CD47 prevents Rac-mediated phagocytosis through Vav1 dephosphorylation

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Summary

CD47 is expressed by viable cells to protect against phagocytosis. CD47 is recognized by SIRPα, an inhibitory receptor expressed by macrophages and other myeloid cells. Activated SIRPα recruits SHP-1 and SHP-2 phosphatases but the inhibitory signaling cascade downstream of these phosphatases is not clear. In this study, we used time lapse imaging to measure how CD47 impacts the kinetics of phagocytosis. We found that targets with IgG antibodies were primarily phagocytosed through a Rac-based reaching mechanism. Targets also containing CD47 were only phagocytosed through a less frequent Rho-based sinking mechanism. Hyperactivating Rac2 eliminated the suppressive effect of CD47, suggesting that CD47 prevents activation of Rac and reaching phagocytosis. During IgG-mediated phagocytosis, the tyrosine kinase Syk phosphorylates the GEF Vav, which then activates the GTPase Rac to drive F-actin rearrangement and target internalization. CD47 inhibited Vav1 phosphorylation without impacting Vav1 recruitment to the phagocytic synapse or Syk phosphorylation. Macrophages expressing a hyperactive Vav1 were no longer sensitive to CD47. Together this data suggests that Vav1 is a key target of the CD47 signaling pathway.

Introduction

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33   The immune system is regulated by a combination of activating signals on

34   pathogenic targets and inhibitory signals that protect healthy tissue[1]. Inhibitory immune

35   receptors are critical for tolerance in healthy tissues, and restoring homeostasis after

36   injury and infection[2–4]. Macrophages, effectors of the innate immune system, protect the

37   body by phagocytosing harmful targets while robustly ignoring healthy cells. CD47 is an

38   inhibitory signal on the surface of viable cells that prevents phagocytosis[5,6]. Compared

39   to their activating counterparts, the inhibitory signals that protect healthy cells remain

40   poorly understood.

41   IgG antibodies bind bacteria, fungi or virally infected cells and trigger

42   phagocytosis[5,7,8]. Antibody dependent phagocytosis is also an important mechanism for

43   many cancer immunotherapies including rituximab (CD20 antibody) and trastuzumab

44   (Her2 antibody)[9–12]. IgG is recognized by the Fc Receptor in macrophages[13]. IgG

45   binding triggers Fc Receptor phosphorylation and recruits the effector kinase Syk. Syk

46   activates several downstream pathways to promote F-actin reorganization and

47   phagocytosis.

48   To avoid phagocytosis of healthy cells, CD47 is a potent "Don't Eat Me" signal

49   expressed by nearly all viable cells in mice and humans[14–16]. CD47 suppresses

50   phagocytosis by activating the inhibitory receptor SIRPα in macrophages[17–21]. Cells

51   lacking CD47 are rapidly cleared from healthy tissues[14,16]. Bacteria mimic CD47 to

52   evade the innate immune system[22–24]. CD47 is also upregulated on many cancer cells,

53   allowing these malignant cells to evade the immune system and proliferate

54   unchecked[14,15,25].

55   Modulating the CD47 signaling pathway has broad therapeutic implications. In

56   cancer treatment, CD47 blockades have shown promising results in several phase I and

57   phase II clinical trials, often administered in combination with a therapeutic IgG antibody

58   that activates phagocytosis of cancer cells[6,26–30]. CD47 blockades promote both

59   phagocytosis of cancer cells by macrophages and cross-presentation of cancer antigen

60   by dendritic cells[15,31,32]. CD47 blockades also improve phagocytosis of diseased

61   vascular tissue to treat atherosclerosis and clearance of viral infections[33,34]. Conversely,

62   activating the CD47 signaling pathway has many potential therapeutic applications.

63   Adding CD47 to transplanted cells or materials is an exciting strategy to protect these

64   materials from macrophage phagocytosis[35–38]. Similarly, activating the CD47 receptor,

65   SIRPα, is a potential therapy for auto-immune disorders to counteract macrophage

66   hyperactivation[39,40].

67   Despite the immense therapeutic interest in CD47 and its receptor SIRPα, very

68   little is known about how this inhibitory signal is transduced within the macrophage.

69   CD47 binding positions SIRPα at the phagocytic synapse between a macrophage and

70   its target, where SIRPα's two intracellular immune tyrosine inhibitory motifs (ITIMs) are

71   phosphorylated by Src family kinases[41,42]. The phosphorylated ITIM motifs recruit SHP-

72   1 and SHP-2 phosphatase, and SHP-1 is required for CD47 to inhibit phagocytosis[17–

73   19,43–45]. The substrates of these phosphatases during phagocytosis have not been

74   identified. Several studies have shown that SHP-1/2 phosphatases do not directly de-

75   activate the IgG-binding Fc Receptor[17,42,46]. Downstream, this inhibitory signaling

76   pathway prevents activation of integrin and myosin II[42,46]. However, there are many

77 signaling events between Fc Receptor phosphorylation and the inside out activation of

78 integrins or myosin II, leaving several potential targets for SIRPα-bound phosphatases[5].

79   In this study, we clarified the downstream signaling cascade enacted by CD47,

80 and determined how this inhibitory pathway affects phagocytosis of antibody-bound

81 targets. We used timelapse microscopy to characterize how CD47 affected each step in

82 the phagocytic process. For IgG opsonized targets, we observed two previously

83 described modes of phagocytosis regulated by different GTPases. Most targets were

84 phagocytosed via Rac-driven reaching phagocytosis and a smaller number though Rho-

85 driven sinking phagocytosis. Targets with CD47 were only phagocytosed through the

86 less frequent Rho-based sinking mechanism. Inhibiting Rho eliminated phagocytosis of

87 synthetic targets and cancer cells with CD47, while inhibiting Rac had little effect. A

88 hyperactive Rac2 mutation (Rac2^{E62K}) bypassed CD47's inhibitory effect. This

89 demonstrates that CD47 inhibits Rac-driven reaching phagocytosis, and suggests that

90 the direct targets of SIRPα-bound phosphatases are upstream of Rac. We found CD47

91 causes dephosphorylation of Vav1, the guanine nucleotide exchange factor (GEF) that

92 activates Rac but not Rho at the phagocytic cup. CD47 did not cause any change in Syk

93 activation or Vav1 recruitment, suggesting that Vav1 is directly regulated by SHP

94 phosphatases. Overall, our study provides a mechanistic model for how CD47

95 suppresses phagocytosis.

96 **Results**

97 **CD47 slows phagocytosis and increases cup retraction and failure**

98   To study how CD47 inhibits IgG-mediated phagocytosis, we used reconstituted,

99 cell-mimicking engulfment targets consisting of silica beads coated with fluorescent

100 supported lipid bilayers (Fig.1A, 1B)[42,47]. To activate Fc Receptor-mediated

101 phagocytosis, we opsonized beads with anti-biotin IgG, which binds biotinylated lipids in

102 the bilayers. We incorporated His-tagged CD47 onto beads via attachment to Ni-NTA-

103 conjugated lipids, so that the CD47 extracellular domain is positioned to activate SIRPα.

