IgG and either CD47 (red) or an inactive mutant CD47 (F37D, T115K; green). The functionalized beads were added to RAW264.7 macrophages and fixed after 30 min. The average number of beads per macrophage was assessed by confocal microscopy and normalized to the maximum average bead eating observed in that replicate. Each dot represents an independent replicate (n$\geq$100 cells analyzed per experiment), and data are compared using a two-way ANOVA. For visualization, the data were fit using the "[Agonist] vs response – variable slope" model in Graphpad Prism. (C) Still images depict the assay described in (B). The supported lipid bilayers contain the fluorescently-labeled lipid atto390-DOPE (green) and the macrophages membranes are labeled with CellMask (magenta). Internalized beads are indicated with a yellow dot. (D) Graph depicts the fraction of cells engulfing the indicated number of beads (pooled data from three independent replicates included in (B)). Macrophages encountering CD47-conjugated beads (red) were less likely to engulf, and those that did engulfed fewer beads. CD47^{F37D,T115K}, a mutant that cannot bind SIRPA, was used as a control (green). (E) Macrophages were incubated with beads coated with a supported lipid bilayer containing 10% phosphatidylserine and either CD47 or the inactive CD47^{F37D,T115K}. Data were normalized to the maximum bead eating observed in that replicate. The complete, pooled data are shown in Supplementary Figure 1E. Dots and error bars denote the mean and standard error of independent replicates. *** indicates p<0.0005 and ** indicates p<0.005 by a Kruskal-Wallis test on the pooled data (D) or a Student's T test on the means of individual replicates (E). Scale bar denotes 5 μm in this and all subsequent figures.

Figure 2: Unligated SIRPA is excluded from the phagocytic synapse

(A) SIRPA-GFP (top; green in merge) is depleted from the base of the phagocytic cup (arrow) when a macrophage engulfs a bead functionalized with IgG and CD47$^{F37D, T115K}$, which cannot bind SIRPA (left; supported lipid bilayer, magenta). SIRPA is not depleted when CD47 is present (IgG+CD47, right). Graph depicts the ratio of SIRPA-GFP at the phagocytic cup/cell cortex for individual phagocytic cups. (B) A schematic shows full length SIRPA on the left and FRB^{ext}-SIRPA on the right. (C) SIRPA-GFP and FRB^{ext}-SIRPA-GFP are shown at cell-bead contacts (arrow). On the right, a graph depicts the ratio of GFP fluorescence at the synapse (arrow) compared to the cortex for the indicated SIRPA chimeras. (D) A schematic shows the synthetic tethers used to control macrophage-bead spacing. 'Max tether length' indicates the predicted height of these semi-rigid proteins if fully extended (Bakalar et al., 2018). Purified FKBP is attached to supported lipid-bilayer-coated beads, and the Fibcon repeat proteins with a N terminal FRB are expressed in the macrophage. In the presence of a rapamyacin analog, FKBP and FRB form a high affinity dimer tethering the bead to the macrophage. SIRPA-mCherry fluorescence was measured at the cell-bead synapse (arrow in E) and the cell cortex. The ratio of SIRPA fluorescence at the synapse compared to the cortex is graphed on the right. Representative images are shown in (E). Short tethers (Fib0FRB and Fib1FRB) drive a depletion of SIRPA at the cell-bead synapse, and long tethers (Fib3FRB and Fib5FRB) drive little or no depletion of SIRPA. In A, C, and D, dots represent individual cups, red lines show mean $\pm$ 95% confidence intervals, and data is pooled from three independent experiments. *** denotes $p<0.0005$, ** denotes $p<0.005$, * denotes $p<0.05$ and n.s. denotes $p>0.05$ as determined by a Student's T test (A, C); or an Ordinary one-way ANOVA with Holm-Sidak multiple comparison test (D).

Figure 3: Localizing SIRPA to the macrophage-target synapse suppresses engulfment

(A) A schematic of the chimeric synapse localized FcR3^{EXT}SIRPA^{INT} construct is shown on the left and representative images of its localization to the cell bead interface are shown in the center. On the right, a graph depicts the ratio of GFP fluorescence at the macrophage-bead synapse compared to the cortex for full length SIRPA,

516   FcR3^{EXT}SIRPA^{INT}, and the chimeric SIRPA construct with the four tyrosines in the ITIM

517   domains mutated to phenylalanine (FcR3^{EXT}SIRPA 4F^{INT}). (B) A graph depicts the

518   average number of internalized IgG beads per macrophage expressing the indicated

519   chimeric SIRPA constructs normalized to the maximum bead eating in that replicate.

520   Mock infected macrophage or macrophages expressing membrane tethered GFP (GFP-

521   CAAX) were used as a control.. (C) Schematic (left) shows a system for inducible

522   recruitment of the SIRPA intracellular domain to the phagocytic cup. Recruiting SIRPA to

523   the phagocytic cup suppresses engulfment compared to soluble SIRPA or compared to

524   wild-type macrophages treated with a rapamycin analog (normalized to maximum bead

525   eating in that replicate). Mutating the ITIM domains of SIRPA (SIRPA 4F) eliminated a

526   rapamycin-induced effect. (D) The graph shows the number of beads engulfed by

527   uninfected, SIRPA-GFP, or FRB^{EXT}-SIRPA expressing macrophages normalized to the

528   maximum observed eating in that replicate. In A, dots represent individual cups, red lines

529   show mean $\pm$ 95% confidence intervals, and data is pooled from three independent

530   experiments. In B, C, and D dots show the average from independent replicates with the

531   error bars denoting SEM for that replicate. *** denotes $p<0.0005$, ** denotes $p<0.005$, *

532   denotes $p<0.05$ and n.s. denotes $p>0.05$ as determined by an Ordinary one-way ANOVA

533   with Holm-Sidak multiple comparison test.

534

535   **Figure 4: CD47 does not affect IgG clustering and Syk recruitment.**

536   (A) TIRF microscopy shows that macrophages are able to form IgG microclusters (left;

537   cyan in merged image) that recruit Syk (middle; magenta in merged image) when landing

538   on bilayers containing IgG + CD47^{F37D,T115K} (top) or IgG + CD47 (bottom). Inset shows

539   the boxed region of the image above. The linescan shows the fluorescent intensity of

540   AlexaFluor 647-IgG and Syk-mCherry at the indicated position (white arrow). Intensity

541   was normalized so that 1 is the highest observed intensity and 0 is background. (B) The

542   fraction of cells forming IgG (left) or Syk (right) was measured in static images of

543   macrophages that had landed on the indicated supported lipid bilayer after 15 min. (C)

544   Size of the IgG clusters (left), and the mean fluorescent intensity of Syk-mCherry

545   colocalizing with these clusters (right) was measured at the same timepoint. (D) TIRF

546   microscopy shows that, in the presence of CD47, SIRPA (green) does not co-localize with

547   IgG clusters (cyan; arrowheads). Inset shows the boxed region in the above image. The

548   linescan shows the fluorescent intensity of AlexaFluor 647-IgG and SIRPA-GFP at the

549   position indicated by a white arrow. The Pearson's Correlation Coefficient (PCC) for Syk

550   and IgG, or SIRPA and IgG in individual cells is indicated in the graph on the right. In B,

551   each dot represents the percent from an independent experiment (n $\geq$ 20 cells per

552   replicate) and the lines denote mean $\pm$ SD. In C and D, each dot represents an individual

553   cell and data was pooled from three independent experiments. The red lines indicate the

554   mean $\pm$ 95% confidence intervals. *** denotes p<0.0005, and n.s. denotes p>0.05 as

555   determined by a Student's T test.