104 We then incubated primary mouse bone marrow-derived macrophages (BMDMs) with

105 IgG or IgG+CD47 beads and measured the number of internalized beads via confocal

106 microscopy (Fig. 1A, 1B). IgG beads were engulfed approximately two times more than

107 IgG+CD47 beads (Fig. 1B), consistent with previous studies[42,48].

108   We next used time-lapse confocal microscopy to determine how CD47 affects

109 the kinetics of phagocytosis. Phagocytosis consists of discrete steps, each associated

110 with unique molecular mediators: (1) target particle binding, (2) initiation of

111 phagocytosis, and (3) completion, as defined by phagocytic cup closure (Fig. 1C, D).

112 We measured the frequency of completing each step for IgG and IgG+CD47 beads. We

113 also measured the time between successfully completing each step (Fig. 1E-I). We

114 found that CD47 did not inhibit target binding (Fig. 1E). We also found no difference in

115 the frequency of initiating phagocytosis or the time between bead binding and initiation

116 of phagocytosis (Fig. 1F, 1G). However, CD47 decreased the frequency of successfully

117 completing phagocytosis (Fig. 1I). Most strikingly, CD47 dramatically slowed the

118 engulfment time, or the time between initiation and completion of phagocytosis (Fig.

6

119   1H). These results indicate that CD47 affects the progression of macrophage

120   membrane around the phagocytic target or phagocytic cup closure, rather than target

121   particle binding or initiation of phagocytosis.

122   **CD47 positive targets are phagocytosed via sinking phagocytosis**

123   Since the engulfment speed of IgG and IgG+CD47 targets was dramatically

124   different, we hypothesized that engulfment of the two bead types may be

125   morphologically distinct. In our timelapse data, we clearly observed two previously

126   described forms of phagocytosis: reaching and sinking phagocytosis[49–52]. Reaching

127   phagocytosis involves rapid extension of F-actin rich protrusions around the phagocytic

128   target[49,52–54]. During sinking phagocytosis, the target is slowly pulled into the cell without

129   membrane extension[49,50,52,55]. We found that IgG beads were almost always engulfed

130   via reaching phagocytosis (Fig. 2A, C, Video S1), whereas IgG+CD47 beads were

131   usually engulfed via sinking phagocytosis (Fig. 2B, C, Video S2). This is consistent with

132   the slower engulfment speed of IgG+CD47 targets. To quantify the frequency of these

133   two modes of phagocytosis more rigorously, we measured the position of the

134   engulfment target relative to the cell cortex when phagocytosis was completed (Fig.

135   2D)[55]. This analysis revealed two populations of engulfed targets: one located outside

136   the cell cortex at the time of cup closure (beads engulfed via reaching phagocytosis),

137   and one located inside the cell cortex (beads engulfed via sinking phagocytosis). For

138   IgG beads, 80% were outside the cell when phagocytosis completed. In contrast, the

139   majority of IgG+CD47 targets were inside the cell when the phagocytic cup closed.

140   We found that the increased failure rate of phagocytosis of CD47 targets was

141   due to increased retraction of reaching phagocytic cups. If the macrophage initiated

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142   reaching phagocytosis of a target with CD47, the phagocytic cup usually retracted

143   before it extended around the entire target, resulting in failed reaching phagocytosis

144   (Fig. 2E, F; Video S3). In contrast, retraction of reaching phagocytic cups was rarely

145   observed for IgG-only targets (Fig. 2E). Together these data indicate that CD47 inhibits

146   reaching phagocytosis, but not sinking phagocytosis.

147   **Rho, not Rac, is required for phagocytosis of CD47 positive targets**

148   Reaching and sinking phagocytosis are characterized by distinct actin

149   cytoskeleton dynamics[52,55]. One measurable difference is that F-actin accumulates at

150   the phagocytic cup rim during reaching but not sinking phagocytosis[55]. To test if CD47

151   changes F-actin organization at the phagocytic cup, we incubated BMDMs with IgG and

152   IgG+CD47 beads, then fixed and stained with phalloidin to visualize F-actin during

153   phagocytosis (Fig. 3A, B). Actin recruitment to phagocytic cup rims was significantly

154   reduced during engulfment of IgG+CD47 targets compared to IgG targets (Fig. 3B). This

155   suggests that CD47 alters actin organization during phagocytosis.

156   The Rho GTPase family (Rac, Rho and Cdc42) are regulators of the actin

157   cytoskeleton during phagocytosis, and different GTPases have been implicated in

158   reaching and sinking phagocytosis. Reaching phagocytosis is regulated by the GTPase

159   Rac while sinking phagocytosis requires the GTPase Rho[52]. Since CD47 inhibits

160   reaching phagocytosis, we hypothesized that CD47 specifically inhibits Rac, but not

161   Rho. Additionally, since IgG+CD47 targets are engulfed via sinking phagocytosis, we

162   hypothesized that Rho is required for phagocytosis of IgG+CD47 target particles. To

163   test these hypotheses, we treated BMDMs with inhibitors of Rac (NSC23766 and MBQ-

164   167; also inhibit the related GTPase CDC42) or an inhibitor of Rho (C3 transferase). We

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165   then incubated the BMDMs with IgG, IgG+CD47, or unopsonized beads (Fig. 3C). Rac

166   inhibition significantly reduced phagocytosis of IgG targets, causing BMDMs to eat the

167   same amount of IgG and IgG+CD47 beads. Rac inhibition did not affect phagocytosis of

168   IgG+CD47 targets. On the other hand, Rho inhibition decreased phagocytosis of both

169   target types, nearly eliminating phagocytosis of IgG+CD47 targets. Together, this data

170   supports the hypothesis that CD47 inhibits Rac-mediated phagocytosis. This data also

171   demonstrates that Rho, not Rac, is required for phagocytosis of IgG+CD47 targets.

172   We next sought to validate these results with cancer cell targets in lieu of

173   synthetic bead targets. We hypothesized that CD47-expressing cancer cells, like

174   IgG+CD47 beads, are phagocytosed through a Rho-dependent mechanism, while CD47

175   knockout cells, like IgG beads, are primarily phagocytosed via Rac and Cdc42. To test

176   this, we measured phagocytosis of L1210 mouse leukemia cells opsonized with an anti-

177   CD20 antibody. This is modeled after the clinical combination of CD20 antibodies and

178   CD47 blockade, which has shown promise in clinical trials[27]. We used CRISPR-Cas9 to

179   generate a monoclonal CD47^{KO} L1210 line (Supp Fig. 3). We confirmed that CD47 was

180   not expressed on these cells by staining for CD47 (Supp Fig. 3). We again treated

181   BMDMs with pharmacological inhibitors of Rac or Rho, then incubated them with the

182   opsonized WT or CD47^{KO} L1210s and monitored phagocytosis via live cell microscopy

183   (Fig. 3D). As expected, untreated BMDMs engulfed more CD47^{KO} L1210 cells than WT

184   L1210 cells. Pharmacological inhibition of Rac decreased phagocytosis of CD47^{KO}

185   L1210 cells, but did not affect phagocytosis of wild type L1210 cells. In contrast, Rho

186   inhibition decreased engulfment of both CD47^{KO} and wild type L1210 cells, completely

187   eliminating phagocytosis of wild type cells. Together, this demonstrates that CD47

188   inhibits Rac-mediated phagocytosis of antibody opsonized cancer cells.

189   **Hyperactive Rac2 overcomes CD47 inhibition**

190   Our results show that inhibiting Rac mirrors the effect of CD47, however the

191   direct targets of SIRPα-bound phosphatases could be upstream or downstream of Rac.

192   We next hypothesized that if the direct targets are upstream of Rac, then

193   hyperactivating Rac could overcome CD47-mediated inhibition of phagocytosis. In

194   contrast, if CD47 inhibits later steps in phagocytosis, hyperactivating Rac could promote

195   phagocytosis but targets with CD47 would still be engulfed less than cells without CD47.

196   To test this, we isolated BMDMs from mice heterozygous for the hyperactive Rac2^{E62K}

197   mutation (Rac2$^{E62K/+}$)[56,57]. This Rac2 mutation is associated with a moderate increase in

198   Rac activity[56]. We found that Rac2$^{E62K/+}$ and wildtype macrophages phagocytosed a

199   similar amount of antibody-opsonized CD47^{KO} L1210 cells. However, Rac2$^{E62K/+}$

200   macrophages were insensitive to CD47 and phagocytosed dramatically more wild type

201   L1210 cells compared to wild type macrophages (Fig. 4A-E; Video S4). This data

202   demonstrates that increasing Rac activity bypasses the inhibitory effect of CD47.

203   **CD47 inhibits Rac through dephosphorylation of Vav1**

204   We next considered the potential direct targets of SIRPα-bound phosphatases.

205   We did not expect SIRPα-bound phosphatases to directly target Rac, as Rac GTPases

206   are primarily regulated by GTP-binding and hydrolysis, rather than phosphorylation.

207   Instead, we hypothesized that the direct target of SIRPα-bound phosphatases is

208   upstream of Rac. Since CD47 does not inhibit Rho-mediated phagocytosis, we

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209   reasoned that the early steps in phagocytosis, required for both sinking and reaching

210   phagocytosis, are also unlikely to be the direct target of SIRPα-bound phosphatases.

211   After IgG binds the Fc Receptor, IgG and Fc Receptor form nanoscale clusters within

212   the plasma membrane[58–60]. At these clusters, the Fc Receptor intracellular ITAMs are

213   phosphorylated and recruit Syk kinase[59–61]. We have previously found that CD47 does

214   not affect Fc Receptor clustering or alter Syk recruitment to these clusters[42]. In addition,

215   other studies have found little or no change in Fc Receptor or Syk phosphorylation for

216   CD47-positive targets[17,46]. To confirm this, we incubated macrophages with

217   unopsonized, IgG, or IgG+CD47 beads, and measured Syk phosphorylation (pY346) by

218   western blot. As expected, IgG beads increased Syk phosphorylation, and the addition

219   of CD47 to IgG beads did not reduce the IgG-mediated Syk phosphorylation (Fig. 5A,

220   B). Taken together, these data suggest that the direct target of SIRPα-bound

221   phosphatases is downstream of Syk but upstream of Rac.

222   As a GTPase, Rac activation is regulated by Guanine nucleotide Exchange

223   Factors (GEFs) that promote the exchange of GDP for GTP. Vav1 is the GEF that

224   activates Rac during phagocytosis[62,63]. Vav1 is an attractive potential target for SIRPα-

225   bound phosphatases for several reasons. First, Vav1 is required for Rac, but not Rho,

226   activity during phagocytosis[64]. Second, Syk phosphorylates Vav1 on tyrosine Y174 to

227   activate Vav1, making Vav1 the direct link between Syk and Rac[65]. Finally, in NK cells,

228   SHP-1 directly dephosphorylates Vav1 to prevent target cell killing, suggesting that this

229   SIRPα-bound phosphatase can directly regulate Vav1[66,67]. Supporting this, the

230   sequence surrounding the Y174 site on Vav1 (DEIpYEDL) closely matches the optimal

231   SHP-1 target sequences from two previous studies ((D/E)X(L/I/V)XpYXX(L/I/V) and

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232   (D/EXpY)) in both mouse and humans[68,69]. We hypothesized that SIRPα-bound

233   phosphatases could target Vav1 to inhibit phagocytosis. We first assayed Vav1

234   phosphorylation using a phosphoVav1 antibody (pY174)[70,71]. We found that

235   macrophages incubated with IgG beads showed increased Vav1 phosphorylation, and

236   adding CD47 to the beads eliminated this increase (Fig. 5A, C). To confirm the phospho

237   antibody staining, we immunoprecipitated Vav1 and used a pan-phosphotyrosine

238   antibody to measure Vav1 phosphorylation (Fig. 5D, E). This confirmed that IgG

239   increased Vav1 phosphorylation, and CD47 eliminated this increase. This data shows

240   that CD47 eliminates IgG-induced Vav1 phosphorylation.

241   **CD47 does not prevent Vav1 recruitment to the phagocytic synapse**

242   We next sought to clarify the mechanism by which CD47 reduces Vav1

243   phosphorylation. We began by examining Vav1 localization in the phagocytic synapse

244   using TIRF microscopy. Vav1 binds directly to Syk, but may have other binding partners

245   at the phagocytic cup[65,72]. In particular, Rac, which directly binds to Vav, is active

246   throughout the actin-rich extensions that surround the phagocytic target[73–75]. We

247   constructed planar supported lipid bilayers on a glass coverslip, then imaged IgG

248   clustering and mCherry-Vav1. We found that on IgG bilayers, Vav1 was enriched at IgG

249   clusters, but also present throughout the phagocytic synapse (Fig. 6A). This is

250   consistent with the reported interaction of Vav1 with Syk, present at IgG clusters, and

251   Rac, present along the sides of the phagocytic cup[59,73].

252   SIRPα-bound phosphatases could reduce Vav1 phosphorylation either by

253   preventing recruitment to IgG clusters where Vav1 is phosphorylated, or by

254   dephosphorylating Vav1 directly. To distinguish between these possibilities, we used

255   TIRF imaging to measure Vav1 recruitment to a phagocytic synapse containing CD47.

256   We verified that CD47 inhibited cell spreading across the bilayer, demonstrating that the

257   CD47 pathway was active (Fig. 6C). IgG cluster size was not affected by CD47,

258   consistent with previous literature (Fig. 6A, B)[42]. CD47 did not reduce Vav1 recruitment

259   to IgG clusters (Fig. 6A, B). In fact, Vav1 localization correlated even more strongly with

260   IgG when CD47 was present (Fig. 6D). Since the Vav1 signal at IgG clusters is similar

261   with IgG or IgG+CD47, this increased colocalization is likely due to a decrease in Vav1

262   outside of the IgG clusters. Overall, this demonstrates that CD47 does not prevent Vav1

263   recruitment to IgG clusters.

264   **Hyperactive Vav1 eliminates the effect of CD47**

265   Since our data suggests that SHP-1 directly dephosphorylates Vav, we

266   hypothesized that constitutive Vav1 activity would be sufficient to bypass the

267   suppressive effect of CD47. Phosphorylation of Y174 on Vav1 displaces this residue

268   from its intramolecular binding pocket, relieving autoinhibition of Vav1[76]. Similarly,

269   mutating this residue to phenylalanine results in a constitutively active Vav1[77]. We used

270   a previously characterized hyperactive Vav1 construct, where Y174 and two other key

271   tyrosines (Y142 and Y160) are mutated to prevent Vav1 autoinhibition[77,78].

272   Macrophages expressing this mutant Vav1 were not sensitive to CD47, phagocytosing a

273   comparable amount of IgG or IgG+CD47 beads (Fig. 6E). Together, this data strongly

274   suggests that Vav1 dephosphorylation is critical for CD47 to inhibit phagocytosis.