556

557   Figure 5: CD47 prevents integrin activation

558   (A) Still images from a TIRF microscopy timelapse show that macrophages form IgG

559   (black) microclusters as they spread across bilayers containing IgG and an inactive

560   CD47$^{F37D, T115K}$ which cannot bind to SIRPA bilayer (top). Adding CD47 to the bilayer

561   inhibits cell spreading (bottom; graphed on right, average area of contact from n$\geq$11 cells

562    $\pm$ SEM, pooled from three separate experiments). (B) TIRF images show the cell

563   membrane (mCherry-CAAX; white) of macrophages engaging with an IgG and inactive

564   CD47$^{F37D, T115K}$ (left), or IgG and CD47 (right) bilayer. Graphs depict the average number

565   of cells seen contacting the bilayer after 10 min (center) and the average area of cell

566   contact (right). Each dot represents an individual field of view (center) or cell (right) pooled

567   from three independent experiments. (C) Blocking integrin activation using a function-

568   blocking antibody (2E6) targeting the β2 integrin subunit decreased the efficiency of

569   engulfment. Graph shows the number of beads engulfed normalized to the maximum

570   observed eating in that replicate. Each data point represents an independent experiment

571   and the error bars denote the SEM for that replicate. (D) Immunofluorescence images

572   show phosphopaxillin (top; green in merge) and F-actin (center; magenta in merge;

573   visualized with phalloidin) at the phagocytic cup of a bead containing IgG and inactive

574   CD47$^{F37D, T115K}$ (left) or an IgG- and CD47-coated bead (right). Graphs show the ratio of

575   phosphopaxilin (center) or actin (right) intensity at the phagocytic cup/cell cortex. Each

576   dot represents an individual phagocytic cup; lines denote the mean $\pm$ 95% confidence

577   intervals. *** denotes p<0.0005, ** denotes p<0.005, and * denotes p<0.05 as determined

578   by Student's T test (A, B, and C) or Ordinary one-way ANOVA with Tukey's Multiple

579   Comparison test (D).

580

581   **Figure 6: Bypassing inside out activation of integrin eliminates the effect of CD47.**

582

583   (A) The schematic shows a simplified signaling diagram. If CD47 and SIRPA act upstream

584   of integrin, then providing an alternate means of integrin activation (Mn^{2+} or ICAM) should

585   eliminate the effect of CD47. (B) Macrophages were treated with 1 mM Mn^{2+} and fed

586   beads with IgG and either CD47 (red) or the non-signaling CD47$^{F37D, T115K}$ (green). Bars

587   denote the average number of beads eaten from the pooled data of three independent

588   replicates $\pm$ SEM. (C) Beads were incubated with the indicated concentration of IgG and

589   added to macrophages. Treatment with Mn^{2+} did not dramatically enhance engulfment

590   (black, compared to grey). Dots represent the average number of beads eaten $\pm$ SEM in

591   one data set representative of three experiments. (D) Immunofluorescence shows that

592   adding ICAM (10 nM coupling concentration) to IgG + CD47 beads rescues

593   phosphopaxillin (top; green in merge, bottom) and actin (middle; magenta in merge) at

594   the phagocytic cup. The quantification of this data is graphed in Figure 3D alongside the

595   appropriate controls. (E) Beads were functionalized with IgG and either CD47 (red) or the

596   non-signaling CD47$^{F37D, T115K}$ (green). Adding ICAM to the beads abrogated the effect of

597   CD47 (center) but did not stimulate engulfment without IgG (right). (F) Complement-

598   opsonized CD47+ mouse red blood cells (RBCs) were fed to control (grey) or 1 mM Mn^{2+}

599   treated (black) macrophages. Unopsonized IgM treated RBCs were used as a negative

600   control. Red blood cell internalization is graphed on the left. An anti-iC3b antibody was

601   added after fixation, but before cell permeabilization to distinguish between engulfed

602   (green) and non-engulfed (green and magenta) RBCs. On the right, representative

603   images show increased adhesion and engulfment of RBCs (all RBCs, green; non-

604   engulfed RBCs, magenta) in macrophages (nuclei stained with DAPI, cyan) treated with

605   Mn^{2+}. The inset (red box) highlights one example macrophage and is shown at higher

606   magnification on the right. The macrophage cortex was determined through CellMask

607   staining and is outlined in red. (G) Schematic shows the DNA-based adhesion system.

608   Macrophages express a synthetic adhesion receptor containing an intracellular GFP, and

609 an extracellular SNAP tag, which is conjugated to benzylguanine DNA. Graph depicts
610 the mean number of bead contacts per cell, using beads functionalized only with
611 neutravidin or with neutravidin and biotinylated ligand DNA (no IgG). Arrows point to cell
612 membrane clinging to the adherent beads. (H) Beads were ligated to IgG, either CD47
613 (red) or the non-signaling CD47$^{F37D, T115K}$ (green), and biotinylated DNA to control
614 adhesion. All cells express the adhesion receptor, which is conjugated to benzylguanine-
615 DNA. Graph depicts the average number of beads engulfed per cell. (I) Bone marrow-
616 derived macrophages expressing a membrane tethered GFP (green) were incubated with
617 L1210 murine leukemia cells expressing H2B-mCherry (magenta). Treating with 100 μM
618 manganese allowed for engulfment of whole cancer cells. These images correspond to
619 frames from Movie S3. The percent of macrophages engulfing a cancer cell during an 8
620 hr timelapse is graphed on the right. Each dot represents an independent replicate, with
621 red lines denoting mean ± SEM. (J) Model figure shows that in the absence of CD47 (left),
622 SIRPA is segregated away from the phagocytic synapse and Fc Receptor binding triggers
623 inside out activation of integrin. When CD47 is present (right), SIRPA localizes to the
624 synapse and inhibits integrin activation. In F, G and I, each dot represents an independent
625 replicate, with red lines denoting mean ± SEM. *** denotes $p<0.0005$, ** denotes $p<0.005$
626 and n.s. denotes $p>0.05$ as determined by a Kruskal-Wallis test (B, E, H), Student's T test
627 (F, G) or an Ordinary one way ANOVA with Holm-Sidak multiple comparison test (I).
628

STAR METHODS

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ron Vale (valer@janelia.hhmi.org).