275 **Discussion**

276   CD47 is a potent "Don't Eat Me" signal that protects healthy cells and cancer

277   cells from macrophage attack. Understanding how CD47 inhibits phagocytosis of

278   antibody-bound targets is particularly important, since combining CD47 blockades and

279   monoclonal antibody therapies is the subject of several ongoing clinical trials[6,27–29]. We

280   examined the inhibitory signaling cascade enacted by CD47. We show that CD47

281   specifically inhibits Rac-mediated reaching phagocytosis, but not Rho-mediated sinking

282   phagocytosis. We consider each step in the phagocytosis signaling cascade leading to

283   Rac activation, and demonstrate that Vav1 is the key target of SIRPα-bound

284   phosphatases (Fig. 6F).

285   We show that Vav1 is dephosphorylated in response to CD47, and that

286   constitutively active Vav1 rescues phagocytosis of CD47-positive targets (Fig. 6). This

287   demonstrates that Vav1 is a key target of the CD47 signaling cascade. There are

288   several reasons to think that Vav1 is directly dephosphorylated by SIRPα-bound

289   phosphatases SHP-1 and SHP-2. After Fc Receptor activation, Syk binds to the Fc

290   Receptor's phosphorylated ITAMs. Vav1 binds to Syk, and is activated by Syk

291   phosphorylation[65,72]. When CD47 is present, we find no difference in IgG cluster size,

292   Syk recruitment or Syk phosphorylation, consistent with previous literature[17,42,46]. This

293   suggests that the phagocytosis signaling cascade upstream of Vav1 is not affected by

294   CD47. We further demonstrate that CD47 does not affect Vav1 recruitment to IgG

295   clusters, arguing against an indirect effect on Vav1 due to upstream changes in

296   signaling preventing Vav1 recruitment to the phagocytic synapse. In addition, Vav1 is

297   directly dephosphorylated by SHP-1 in NK cells[66,67]. SHP-1 also dephosphorylates Vav1

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298   to prevent Fas internalization and cell death in B cells[79]. We propose SHP-1 can also

299   dephosphorylate Vav1 at the phagocytic synapse.

300   Our findings explain a number of results in the current literature, which could be

301   downstream consequences of Vav1 inactivation. First, we have previously shown that

302   CD47 prevents inside out activation of integrin[42]. Vav family GEFs are required for

303   integrin activation during many immune cell processes including phagocytosis, cell

304   migration and cell spreading[80–83,83–85]. Another study found that CD47 prevents myosin

305   II phosphorylation, which is often required for phagocytosis[46]. This could be a

306   downstream effect of Rac inhibition, since Rac promotes myosin II activation, or

307   because myosin II is required for later steps in the phagocytosis program than Rac[86,87].

308   While it is possible that SIRPα-bound phosphatases target multiple proteins at the

309   phagocytic synapse including both myosin II and Vav1, our finding that Vav1 activation

310   rescues phagocytosis of CD47-positive targets supports the idea that Vav1 is the

311   primary target of SIRPα-bound phosphatases.

312   Our work also provides some insight into Vav1 function during phagocytosis.

313   There are very compelling studies showing that Vav1 binds directly to Syk via its SH2

314   domain, and this interaction promotes Vav1 phosphorylation both with purified proteins

315   and in cells[65,72]. Vav1 phosphorylation relieves Vav1 autoinhibition[76]. Once

316   phosphorylated via this transient interaction, Vav1 remains in the active conformation

317   until dephosphorylated[72]. During phagocytosis, we envision that Vav1 is activated by

318   Syk at IgG clusters, but moves on to activate Rac at other locations in the phagocytic

319   cup. Rac is active along the sides and base of the phagocytic cup[73]. Vav1 may be

320   targeted by SIRPα-bound phosphatases after leaving IgG clusters, since we have

321 previously seen that SIRPα does not colocalize with IgG clusters[42]. This would explain

322 our TIRF data showing a reduction in Vav1 at the phagocytic synapse outside of IgG

323 clusters when CD47 is also present.

324   One question raised by our study is whether the mechanism of phagocytosis

325 (reaching vs sinking phagocytosis) affects phagosome processing. Other studies have

326 suggested that the mode of target entry can affect what proteins are recruited to the

327 phagosome[52,88,89]. Rac and Vav1 also have known roles in phagosome processing[64].

328 Interestingly, CD47 blockades enhance cross presentation, which could be due to a

329 change in phagocytosis by dendritic cells, a change in the subsequent processing of the

330 phagocytosed material to promote cross presentation, or a separate signaling pathway

331 in dendritic cells[32,90,91].

332   Our work has broader implications for understanding inhibitory immune

333 receptors. In macrophages, there are several other inhibitory immune receptors that

334 regulate phagocytosis[92–96]. Future studies will need to examine whether these function

335 through a shared mechanism, or whether each inhibitory signaling pathway has unique

336 targets. An interesting implication of our work is that CD47 inhibits some, but not all,

337 parts of the Fc Receptor signaling pathway. Inhibitory receptors like SIRPα are often

338 described as the counterbalance to activating receptors like the Fc Receptors. This

339 gives the impression of a single dial that can be turned up or turned down. Instead, we

340 see that CD47 strongly inhibits Rac, but has no effect on Rho. This suggests a

341 potentially more complicated situation where inhibitory receptors target specific

342 downstream signals to sculpt the macrophage response.

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343   Our work has several therapeutic implications. Our finding that CD47 does not

344   inhibit Rho-mediated phagocytosis suggests that inhibitors of Rho-mediated

345   phagocytosis would synergize with CD47. In the cases where CD47 is currently used to

346   protect transplanted cells or particles, a secondary signal that inhibits Rho could be very

347   potent. This also suggests that CD47 would be more protective for targets that are

348   phagocytosed via a Rac-based mechanism than targets that are phagocytosed via a

349   Rho-based mechanism. Cancer cells can express a variety of different "Eat Me" signals,

350   and the phagocytic receptors that regulate engulfment can vary for different cancer

351   populations[5]. Our work suggests that cancer cell CD47 could potently regulate

352   phagocytosis if the signals on the cancer cell activate Rac. Finally, this work also

353   suggests that macrophages with hyperactive Rac are insensitive to CD47, and adept at

354   phagocytosing cancer cells[57].

355

Figure legends

Figure 1: CD47 decreases the probability of completing engulfment and slows the engulfment process. (A) Schematic depicts the supported lipid bilayer system used to study phagocytosis. Silica beads are coated with a supported lipid bilayer containing fluorescent atto647 lipids. Anti-biotin IgG binds to biotinylated lipids in the bilayer. 10xHis-tagged CD47 extracellular domain is attached to beads via a Ni^{2+}-chelating DGS-NTA lipid, so that the SIRPα binding domain is positioned outwards. (B) Beads (magenta) conjugated with IgG or IgG+CD47 were incubated with mouse bone marrow derived macrophages (BMDMs) expressing membrane tethered GFP (GFP-CAAX; green) for 30 minutes then imaged with confocal microscopy. The average number of beads engulfed per macrophage was counted and normalized to the maximum average number of beads engulfed per macrophage for that experiment to control for batch to batch variability in macrophage appetite. (C) Schematic depicts the stages of phagocytosis: target particle binding, initiation of phagocytosis, and completion (phagocytic cup closure). (D) Representative images of each stage. BMDMs are expressing GFP-CAAX (green, top; greyscale, bottom) and the bead supported lipid bilayer is labeled with atto647 (magenta, top). Box in the top panel shows area of inset below. Timelapse confocal microscopy was used to quantify the fraction of beads bound to a macrophage (E); the time from binding to initiation of phagocytosis, if it occurred (F); the fraction of bound beads that proceed to the initiation step (G); the engulfment time, which is the time from initiation to completion, if it completed (H); and the fraction of beads that proceeded from the initiation to completion (I). For (F) and (H), the large

379   filled data points represent the mean of an independent replicate, while the smaller

380   unfilled data points of the same shape indicate individual cells quantified on the same

381   day for that replicate. For (B) each dot represents an independent replicate composed

382   of at least 100 macrophages per condition; data was compared using an unpaired t test.

383   For (E)-(I) each independent replicate composed of at least 15 engulfment events. The

384   means from four independent replicates were compared using an unpaired t test. In all

385   graphs, bars denote the mean ± SEM. * denotes p<0.05, ** denotes p<0.005, ***

386   denotes p<0.0005. Scale bars are 10μm.

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388   **Figure 2: CD47 shifts primary mode of phagocytosis from reaching to sinking.** (A)

389   Timelapse images show reaching phagocytosis of an IgG bead target by a BMDM.

390   White arrowheads point to extension of phagocyte membrane (GFP-CAAX, green) out

391   around the target (atto647 lipid; magenta). White arrow denotes subsequent closure of

392   the phagocytic cup while the bead remains outside the phagocyte cortex. Images

393   correspond to Video S1 (B) Timelapse images show sinking phagocytosis of IgG+CD47

394   bead. Yellow arrows show closure of the phagocytic cup when the bead is within the

395   macrophage. Images correspond to Video S2. (C) Graph depicts the fraction of

396   successful phagocytosis that occurred via sinking phagocytosis based on the apparent

397   morphology in timelapse confocal microscopy data. (D) Graph depicts the position of

398   the bead centroid relative to the phagocyte cortex when phagocytosis was completed.

399   (E) Graph depicts the fraction of retracted reaching cups out of the total number of

400   initiated reaching cups. Retracted cups were defined as cases in our timelapse data set

401   where membrane extensions grew around the target but were subsequently

402   disassembled. (F) Images show an example of failed reaching phagocytosis of an

403   IgG+CD47 bead. White arrowheads highlight extension of the macrophage membrane,

404   which is subsequently retracted. Images correspond to Video S3. For (C) and (E), the

405   averages from four independent experiments were plotted and compared using an

406   unpaired t test. Bars represent the mean ± SEM. For (D), each data point represents an

407   individual bead (*n*=60 targets per condition from 4 independent experiments), and data

408   was compared using an unpaired t test. ** denotes $p<0.005$, *** denotes $p<0.0005$.

409   Scale bars are 10μm.

410

411   **Figure 3: CD47 targets distinct actin regulators.** (A, B) BMDMs were incubated with

412   IgG or IgG+CD47 beads then fixed and stained with 488 phalloidin (greyscale) to

413   visualize F-actin during phagocytosis. Enrichment of F-actin at late-stage (>50%

414   completed) phagocytic cup rims was measured by comparing the mean fluorescent

415   intensity (MFI) of phalloidin at the cup rim (red dot) to the MFI of phalloidin at the cell

416   cortex (blue line). Bead position is indicated with a dashed yellow line. (B) Graph

417   depicts data quantification described in (A). The large filled data points represent the

418   mean of an independent replicate, while the smaller unfilled data points of the same

419   shape indicate individual cells collected on that day for that replicate. Dashed line at 1

420   corresponds to no F-actin enrichment at cup rims. (C) BMDMs were treated with

421   pharmacological inhibitors of Rac and Cdc42 (NSC23766 and MBQ-167) or Rho (C3

422   transferase) for 24 hours, then incubated with IgG, IgG+CD47, or unopsonized

423   supported lipid bilayer coated beads. Phagocytosis was measured by confocal

424   microscopy. The average number of beads phagocytosed was normalized to the

20

425   maximum phagocytosis in that replicate. (D) BMDMs were treated with pharmacological

426   inhibitors of Rac and Cdc42 (NSC23766 and MBQ-167) or Rho (C3 transferase) for 24

427   hours, then the inhibitors were removed and replaced with fresh media. WT or CD47^{KO}

428   mouse L1210 leukemia cells were dyed with CellTrace Far Red then opsonized with an

429   anti-murine CD20 monoclonal antibody and added to the BMDMs. Phagocytosis was

430   monitored for 10 hours via timelapse microscopy. The percent of BMDMs that engulfed

431   was quantified, and data was normalized to maximum for that experiment. For (B), the

432   means of 4 independent experiments were compared using an unpaired t test. For (C)

433   and (D), each data point represents an independent experiment including quantification

434   of at least 100 macrophages. Data was compared using one-way ANOVA with Holm

435   Sidak multiple comparison test. In all graphs, bars represent the mean ± SEM. *

436   denotes $p < 0.05$, ** denotes $p < 0.005$, *** denotes $p < 0.0005$, **** denotes $p < 0.00005$.

437   Scale bars are 10µm.

438

439   **Figure 4: Hyperactive Rac bypasses CD47.** (A-E) WT or CD47^{KO} mouse L1210

440   leukemia cells were dyed with CellTrace Far Red (magenta) then opsonized with an

441   anti-murine CD20 monoclonal antibody and added to the BMDMs (green) from WT or

442   Rac2$^{E62K/+}$ mice. (A) Stills from a representative timelapse showing WT BMDMs

443   incubated with CD47^{KO} L1210 cells. Yellow arrow indicates phagocytosis. (B) Stills from

444   a representative timelapse showing Rac2$^{E62K/+}$ BMDMs incubated with CD47^{KO} L1210

445   cells. Yellow arrow indicates phagocytosis. (C) Stills from a representative timelapse

446   showing WT BMDMs incubated with CD47-positive WT L1210 cells. (D) Stills from a

447   representative timelapse showing Rac2$^{E62K/+}$ BMDMs incubated with CD47-positive WT

448   L1210 cells. Yellow arrow indicates phagocytosis. (E) Graphs show the phagocytosis of

449   L1210 cells during a 10 hour timelapse, normalized to the maximum phagocytosis on

450   each day. Data compared using one-way ANOVA with Holm Sidak multiple comparison

451   test. Line and bars denote mean and SEM. * denotes $p<0.05$, ** denotes $p<0.005$, ***

452   denotes $p<0.0005$. Scale bars are 10μm.

453

454   **Figure 5: CD47 inhibits IgG-mediated phosphorylation of Vav.** (A) BMDMs were

455   incubated with unopsonized (--), IgG, or IgG+CD47 beads for 10 minutes, then lysed

456   directly in 2X Laemmli sample buffer. Whole cell lysates were immunoblotted with the

457   indicated antibodies. Representative of $n = 3$ independent replicates. (B, C) The ratio of

458   phosphorylated Syk to total Syk or phosphorylated Vav1 to total Vav1 was quantified

459   and normalized to the IgG condition for that replicate. (D) BMDMs were incubated with

460   unopsonized (--), IgG, or IgG+CD47 beads for 10 minutes, then lysed in LB1 lysis

461   buffer. Vav1 was immunoprecipitated, then probed for tyrosine phosphorylation.

462   Representative of $n = 2$ independent immunoprecipitations. For (B) and (C), data was

463   compared with one-way ANOVA with Holm Sidak multiple comparison test. Error bars

464   denote SEM. * denotes $p<0.05$.