Materials Availability

Plasmids generated in this study have been deposited to Addgene or can be obtained from the Lead Contact.

Data and Code Availability

All code used to acquire or analyze data in this paper is publicly available through µManager (micro-manager.org) or Fiji (fiji.sc). Complete imaging data sets are available from the Lead Contact.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture

RAW264.7 macrophages were provided by the ATCC and certified mycoplasma-free. The cells were cultured in DMEM (Gibco, Catalog #11965–092) supplemented with 1 x Pen-Strep-Glutamine (Corning, Catalog #30–009 CI), 1 mM sodium pyruvate (Gibco, Catalog #11360-070) and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Catalog #S11150H). To keep variation to a minimum, cells were discarded after 20 passages. L1210 cells were also acquired from the ATCC.

J774A.1 macrophages were provided by the UCSF cell culture facility. J774A.1 and 293T cells were tested for mycoplasma using the Lonza MycoAlert Detection Kit (Lonza, Catalog# LT07-318) and control set (Lonza, Catalog #LT07-518).

Bone marrow-derived macrophages were generated from the hips and long bones of C57BL/6J mice as previously described (Weischenfeldt and Porse, 2008) except that purified 25 ng/ml M-CSF (Peprotech, Catalog # 315–02) was used.

METHOD DETAILS

Lentivirus production and infection

All constructs were expressed in RAW264.7 using lentiviral infection. Lentivirus was produced in HEK293T cells transfected with pMD2.G (a gift from Didier Tronon, Addgene plasmid # 12259 containing the VSV-G envelope protein), pCMV-dR8.91 (since replaced by second generation compatible pCMV-dR8.2, Addgene plasmid #8455), and a lentiviral backbone vector containing the construct of interest (derived from pHRSIN-CSGW, see STAR methods) using lipofectamine LTX (Invitrogen, Catalog # 15338–100). Constructs are described in detail in the Key Resources Table. The media was harvested 72 hr post-transfection, filtered through a 0.45 µm filter and concentrated using LentiX (Takara Biosciences). After addition of the concentrated virus, cells were centrifuged at 2000xg for 45 min at 37°C. Cells were analyzed a minimum of 60 hr later, and maintained for a maximum of one week.

Supported lipid bilayer assembly

SUV preparation

The following chloroform-suspended lipids were mixed and desiccated overnight to remove chloroform: 96.8% POPC (Avanti, Catalog # 850457), 2% Ni^{2+}-DGS-NTA (Avanti, Catalog # 790404), 1% biotinyl cap PE (Avanti, Catalog # 870273), 0.1% PEG5000-PE (Avanti, Catalog # 880230, and 0.1% atto390-DOPE (ATTO-TEC GmbH, Catalog # AD 390–161). The lipid sheets were resuspended in PBS, pH7.2 (Gibco, Catalog # 20012050) and stored under argon. The lipids were broken into small unilamellar vesicles via several rounds of freeze-thaws. The mixture was cleared using ultracentrifugation (TLA120.1 rotor, 35,000 rpm / 53,227 x g, 35 min, 4°C). The lipids were then stored at 4°C under argon for up to two weeks.

Planar bilayer preparation for TIRF microscopy

Ibidi coverslips (catolog #10812) were RCA cleaned. Supported lipid bilayers were assembled in custom plasma cleaned PDMS (Dow Corning, catalog # 3097366-0516 and 3097358-1004) chambers at room temperature for 1 hour. Bilayers were blocked with 0.2% casein (Sigma, catalog # C5890) in PBS. Proteins were coupled to the bilayer for

691   45 min. Imaging was conducted in HEPES buffered saline (20 mM HEPEs, 135 mM NaCl,

692   4 mM KCl, 10 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$). Bilayers were assessed for

693   mobility by either photobleaching or monitoring the mobility of single particles.

694

695   *Bead preparation*

696   8.6×10^8 silica beads with a 5.02 µm diameter (10 µl of 10% solids, Bangs Labs, Catalog

697   # SS05N) were washed three times with PBS, mixed with 1mM SUVs in PBS and

698   incubated at room temperature for 0.5-2 hr with end-over-end mixing to allow for bilayer

699   formation. Beads were then washed three times with PBS to remove excess SUVs and

700   incubated in 100 µl of 0.2% casein (Sigma, catalog # C5890) in PBS for 15 min before

701   protein coupling. Unless otherwise indicated, anti-biotin AlexaFluor647-IgG (Jackson

702   ImmunoResearch Laboratories Catalog # 200-602-211, Lot # 137445) was added

703   between 3 and 30 nM, always using the lowest IgG concentration that triggered

704   engulfment. Purified $CD47^{ext}$-His_{10} was added at 1 nm. Proteins were coupled to the

705   bilayer for 1 hr at room temperature with end-over-end mixing.

706

707   *Protein density estimation*

708   Given the high affinity of His_{10} for Ni^{2+}-DGS-NTA (0.6 nM (Hui and Vale, 2014)), and

709   antibody-antigen interactions, we expect close to 100% coupling efficiency (Hui and Vale,

710   2014). Complete coupling would result in 600 molecules/$µm^2$ CD47 and 300

711   molecules/$µm^2$ IgG for the 3 nM coupling condition. This is well within the range of CD47

712   on the surface of a cancer cell (Figure S1). In addition, to estimate the amount of IgG

713   bound to each bead, we compared the fluorescence of IgG on the bead surface to

714   calibrated fluorescent beads (Quantum AlexaFluor 647, Bangs Lab) using confocal

715   microscopy. Using this method, we measured 200-360 molecules/$µm^2$ of IgG, which is

716   consistent with the theoretical prediction of near-complete coupling.

717

718   **Protein Purification**

719      $CD47^{ext}$-His_{10}, $CD47^{ext\ F37D,\ T115K}$-His_{10} (aa40-182; Uniprot Q61735) and ICAM-tagBFP-

720   His_{10} (O'Donoghue et al., 2013) were expressed in SF9 or HiFive cells using the Bac-to-

721   Bac baculovirus system as described previously (Hui and Vale, 2014). Briefly, the N-

722   terminal extracellular domain of CD47 was cloned into a modified pFastBac HT A with an

723   upstream signal peptide from chicken RPTPσ (Chang et al., 2016). Insect cell media

724   containing secreted proteins was harvested 72 hr after infection with baculovirus. His$_{10}$

725   proteins were purified by using Ni-NTA agarose (Qiagen, Catalog # 30230), followed by

726   size exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare,

727   Catalog # 17517501). The purification buffer was 30 mM HEPES pH 7.4, 150 mM NaCl,

728   2 mM MgCl$_2$, 5% glycerol (CD47) or 150 mM NaCl, 50 mM HEPES pH 7.4, 5% glycerol,

729   2 mM TCEP (ICAM).

730

731   ***Microscopy and analysis***

732   Images were acquired on a spinning disc confocal microscope (Nikon Ti-Eclipse

733   inverted microscope with a Yokogawa spinning disk unit and an Andor iXon EM-CCD

734   camera) equipped with a 40 × 0.95 NA air and a 100 × 1.49 NA oil immersion objective.

735   The microscope was controlled using µManager (Edelstein et al., 2010). For TIRF

736   imaging, images were acquired on the same microscope with a motorized TIRF arm, but

737   using a Hamamatsu Flash 4.0 camera and the 100x 1.49 NA oil immersion objective.

738   Data was analyzed in ImageJ (Rueden et al., 2017; Schindelin et al., 2012).

739

740   **Quantification of engulfment**

741   30,000 macrophages were plated in one well of a 96-well glass bottom MatriPlate

742   (Brooks, Catalog # MGB096-1-2-LG-L) between 12 and 24 hr prior to the experiment.

743   Unless otherwise noted, macrophages remained in culture media (DMEM with 10% heat

744   inactivated serum) throughout the experiment. ~8 x 10^5 beads were added to well and

745   engulfment was allowed to proceed for 30 min. Cells were fixed with 4% PFA and stained

746   with CellMask (ThermoFisher, catalog # C10045) without membrane permeabilization to

747   label cell boundaries. Images were acquired using the High Content Screening (HCS)

748   Site Generator plugin in µManager (Edelstein et al., 2010). For Figure 1B-D, 2G-H, 4F-I,

749   S3D, and S4, the analyzer was blinded during engulfment scoring using the position

750   randomizer plug-in in µManager.

751

752   **TIRF imaging**

753 Macrophages were removed from their culture dish using 5% EDTA in PBS, two times

754 washed and resuspended in the HEPES imaging buffer (20 mM HEPES, 135 mM NaCl,

755 4 mM KCl, 10 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) before being added to the TIRF

756 chamber.

757

758 *Quantification of IgG clusters and Syk recruitment*

759 After 15 minutes of interacting with the bilayer, cells that had spread on the bilayer

760 surface were selected for analysis. Otsu thresholding in ImageJ was used to select IgG

761 clusters in an unbiased manner. This selection was used to generate an ROI that was

762 then applied to the Syk-mCherry channel. The area of the ROI (area of IgG clusters) and

763 the mean Syk intensity within that ROI were measured.

764

765 *Pearson's Correlation Coefficient*

766 The region of cell-bilayer contact was manually selected in ImageJ and the Pearson's

767 correlation coefficient between AlexaFluor647-IgG and either SIRPA-GFP or Syk-

768 mCherry for this ROI was measured using the Coloc2 plugin (Schindelin et al., 2012).

769

770 *Quantification of cell-bilayer contact area*

771 For 3A the area of the cell contacting the bilayer was traced in ImageJ beginning with

772 the first frame where the cell can be detected. Only cells with mobile IgG clusters were

773 included. For 3B, the number of macrophage-bilayer contacts and the area was quantified

774 in still images of live cells between 10 and 15 min after cells were added to the bilayer.

775 The macrophage membrane was labeled with mCherry-CAAX. All cells were included.

776

777 **Phosphopaxillin staining**

778 Macrophages were fixed in 4% PFA for 15 min, then permeabilized and blocked with

779 0.1% BSA in PBS with 0.5% Tween 20. The cells were incubated with the phosphopaxillin

780 antibody at 1:50 dilution at 4° C overnight before incubating with Alexa Fluor 555 anti-

781 rabbit secondary (21428), Alexa Fluor 488 phalloidin (A12379).

782

783 **Quantification of synapse intensity of phosphoPaxillin, actin and SIRPA constructs**

784   Phagocytic cups were selected for analysis based on the presence of clustered IgG at
785   the cup base (SIRPA chimeras) or clear initiation of membrane extensions around the
786   phagocytic target (actin, phosphopaxillin). The phagocytic cup and the cell cortex were
787   traced with a line 3 pixels wide at the Z-slice with the clearest cross section of the cup.
788   The average background intensity was measured in an adjacent region and subtracted
789   from each measurement.
790

791   **Integrin block and Fab generation**
792   To disrupt integrin function, the blocking antibodies or isotype control indicated in the
793   "Key Reagents" table were added to macrophages at 10 µg/ml 30 minutes before IgG-
794   opsonized beads. To eliminate any effects of the Fc domain, we generated Fabs of the
795   β2 blocking antibody and isotype control using the Pierce Fab separation kit
796   (ThermoFisher 44985). In figures 3 and S4C, the antibodies and beads were added to
797   macrophages in complete media containing heat inactivated serum. In figure S4B,
798   macrophages were washed into serum-free HEPES imaging buffer (20 mM HEPES,
799   135 mM NaCl, 4 mM KCl, 10 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) prior to antibody
800   treatment to eliminate any potential serum components that may serve as integrin ligands.
801

802   **DNA tether experiments**
803   For the DNA adhesion experiments, bilayers were assembled on silica beads, blocked
804   with 0.2% casein, and coupled to IgG at a 3 nM concentration as described above. After
805   15 min of IgG coupling, 1 µg/ml neutravidin was added for 20 minutes. After washing out
806   the neutravidin, 100 nM DNA ligand strand (5' biotin, see Key Reagents table) and 1 nM
807    $CD47^{ext}$-His_{10} were added and coupled for 45 min. During this time, macrophages plated
808   in a 96 well imaging plate expressing a DNA chimeric adhesion receptor (extracellular
809   SNAP - CD86 transmembrane domain - intracellular EGFP) were incubated with 1 uM
810   receptor DNA strand (5' benzylyguanine, see Key Reagents table, Farlow et al., 2013) in
811   100 ul buffer for 10 minutes. Cells and beads were both washed 4 times. ~8 x 10^5 beads
812   were added to well and engulfment was allowed to proceed for 30 min in HEPES imaging
813   buffer. The DNA ligand and receptor strand sequences along with their modifications can
814   be found in the key resources table.

815

Whole cell internalization assay

For timelapse imaging, 30,000 primary bone marrow derived macrophages infected with GFP-CAAX were plated in a 96-well glass bottom MatriPlate (Brooks, Catalog # MGB096-1-2-LG-L). 2 hours prior to imaging, cells were washed into serum-free, phenol free DMEM for imaging. Manganese (SigmaAldrich, M8054) was added at 100 μM 30 min prior to imaging. When indicated, CD47 function-blocking antibody clone miap301 (Biolegend, 127520) was used at 10 mg/ml. 100,000 H2B-mCherry expressing L1210 cells were added and the co-culture was imaged for 8 hr.