465

466   **Figure 6: Vav1 is key target of CD47-mediated inhibition of phagocytosis.** (A) TIRF

467   images show IgG (AlexaFluor 488-IgG, green) and mCherry-Vav1 (magenta) as

468   BMDMs interact with an IgG (top) or IgG+CD47 (bottom) bilayer. The linescan shows

469   the fluorescent intensity of AlexaFluor 488-IgG and mCherry-Vav1 at the indicated

470   position (white arrow in images). Intensity was normalized so that 1 is the highest

471   observed intensity and 0 is the lowest observed intensity. (B) The mean fluorescent

472   intensity of mCherry-Vav1 at IgG clusters (left) and the area of the IgG clusters (right)

473   was measured for n=30 cells landing on IgG or IgG+CD47 bilayers, pooled from 3

474   independent experiments. (C) Images from TIRF microscopy timelapse show IgG

475   (black) clustering as BMDMs land and spread on a bilayer with IgG (top) or IgG+CD47

476   (bottom). Graph depicts the average area of contact from n≥16 cells pooled from 3

477   separate experiments. (D) The Pearson's Correlation Coefficient was calculated for

478   mCherry-Vav1 and AlexaFluor 488-IgG in the footprint of n≥38 cells from 3 separate

479   experiments landing on IgG or IgG+CD47 bilayers. (E) BMDMs expressing membrane-

480   tethered mCherry (mCherry-CAAX) or a hyperactive Vav1 mutant (mCherry-Vav3F)

481   were incubated with unopsonized (--), IgG, or IgG+CD47 beads for 30 minutes, then

482   visualized via confocal microscopy. The average number of beads engulfed per

483   macrophage was quantified and normalized to the highest average for that experiment.

484   Points denote averages from each of the 3 replicates comprising at least 100

485   macrophages. (F) Diagram shows the proposed pathway for CD47 signaling. Data was

486   compared using an unpaired t test (B, D) or one way ANOVA with Holm Sidak multiple

487   comparison test. (E). Line and error bars denote mean and SEM.* denotes p<0.05, **

488   denotes p<0.005, *** denotes p<0.0005. Scale bars are 10μm.

Supplemental figure legends

Video S1: Reaching phagocytosis of an IgG bead, related to Figure 2.

Representative video of a BMDM (green) engulfing an IgG bead (magenta) via reaching

phagocytosis. Images are maximum projections of a confocal z-stack taken every 15

seconds. Scale bar is 10μm.

Video S2: Sinking phagocytosis of an IgG+CD47 bead, related to Figure 2.

Representative image of a BMDM (green) engulfing an IgG+CD47 bead (magenta) via

sinking phagocytosis. Images are maximum projections of a confocal z-stack taken

every 15 seconds. Scale bar is 10μm.

Video S3: Failed reaching phagocytosis of IgG+CD47 bead, related to Figure 2.

Representative image of a BMDM (green) attempting to engulf an IgG+CD47 bead

(magenta) via reaching phagocytosis. IImages are maximum projections of a confocal z-

stack taken every 15 seconds. Scale bar is 10μm.

**Video S4: Rac2$^{E62K/+}$ macrophage phagocytoses a wild type L1210 cell, related to
Figure 4.**

Representative video of a BMDM (green) phagocytosing a L1210 cell (CellTrace Far

Red; magenta). Images were taken every 3 minutes. Scale bar is 10μm.

Supplementary Figure 1: Validation of Rho GTPase inhibitors, related to Figure 3.

To validate that the GTPase inhibitors used in Figure 3 had the expected effects on

25

512   macrophage actin dynamics, BMDMs were incubated for 24 hours in DMEM

513   (untreated), or DMEM supplemented with 1mM NSC23766, 500nM MBQ-167, or

514   0.5µg/mL C3 transferase, then fixed and stained with GFP-conjugated phalloidin.

515   NSC23766 and MBQ-167 had similar effects on macrophage morphology, as both

516   induced cell rounding, shrinkage, and reduction of lamellipodia and cell polarity,

517   characteristic of Rac inhibition. On the other hand, C3 transferase induced loss of stress

518   fibers, protrusion of dendritic extensions and collapse of the cell body, as expected with

519   RhoA inhibition. Scale bars are 20µm.

520

521   **Supplementary Figure 2: Validation of CD47 knockout cell line, related to Figure 3**

522   To validate knockout of CD47 in L1210s, WT and CD47^{KO} L1210s were stained with

523   APC-conjugated anti-CD47 and isotype control antibodies. To the left, histograms with

524   APC signal are shown. To the right, mean fluorescent intensity of APC signal in the

525   stained populations is graphed.

526

527   **Supplementary Figure 3: Uncropped Western blots, related to Figure 5:** (A)

528   Uncropped Syk and phospho-Syk blots used in Figure 5A. (B) Uncropped total Vav and

529   phospho-Vav blots used in Figure 5A. (C) To validate efficacy of Vav

530   immunoprecipitation, the following samples were collected, run on an SDS-PAGE gel

531   and probed for total Vav: whole cell emulsion, cell pellet, cell lysate (input used for IP),

532   flow through, first wash, and elution (containing immunoprecipitated Vav). To the right,

533   uncropped blots used in Figure 5D are shown.

534

535 **Supplementary Figure 4: Vav3F has morphological effects consistent with Vav1**

536 **activation, related to Figure 6**

537 Two representative BMDMs expressing Vav3F show distinct morphology, characteristic

538 of Vav1 hyperactivation. Vav3F-expressing cells are more spread and have increased

539 lamellipodia and ruffles, as shown previously[78]. Vav3F-expressing cells also have large

540 numbers of macropinosomes, as expected with increased Vav-dependent

541 macropinocytosis. Scale bars are 20μm.

542 **Methods**

543 **Key Resources Table**

544 **Resource Availability**

545 Lead Contact

546 Further information and requests for reagents and resources should be directed to and

547 will be fulfilled by the Lead Contact, Meghan Morrissey (morrissey@ucsb.edu)

548 Materials Availability

549 Plasmids generated in this study have been deposited to Addgene or can be obtained

550 from Lead Contact.

551 Data and Code Availability

552 Complete imaging datasets are available from the lead contact upon request. Any

553 additional information required to reanalyze the data reported in this paper is also

554 available from the lead contact upon request.

555 **Experimental Model and Study Participant Details**

556 Cell lines

557 L1210 cells were obtained from the UCSF cell culture facility and certified mycoplasma

558 and pathogen free (IMPACT III test, IDEXX BioAnalytics). The cells were cultured in

559 DMEM (GIBCO, Catalog #11965-092) supplemented with 1% Pen-Strep-Glutamine

28

560   (Corning, Catalog #30-009 CI) and 10% heat-inactivated fetal bovine serum (Atlanta

561   Biologicals, Catalog #S11150H) at 37°C. Lenti-X 293T cells (Takara Biosciences, Cat#

562   632180) were cultured in DMEM, 10% FBS, 1% PSG media at 37°C.