For the end-point analysis, 30,000 primary bone marrow derived macrophages were plated in a 96-well glass bottom MatriPlate. The following morning, the macrophages were serum starved for 2 hours. Then 60,000 L1210 dyed with CFSE (ThermoFisher, C34570) were added to the well. Engulfment was allowed to proceed for 4 hr, then cells were fixed and stained with DAPI to indicate nuclei and F4/80 to label macrophages. A blinded analyzer counted the fraction of F4/80+ cells containing CFSE+DAPI+ particles in each condition.

Mouse red blood cell internalization assay

For the mouse red blood cell internalization assay, 30,000 RAW264.7 macrophages (ATCC) were plated in one well of a 96-well glass bottom MatriPlate (Brooks, MGB096-1-2-LG-L) between 12 and 24 hr prior to the experiment. Mouse red blood cells (RBCs) (Innovative Research, 88R-M001) were washed into PBS and stained with CSFE (ThermoFisher, C34554) or Alexa Fluor 488 NHS Ester (ThermoFisher, A20000) for 1hr at room temperature. RBCs were then opsonized with C3bi as previously described (Chow et al., 2004). Briefly, RBCs were incubated with anti-mouse IgM (MyBioSource, MBS524107) for 1 hour at 37C. A portion of RBCs were separated for IgM controls, and the remaining RBCs were incubated with C5 deficient serum (Sigma-Aldrich, C1163) for 1hr at 37C. Macrophages were washed into serum-free HEPES imaging buffer and incubated with 150 ng/mL PMA and 1 mM Manganese or water. ~1 x 10^6 RBCs were added to each well and engulfment was allowed to proceed for 1 hour in incubator. Cells were washed with PBS, unengulfed RBCs were lysed with water for 2 min, and cells were

846 fixed with 4% PFA. Remaining non-lysed, non-engulfed RBCs were stained with APC

847 anti-iC3b antibody (Biolegend, 1:200 dilution, 846106) for 30 min. Macrophages were

848 stained with CellMask orange (ThermoFisher, 1:5,000 dilution) and Hoechst (Invitrogen,

849 H3570, 1:1,000 dilution) for 15 min. Images were acquired using the High Content

850 Screening (HCS) Site Generator plugin in µManager (Edelstein et al., 2010). The analyzer

851 was blinded during engulfment scoring using the position randomizer plug-in in

852 µManager.

853

854 **QUANTIFICATION AND STATISTICAL ANALYSIS**

855 Statistical analysis was performed in Prism 8 (GraphPad, Inc). The statistical test used

856 is indicated in the relevant figure legend. Sample sizes were predetermined and indicated

857 in the relevant figure legend. In general the analyzer was blinded during analysis using

858 either manual renaming of the files or the data randomizer plugin in µManager. The details

859 of each quantification method and blinding strategy are included in the Methods section.

860

Supplemental Figure Legends

Movie S1, related to Figure 5: Macrophage encounters IgG bound to a supported lipid bilayer.

TIRF imaging (see schematic in Figure S2) shows Alexa Fluor 647-IgG (black) in the supported lipid bilayer as a macrophage engages with an IgG-bound target. Frames were acquired every 20 sec and time is indicated in the top left. Scale bar denotes 5 μm.

Movie S2, related to Figure 5: Macrophage encounters IgG and CD47 bound to a supported lipid bilayer.

TIRF imaging shows Alexa Fluor 647-IgG (black) in the supported lipid bilayer as a macrophage engages with an IgG and CD47-bound target. Frames were acquired every 20 sec and time is indicated in the top left. Scale bar denotes 5 μm.

Movie S3, related to Figure 6: An Mn^{2+}-treated macrophage encounters an L1210 leukemia cell

A macrophage infected with GFP-CAAX encounters an L1210 leukemia cell labeled with H2B-mCherry. In the presence of 100 μM Mn^{2+} the macrophage is able to engulf the cancer cell. Images were acquired every 5 min for 140 min. The field of view is 53 μm by 53 μm.

References

884 **References**

885 Advani, R., Flinn, I., Popplewell, L., Forero, A., Bartlett, N.L., Ghosh, N., Kline, J.,

886 Roschewski, M., LaCasce, A., Collins, G.P., et al. (2018). CD47 Blockade by Hu5F9-G4

887 and Rituximab in Non-Hodgkin's Lymphoma. N. Engl. J. Med. *379*, 1711–1721.

888 Alenghat, F.J., Baca, Q.J., Rubin, N.T., Pao, L.I., Matozaki, T., Lowell, C.A., Golan,

889 D.E., Neel, B.G., and Swanson, K.D. (2012). Macrophages require Skap2 and Sirpα for

890 integrin-stimulated cytoskeletal rearrangement. J. Cell Sci. *125*, 5535–5545.

891 Bakalar, M.H., Joffe, A.M., Schmid, E.M., Son, S., Podolski, M., and Correspondence,

892 D.A.F. (2018). Size-Dependent Segregation Controls Macrophage Phagocytosis of

893 Antibody-Opsonized Targets. Cell *174*, 131–142.

894 Barclay, A.N., and Brown, M.H. (2006). The SIRP family of receptors and immune

895 regulation. Nat. Rev. Immunol. *6*, 457–464.

896 Birge, R.B., Boeltz, S., Kumar, S., Carlson, J., Wanderley, J., Calianese, D., Barcinski,

897 M., Brekken, R.A., Huang, X., Hutchins, J.T., et al. (2016). Phosphatidylserine is a

898 global immunosuppressive signal in efferocytosis, infectious disease, and cancer. Cell

899 Death Differ. *23*, 962–978.

900 Campbell, H., Heidema, C., Pilarczyk, D.G., and DeMali, K.A. (2018). SHP-2 is

901 activated in response to force on E-cadherin and dephosphorylates vinculin Y822. J.

902 Cell Sci. *131*, jcs216648.

903 Caron, E., Self, A.J., and Hall, A. (2000). The GTPase Rap1 controls functional

904 activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory

905 mediators. Curr. Biol. *10*, 974–978.

906 Cham, L.B., Bear, L., Dulgeroff, T., Caspi, M., Weissman, I.L., Lang, K.S., and

907 Correspondence, K.J.H. (2020). Immunotherapeutic Blockade of CD47 Inhibitory

908 Signaling Enhances Innate and Adaptive Immune Responses to Viral Infection.

909 CellReports *31*, 107494.

910 Chang, V.T., Fernandes, R.A., Ganzinger, K.A., Lee, S.F., Siebold, C., McColl, J.,

911 Jönsson, P., Palayret, M., Harlos, K., Coles, C.H., et al. (2016). Initiation of T cell

912 signaling by CD45 segregation at "close contacts". Nat. Immunol. *17*, 574–582.