563   ## Bone-marrow derived macrophage cell culture

564   Six- to ten-week old male and female C57BL/6 mice were sacrificed by CO_2 inhalation.

565   Hips and femurs were dissected and bone marrow was harvested as previously

566   described[97]. Macrophage progenitors were differentiated for seven days in RPMI-1640

567   (GIBCO, Catalog # 72400120) supplemented with 10% heat-inactivated fetal bovine

568   serum, 1% Pen-Strep-Glutamine, and 20% L929-conditioned media. Macrophage

569   differentiation was confirmed by flow cytometry identifying CD11b and F4/80 double

570   positive cells. Differentiated BMDMs were used for experiments from days 7 to 11.

571   **Method Details**

572   ## Lentivirus production and infection

573   All constructs were expressed in either BMDMs or L1210s using lentiviral infection.

574   Lentivirus was produced in Lenti-X 293T cells transfected with pMD2.g (gift from Didier

575   Trono, Addgene plasmid #12259 containing VSV-G envelope protein), pCMV-dR8.2 (gift

576   from Bob Weinberg, Addgene plasmid #8455)[98], and a lentiviral backbone containing

577   the construct of interest using lipofectamine LTX (Invitrogen, Cat #15338-100). The

578   media was harvested 72 h post-transfection, filtered through a 0.45 μm filter (Millapore,

579   Cat# SLHVM33RS) and concentrated using LentiX (Takara Biosciences, Cat# 631232).

580 For BMDMs, concentrated lentivirus was added to cells on day 4 of differentiation, and

581 cells were analyzed between days 7-11.

582 Supported lipid bilayer assembly

583 SUV preparation

584 For beads, the following chloroform-suspended lipids were mixed and desiccated

585 overnight to remove chloroform: 95.3% POPC (Avanti, Cat# 850457), 2.5% biotinyl cap

586 PE (Avanti, Cat# 870273), 2% DGS-NTA (Avanti, Cat# 790404), 0.1% PEG5000-PE

587 (Avanti, Cat# 880230), and either 0.1% atto390-DOPE (ATTO-TEC GmbH, Cat# AD

588 390-161) or 0.1% atto647-DOPE (ATTO-TEC GmbH, Cat# AD 647–151). For planar

589 bilayers, the following chloroform-suspended lipids were mixed and desiccated

590 overnight to remove chloroform: 97.8% POPC (Avanti, Cat# 850457), 1% biotinyl cap

591 PE (Avanti, Cat# 870273), 1% DGS-NTA (Avanti, Cat# 790404), 0.1% PEG5000-PE

592 (Avanti, Cat# 880230), and 0.1% atto647-DOPE (ATTO-TEC GmbH, Cat# AD 647–

593 151). The lipid sheets were resuspended in PBS, pH7.2 (GIBCO, Cat# 20012050) at

594 10mM concentration and stored under inert nitrogen gas. The lipids were broken into

595 small unilamellar vesicles via several rounds of freeze-thaws. The lipids were then

596 stored at -80°C under nitrogen. To remove aggregated lipids, the solution was diluted to

597 2 mM and filtered through a 0.22 uM filter (Millapore, Cat# SLLG013SL) immediately

598 prior to use.

30

599 Planar bilayer preparation for TIRF microscopy

600 Ibidi coverslips (Cat# 10812) were piranha cleaned. Supported lipid bilayers were

601 assembled in custom PDMS (Dow Corning, cat# 3097366-0516 and 3097358-1004)

602 chambers at room temperature for 1 h. Assembled bilayers were washed 6x with PBS,

603 then blocked with 0.2% casein (Sigma, cat# C5890) in PBS for 15 minutes. Anti-biotin

604 IgG (Jackson ImmunoResearch Laboratories Cat# 200-002-211) was added at 1 nM,

605 and 10x-His tagged CD47 (Sino Biological, Cat# 57231-M49H-B) was added at 10 nM.

606 Proteins were coupled to the bilayer for 45 min. Bilayers were then washed 4x with

607 0.2% casein in PBS, then 2x with serum-free RPMI at RT. Imaging was conducted in

608 serum-free RPMI at RT. Bilayers were assessed for mobility by either photobleaching or

609 monitoring the mobility of single particles.

610 Bead preparation

611 Silica beads with a 5.01 µm diameter (9.79% solids, Bangs Laboratories, Cat#

612 SS05003, Lot #16595) were washed several times with PBS, mixed with 1mM SUVs in

613 PBS and incubated at room temperature for 30 min - 2 hrs with end-over-end mixing to

614 allow for bilayer formation. Beads were then washed with PBS to remove excess SUVs

615 and incubated in 0.2% casein (Sigma, Cat# C5890) in PBS for 15 min before protein

616 coupling. Anti-biotin IgG (Jackson ImmunoResearch Laboratories Cat# 200-002-211)

617 was added at 1 nM, and 10x-His tagged CD47 (Sino Biological, Cat# 57231-M49H-B)

618 was added at 10 nM. We estimated the amount of IgG coupling to the beads by

619 comparing the fluorescence of AlexaFluor-647 IgG to calibrated fluorescent beads

620 (Quantum AlexaFluor647, Bangs Lab) and measured 200-360 molecules/um^2 [99]. The

621 CD47 density was selected to give a consistent, strong suppression of phagocytosis.

622   Proteins were coupled to the bilayer for 30 min at room temperature with end-over-end

623   mixing.

624   Phagocytosis assays

625   Bead engulfment

626   50,000 BMDMs were plated per well in a 96-well glass bottom MatriPlate (Brooks, Cat#

627   MGB096-1-2-LG-L) between 24-48 h prior to the experiment. ~8 × 10^5 beads were

628   added to wells and engulfment was allowed to proceed for 30 min. The cells were

629   imaged using spinning disc microscopy (40 × 0.95 NA Plan Apo air). Internalized

630   particles were identified by their fluorescent supported lipid bilayer, and counted in

631   ImageJ by a blinded analyzer using Blind-Analysis-Tools-1.0 ImageJ plug in[61]. For each

632   well, at least 100 macrophages were scored. When Rho GTPase inhibitors were used,

633   BMDMs plated 24 h earlier were incubated with either 1mM NSC23766 (APExBIO, Cat#

634   A1952), 500nM MBQ-167 (MedChemExpress, Cat# HY-112842), or 0.5ug/mL C3

635   Transferase (Cytoskeleton, Cat# CT04-A) for 24 hours before beads were added to

636   cells.

637   L1210 engulfment

638   40,000 WT or Rac2$^{+/E62K}$ BMDMs were plated per well in a 96-well glass bottom plate

639   24-48 hours prior to the experiment. WT or CD47^{KO} L1210 cells were dyed with

640   CellTrace Far Red (Thermo, Cat# C34572), incubated with an anti-mouse CD20

641   antibody (Genentech, clone 5D2) at 5ug/mL, then added to wells at 80,000 cells per

642   well and imaged every 3 min for 10 hours. For each well, at least 100 macrophages

643 were scored by a blind analyzer. Phagocytic macrophages were characterized as

644 BMDMs that engulfed 1 or more whole, viable L1210 cell targets. When Rho GTPase

645 inhibitors were used, BMDMs plated 24 h earlier were incubated with either 1mM

646 NSC23766 (APExBIO, Cat# A1952), 500nM MBQ-167 (MedChemExpress, Cat# HY-

647 112842), or 0.5ug/mL C3 Transferase (Cytoskeleton, Cat# CT04-A) for 24 hours.