913 Chao, M.P., Alizadeh, A.A., Tang, C., Myklebust, J.H., Varghese, B., Gill, S., Jan, M.,

914 Cha, A.C., Chan, C.K., Tan, B.T., et al. (2010a). Anti-CD47 Antibody Synergizes with

915   Rituximab to Promote Phagocytosis and Eradicate Non-Hodgkin Lymphoma. Cell *142*,

916   699–713.

917   Chao, M.P., Jaiswal, S., Weissman-Tsukamoto, R., Alizadeh, A.A., Gentles, A.J.,

918   Volkmer, J., Weiskopf, K., Willingham, S.B., Raveh, T., Park, C.Y., et al. (2010b).

919   Calreticulin Is the Dominant Pro-Phagocytic Signal on Multiple Human Cancers and Is

920   Counterbalanced by CD47. Sci. Transl. Med. *2*, 63ra94-63ra94.

921   Chao, M.P., Weissman, I.L., and Majeti, R. (2012). The CD47-SIRPα pathway in cancer

922   immune evasion and potential therapeutic implications. Curr. Opin. Immunol. *24*, 225–

923   232.

924   Chen, J., Zhong, M.-C., Guo, H., Davidson, D., Mishel, S., Lu, Y., Rhee, I., Pérez-

925   Quintero, L.-A., Zhang, S., Cruz-Munoz, M.-E., et al. (2017). SLAMF7 is critical for

926   phagocytosis of haematopoietic tumour cells via Mac-1 integrin. Nature *544*, 493–497.

927   Chow, C.-W., Downey, G.P., and Grinstein, S. (2004). Measurements of Phagocytosis

928   and Phagosomal Maturation. Curr. Protoc. Cell Biol. *22*, 15.7.1-15.7.33.

929   Dheilly, E., Moine, V., Broyer, L., Salgado-Pires, S., Johnson, Z., Papaioannou, A.,

930   Cons, L., Calloud, S., Majocchi, S., Nelson, R., et al. (2017). Selective Blockade of the

931   Ubiquitous Checkpoint Receptor CD47 Is Enabled by Dual-Targeting Bispecific

932   Antibodies. Mol. Ther. *25*, 523–533.

933   Dransfield, I., Cabanas, C., Craig, A., and Hogg, N. (1992). Divalent Cation Regulation

934   of the Function of the Leukocyte Integrin LFA1.

935   Dupuy, A.G., and Caron, E. (2008). Integrin-dependent phagocytosis: spreading from

936   microadhesion to new concepts. J. Cell Sci. *121*, 1773–1783.

937   Edelstein, A., Amodaj, N., Hoover, K., Vale, R., and Stuurman, N. (2010). Computer

938   control of microscopes using manager. Curr. Protoc. Mol. Biol. *Chapter 14*, Unit14 20.

939   Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson,

940   P.M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes

941   triggers specific recognition and removal by macrophages. J. Immunol. *148*, 2207–

942   2216.

943   Farlow, J., Seo, D., Broaders, K.E., Taylor, M.J., Gartner, Z.J., and Jun, Y.W. (2013).

944   Formation of targeted monovalent quantum dots by steric exclusion. Nat. Methods.

945   Freeman, S.A., and Grinstein, S. (2014). Phagocytosis: Receptors, signal integration,

946   and the cytoskeleton. Immunol. Rev. *262*, 193–215.

947   Freeman, S.A., Goyette, J., Furuya, W., Woods, E.C., Bertozzi, C.R., Bergmeier, W.,

948   Hinz, B., Van Der Merwe, P.A., Das, R., and Grinstein, S. (2016). Integrins Form an

949   Expanding Diffusional Barrier that Coordinates Phagocytosis. Cell *164*, 128–140.

950   Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsuda,

951   M., Takada, T., and Kasuga, M. (1996). A novel membrane glycoprotein, SHPS-1, that

952   binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to

953   mitogens and cell adhesion. Mol. Cell. Biol. *16*, 6887–6899.

954   Gardai, S.J., McPhillips, K.A., Frasch, S.C., Janssen, W.J., Starefeldt, A., Murphy-

955   Ullrich, J.E., Bratton, D.L., Oldenborg, P.-A.A., Michalak, M., and Henson, P.M. (2005).

956   Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-

957   activation of LRP on the phagocyte. Cell *123*, 321–334.

958   Gardner, B., Anstee, D.J., Mawby, W.J., Tanner, M.J., and von dem Borne, A.E. (1991).

959   The abundance and organization of polypeptides associated with antigens of the Rh

960   blood group system. Transfus. Med. *1*, 77–85.

961   Geiger, B., Spatz, J.P., and Bershadsky, A.D. (2009). Environmental sensing through

962   focal adhesions. Nat. Rev. Mol. Cell Biol. *10*, 21–33.

963   Gholamin, S., Mitra, S.S., Feroze, A.H., Liu, J., Kahn, S.A., Zhang, M., Esparza, R.,

964   Richard, C., Ramaswamy, V., Remke, M., et al. (2017). Disrupting the CD47-SIRPα

965   anti-phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for

966   malignant pediatric brain tumors. Sci. Transl. Med. *9*, eaaf2968.

967   Goodridge, H.S., Reyes, C.N., Becker, C.A., Katsumoto, T.R., Ma, J., Wolf, A.J., Bose,

968   N., Chan, A.S.H., Magee, A.S., Danielson, M.E., et al. (2011). Activation of the innate

969   immune receptor Dectin-1 upon formation of a 'phagocytic synapse.' Nature *472*, 471–

970   475.

971   Hatherley, D., Graham, S.C., Turner, J., Harlos, K., Stuart, D.I., and Barclay, A.N.

972   (2008). Paired Receptor Specificity Explained by Structures of Signal Regulatory

973   Proteins Alone and Complexed with CD47. Mol. Cell *31*, 266–277.

974   Hui, E., and Vale, R.D. (2014). In vitro membrane reconstitution of the T-cell receptor

975   proximal signaling network. Nat. Struct. Mol. Biol. *21*, 133–142.

976   Inagaki, K., Yamao, T., Noguchi, T., Matozaki, T., Fukunaga, K., Takada, T., Hosooka,

T., Akira, S., and Kasuga, M. (2000). SHPS-1 regulates integrin-mediated cytoskeletal reorganization and cell motility. EMBO J. *19*, 6721–6731.

Jacobs, S.A., Diem, M.D., Luo, J., Teplyakov, A., Obmolova, G., Malia, T., Gilliland, G.L., and O'Neil, K.T. (2012). Design of novel FN3 domains with high stability by a consensus sequence approach. Protein Eng. Des. Sel. *25*, 107–117.

Jaiswal, S., Jamieson, C.H.M., Pang, W.W., Park, C.Y., Chao, M.P., Majeti, R., Traver, D., van Rooijen, N., and Weissman, I.L. (2009). CD47 Is Upregulated on Circulating Hematopoietic Stem Cells and Leukemia Cells to Avoid Phagocytosis. Cell *138*, 271–285.

James, J.R., and Vale, R.D. (2012). Biophysical mechanism of T-cell receptor triggering in a reconstituted system. Nature *487*, 64–69.

Jiang, P., Lagenaur, C.F., and Narayanan, V. (1999). Integrin-associated protein is a ligand for the P84 neural adhesion molecule. J. Biol. Chem. *274*, 559–562.

Jones, S.L., Knaus, U.G., Bokoch, G.M., and Brown, E.J. (1998). Two signaling mechanisms for activation of alphaM beta2 avidity in polymorphonuclear neutrophils. J. Biol. Chem. *273*, 10556–10566.

Kharitonenkov, A., Chen, Z., Sures, I., Wang, H., Schilling, J., and Ullrich, A. (1997). A family of proteins that inhibit signalling through tyrosine kinase receptors. Nature *386*, 181–186.

Kojima, Y., Volkmer, J.-P., McKenna, K., Civelek, M., Lusis, A.J., Miller, C.L., Direnzo, D., Nanda, V., Ye, J., Connolly, A.J., et al. (2016). CD47-blocking antibodies restore phagocytosis and prevent atherosclerosis. Nature *536*, 86–90.

Lehrman, E.K., Wilton, D.K., Litvina, E.Y., Welsh, C.A., Chang, S.T., Frouin, A., Walker, A.J., Heller, M.D., Umemori, H., Chen, C., et al. (2018). CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development. Neuron *100*, 120-134.e6.

Lin, J., Kurilova, S., Scott, B.L., Bosworth, E., Iverson, B.E., Bailey, E.M., and Hoppe, A.D. (2016). TIRF imaging of Fc gamma receptor microclusters dynamics and signaling on macrophages during frustrated phagocytosis. BMC Immunol. *17*, 5.

Liu, D.Q., Li, L.M., Guo, Y.L., Bai, R., Wang, C., Bian, Z., Zhang, C.Y., and Zen, K. (2008). Signal regulatory protein α negatively regulates β 2 integrin-mediated monocyte adhesion, transendothelial migration and phagocytosis. PLoS One *3*.

1008 Liu, X., Pu, Y., Cron, K., Deng, L., Kline, J., Frazier, W.A., Xu, H., Peng, H., Fu, Y.-X.,

1009 and Xu, M.M. (2015). CD47 blockade triggers T cell–mediated destruction of

1010 immunogenic tumors. Nat. Med. *21*, 1209–1215.

1011 Lu, J., Ellsworth, J.L., Hamacher, N., Oak, S.W., and Sun, P.D. (2011). Crystal

1012 Structure of Fcγ Receptor I and Its Implication in High Affinity γ-Immunoglobulin

1013 Binding. J. Biol. Chem. *286*, 40608–40613.

1014 Majeti, R., Chao, M.P., Alizadeh, A.A., Pang, W.W., Jaiswal, S., Gibbs, K.D., van

1015 Rooijen, N., and Weissman, I.L. (2009). CD47 Is an Adverse Prognostic Factor and

1016 Therapeutic Antibody Target on Human Acute Myeloid Leukemia Stem Cells. Cell *138*,

1017 286–299.

1018 Matlung, H.L., Babes, L., Zhao, X.W., van Houdt, M., Treffers, L.W., van Rees, D.J.,

1019 Franke, K., Schornagel, K., Verkuijlen, P., Janssen, H., et al. (2018). Neutrophils Kill

1020 Antibody-Opsonized Cancer Cells by Trogoptosis. Cell Rep. *23*, 3946-3959.e6.

1021 Michaels, A.D., Newhook, T.E., Adair, S.J., Morioka, S., Goudreau, B.J., Nagdas, S.,

1022 Mullen, M.G., Persily, J.B., Bullock, T.N.J., Slingluffjr, C.L., et al. (2017). Cancer

1023 Therapy: Preclinical CD47 Blockade as an Adjuvant Immunotherapy for Resectable

1024 Pancreatic Cancer.

1025 Motegi, S. -i., Okazawa, H., Ohnishi, H., Sato, R., Kaneko, Y., Kobayashi, H.,

1026 Tomizawa, K., Ito, T., Honma, N., Bühring, H., et al. (2003). Role of the CD47-SHPS-1

1027 system in regulation of cell migration. EMBO J. *22*, 2634–2644.

1028 Mouro-Chanteloup, I., Delaunay, J., Gane, P., Nicolas, V., Johansen, M., Brown, E.J.,

1029 Peters, L.L., Van Kim, C. Le, Cartron, J.P., and Colin, Y. (2003). Evidence that the red

1030 cell skeleton protein 4.2 interacts with the Rh membrane complex member CD47. Blood

1031 *101*, 338–344.

1032 Noguchi, T., Matozaki, T., Fujioka, Y., Yamao, T., Tsuda, M., Takada, T., and Kasuga,

1033 M. (1996). Characterization of a 115-kDa protein that binds to SH-PTP2, a protein-

1034 tyrosine phosphatase with Src homology 2 domains, in Chinese hamster ovary cells. J.

1035 Biol. Chem. *271*, 27652–27658.

1036 O'Donoghue, G.P., Pielak, R.M., Smoligovets, A.A., Lin, J.J., and Groves, J.T. (2013).

1037 Direct single molecule measurement of TCR triggering by agonist pMHC in living

1038 primary T cells. Elife *2*.

1039   Okazawa, H., Motegi, S. -i., Ohyama, N., Ohnishi, H., Tomizawa, T., Kaneko, Y.,

1040   Oldenborg, P.-A., Ishikawa, O., and Matozaki, T. (2005). Negative Regulation of

1041   Phagocytosis in Macrophages by the CD47-SHPS-1 System. J. Immunol. *174*, 2004–

1042   2011.

1043   Oldenborg, P.-A., Gresham, H.D., and Lindberg, F.P. (2001). Cd47-Signal Regulatory

1044   Protein α (Sirpα) Regulates Fcγ and Complement Receptor–Mediated Phagocytosis. J.

1045   Exp. Med. *193*, 855–862.

1046   Oldenborg, P.A., Zheleznyak, A., Fang, Y.F., Lagenaur, C.F., Gresham, H.D., and

1047   Lindberg, F.P. (2000). Role of CD47 as a marker of self on red blood cells. Science *288*,

1048   2051–2054.

1049   Panni, R.Z., Herndon, J.M., Zuo, C., Hegde, S., Hogg, G.D., Knolhoff, B.L., Breden,

1050   M.A., Li, X., Krisnawan, V.E., Khan, S.Q., et al. (2019). Agonism of CD11b reprograms

1051   innate immunity to sensitize pancreatic cancer to immunotherapies. Sci. Transl. Med.

1052   *11*, eaau9240.

1053   Poon, I.K.H., Lucas, C.D., Rossi, A.G., and Ravichandran, K.S. (2014). Apoptotic cell

1054   clearance: basic biology and therapeutic potential. Nat. Rev. Immunol. *14*, 166–180.

1055   Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., and

1056   Eliceiri, K.W. (2017). ImageJ2: ImageJ for the next generation of scientific image data.

1057   BMC Bioinformatics *18*, 529.

1058   Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T.,

1059   Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: An open-source

1060   platform for biological-image analysis. Nat. Methods *9*, 676–682.

1061   Schmid, E.M., Bakalar, M.H., Choudhuri, K., Weichsel, J., Ann, H.S., Geissler, P.L.,

1062   Dustin, M.L., and Fletcher, D.A. (2016). Size-dependent protein segregation at

1063   membrane interfaces. Nat. Phys. *12*, 704–711.

1064   Schmid, M.C., Khan, S.Q., Kaneda, M.M., Pathria, P., Shepard, R., Louis, T.L., Anand,

1065   S., Woo, G., Leem, C., Faridi, M.H., et al. (2018). Integrin CD11b activation drives anti-

1066   tumor innate immunity. Nat. Commun. *9*, 5379.

1067   Seiffert, M., Cant, C., Chen, Z., Rappold, I., Brugger, W., Kanz, L., Brown, E.J., Ullrich,

1068   A., and Bühring, H.J. (1999). Human signal-regulatory protein is expressed on normal,

1069   but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving

1070   its counterreceptor CD47. Blood *94*, 3633–3643.

1071   Spencer, D.M., Wandless, T.J., Schreiber, S.L., and Crabtree, G.R. (1993). Controlling

1072   signal transduction with synthetic ligands. Science *262*, 1019–1024.

1073   Springer, T.A., and Dustin, M.L. (2011). Integrin inside-out signaling and the

1074   immunological synapse. Curr. Opin. Cell Biol. *24*, 107–115.

1075   Subramanian, S., Parthasarathy, R., Sen, S., Boder, E.T., and Discher, D.E. (2006).

1076   Species-and cell type–specific interactions between CD47 and human SIRP␣. Blood

1077   *107*, 2548–2556.

1078   Swaminathan, V., Kalappurakkal, J.M., Mehta, S.B., Nordenfelt, P., Moore, T.I., Koga,

1079   N., Baker, D.A., Oldenbourg, R., Tani, T., Mayor, S., et al. (2017). Actin retrograde flow

1080   actively aligns and orients ligand-engaged integrins in focal adhesions. Proc. Natl.

1081   Acad. Sci. *114*, 10648–10653.

1082   Tamada, M., Sheetz, M.P., and Sawada, Y. (2004). Activation of a Signaling Cascade

1083   by Cytoskeleton Stretch. Dev. Cell *7*, 709–718.

1084   Tsai, R.K., and Discher, D.E. (2008). Inhibition of "self" engulfment through deactivation

1085   of myosin-II at the phagocytic synapse between human cells. J. Cell Biol. *180*, 989–

1086   1003.

1087   Tseng, D., Volkmer, J.-P.J.-P., Willingham, S.B., Contreras-Trujillo, H., Fathman, J.W.,

1088   Fernhoff, N.B., Seita, J., Inlay, M.A., Weiskopf, K., Miyanishi, M., et al. (2013). Anti-

1089   CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective

1090   antitumor T-cell response. Proc. Natl. Acad. Sci. *110*, 11103–11108.

1091   Tsuda, M., Matozaki, T., Fukunaga, K., Fujioka, Y., Imamoto, A., Noguchi, T., Takada,

1092   T., Yamao, T., Takeda, H., Ochi, F., et al. (1998). Integrin-mediated tyrosine

1093   phosphorylation of SHPS-1 and its association with SHP-2. Roles of Fak and Src family

1094   kinases. J. Biol. Chem. *273*, 13223–13229.

1095   Utsugi, T., Schroit, A.J., Connor, J., Bucana, C.D., and Fidler, I.J. (1991). Elevated

1096   expression of phosphatidylserine in the outer membrane leaflet of human tumor cells

1097   and recognition by activated human blood monocytes. Cancer Res. *51*, 3062–3066.

1098   Veillette, A., Thibaudeaut, E., and Latour, S. (1998). High expression of inhibitory

1099   receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in

1100   macrophages. J. Biol. Chem. *273*, 22719–22728.

1101 Weischenfeldt, J., and Porse, B. (2008). Bone Marrow-Derived Macrophages (BMM):

1102 Isolation and Applications. CSH Protoc. *2008*, pdb.prot5080.

1103 Willingham, S.B., Volkmer, J.-P., Gentles, A.J., Sahoo, D., Dalerba, P., Mitra, S.S.,

1104 Wang, J., Contreras-Trujillo, H., Martin, R., Cohen, J.D., et al. (2012). The CD47-signal

1105 regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid

1106 tumors. Proc. Natl. Acad. Sci. *109*, 6662–6667.

1107 Wong, H.S., Jaumouillé, V., Freeman, S.A., Doodnauth, S.A., Schlam, D., Canton, J.,

1108 Mukovozov, I.M., Saric, A., Grinstein, S., and Robinson, L.A. (2016). Chemokine

1109 Signaling Enhances CD36 Responsiveness toward Oxidized Low-Density Lipoproteins

1110 and Accelerates Foam Cell Formation. Cell Rep. *14*, 2859–2871.

1111 Wu, J., Wu, H., An, J., Ballantyne, C.M., and Cyster, J.G. (2018). Critical role of integrin

1112 CD11c in splenic dendritic cell capture of missing-self CD47 cells to induce adaptive

1113 immunity. Proc. Natl. Acad. Sci. 201805542.

1114 Yi, T., Li, J., Chen, H., Hu, Y., Lowell, C.A., and Cyster Correspondence, J.G. (2015).

1115 Splenic Dendritic Cells Survey Red Blood Cells for Missing Self-CD47 to Trigger

1116 Adaptive Immune Responses. Immunity *43*, 764–775.

1117 Yu, D.H., Qu, C.K., Henegariu, O., Lu, X., and Feng, G.S. (1998). Protein-tyrosine

1118 phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. J. Biol.

1119 Chem. *273*, 21125–21131.

1120

1121