648 Inhibitors were then washed out before adding L1210 cells.

Kinetics of engulfment

650 GFP-CAAX expressing BMDMs were plated as described in the bead engulfment assay

651 24-48 hours prior to the experiment. Using ND acquisition in Elements, 2-3 positions per

652 well were manually selected. Approximately 4×10^5 beads were added and

653 phagocytosis was imaged at 15 s intervals with 1um z-steps for 30 min. Bead binding

654 was determined by counting the number of beads that came into and remained in

655 contact with the cells throughout the imaging time, and is shown as a percentage of

656 total beads. Initiation was identified by the frame in which the process of bead

657 internalization began, indicated by membrane deforming or extending around the bead.

658 Engulfment completion was identified by complete internalization of the bead by the

659 macrophage. The initiation time was quantified as the amount of time between bead

660 contact (the first frame in which the bead contacted the macrophage) and engulfment

661 initiation (the first frame in which bead internalization was visualized) and was only

662 measured for beads that were completely internalized by the end of the imaging time.

663 The engulfment time was quantified as the amount of time between engulfment initiation

664 and engulfment completion (the first frame in which the bead has been fully internalized

665 by the cell). In Fig. 2C, an engulfment event was classified as reaching phagocytosis if

666   the target was engulfed via membrane protrusions that extended away from the cell

667   body of the macrophage, out around the target. Alternatively, an engulfment event was

668   classified as sinking phagocytosis if the target was slowly pulled into the cell, in the

669   absence of any such membrane protrusions. Analysis was blinded using Blind-Analysis-

670   Tools-1.0 ImageJ plug in[61].

TIRF imaging

672   After assembling bilayers in TIRF chambers as described earlier, BMDMs were

673   removed from their culture dish using 5% EDTA in PBS and resuspended in serum-free

674   RPMI before being added to the TIRF chamber for imaging.

Quantification of IgG clusters and Vav1 recruitment

676   After 15 min of interacting with the bilayer, cells that had spread on the bilayer surface

677   were selected for analysis. Otsu thresholding in ImageJ was used to select IgG clusters

678   in an unbiased manner. This selection was used to generate an ROI that was then

679   applied to the Vav-mCherry channel. The area of the ROI (area of IgG clusters) and the

680   mean Vav1 intensity within that ROI were measured.

Pearson's Correlation Coefficient

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

683   correlation coefficient between AlexaFluor488-IgG and Vav-mCherry was measured

684   using the JaCoP plugin[100].

Quantification of cell-bilayer contact area

686 For 6C the area of the cell contacting the bilayer was traced in ImageJ beginning with

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

688 included.

Measuring actin recruitment to cup rims

690 50,000 BMDMs were plated per well in a 96-well glass bottom MatriPlate between 24-

691 48 h prior to the experiment. ~8 × 10^5 beads were added to wells and engulfment was

692 allowed to proceed for 15 minutes. Cells were then fixed with 4% PFA for 10 minutes,

693 permeabilized with 0.1% Triton-X, and stained with 14nM acti-stain 488 phalloidin

694 (Cytoskeleton, Cat# PHDG1). Cups that were between 50-100% complete were

695 analyzed.

Generation and validation of CD47^{KO} L1210 cell line

697 To generate stable L1210 CD47^{KO} lines, WT L1210s were infected with a 3rd

698 generation lentiviral backbone encoding Cas9[101] and sgRNAs (sequence:

699 GATAAGCGCGATGCCATGG) targeting the 2nd exon of mouse CD47. Single cells

700 expressing Cas9 and guide RNAs were sorted for monoclonal populations. To validate

701 knockout of CD47, surface expression of CD47 was assessed via FACS (Fig. S1).

702 Single-cell sorted monoclonal populations, as well as WT L1210s, were washed 2x with

703 FACS blocking buffer (PBS, 0.5% BSA, 2mM EDTA), incubated in blocking buffer on ice

704 for 10 minutes, then incubated with an APC-conjugated anti-CD47 antibody (BioLegend,

705 cat# 127514) or an APC-conjugated isotype control antibody (BioLegend, cat# 400511)

706   at 10 ug/mL for 30 minutes on ice in the dark. Cells were then washed 2x with blocking

707   buffer and analyzed using an Attune NxT (Invitrogen).

708   Immunoblotting

709   ~1.6×10^7 beads were added to 1 million BMDMs in one well of a 6-well plate, then 10

710   minutes later cells were washed with PBS and lysed directly in culture plates with 100ul

711   2X Laemmli sample buffer containing 2-mercaptoethanol. Cells were scraped and

712   collected, then boiled for 3 minutes at 95°C and sonicated for 30sec at 50% amplitude

713   (Branson). Proteins were resolved on 4-20% SDS-PAGE gels (Bio-Rad #4561095)

714   before being transferred to 0.45 μm LF PVDF membranes using a wet-transfer method

715   with Towbin transfer buffer (192 mM Glycine, 25 mM Tris-base, 20% methanol).

716   Membranes were blocked in 3% BSA (Fisher Scientific #BP1605-100) in TBST (0.1%

717   Tween-20) for 1 hour, then probed with desired primary antibody in 3% BSA in TBST

718   overnight at 4°C with gentle agitation. The following day, membranes were washed 3x5

719   minutes with TBST before probing with species-specific HRP-conjugated secondary

720   antibodies (Bio-Rad, Cat# 1706515) dissolved in 3% BSA in TBST for 1 hour.

721   Membranes were then washed 3x5 minutes with TBST, followed by one wash with TBS

722   before visualization of chemiluminescence using either Pierce ECL2 Western Blotting

723   Substrate (Thermo Scientific #PI80196) or Pierce SuperSignal West Femto Substrate

724   (Thermo Scientific #34095) on a ChemiDoc MP imaging system (Bio-Rad). The

725   antibodies used for immunoblotting in this study are as follows: anti-Vav1 (CST,

726   Cat#2502S), anti-phosphoVav1 (Abcam, Cat#ab76225), anti-Syk (CST, Cat#13198),

727   anti-phosphoSyk (CST, Cat#2717), anti-phosphoTyrosine (CST, Cat#8954).

36

Immunoprecipitation

729   ~8 × 10^7 beads were added to 10-15 million BMDMs in a 10cm plate, then 10 minutes

730   later cells were washed with PBS and lysed with buffer LB1 (50 mM HEPES pH 7.6,

731   100 mM NaCl, 50 mM KCl, 10 mM MgCl$_2$, 0.5 % IPEGAL CA-630, 0.1% Na-

732   deoxycholate, 2 mM Na$_3$VO$_4$, and 20 mM NaF) containing 1X protease inhibitor

733   (ThermoFisher, Cat# 78430), benzonase (EMD-Millipore, Cat# 101697), and 0.5 mM

734   EDTA directly in 10cm plates. Cells were scraped and collected. Cells were incubated

735   on ice for 15 minutes, passed 5x through a 25G hypodermic needle to shear cells and

736   genomic DNA, then incubated another 15 minutes on ice with mixing by inversion. The

737   resulting lysate was then spun 15,000g x 20 minutes at 4°C and the supernatant was

738   collected. Lysate was then pre-cleared with Protein A agarose beads (Cell Signaling

739   Technology, Cat# 9863S), that were pre-washed twice in LB1, for 30 minutes at 4°C to

740   reduce non-specific binding. Beads were then spun down 5000g x 1 min and

741   supernatant collected. 0.7 ug of Vav1 antibody (CST, Cat# 2502S) was added directly

742   to the lysate and incubated for 2 hours at 4°C with gentle inversion. Protein A agarose

743   slurry, that was pre-washed twice in buffer LB1, was added to each lysate + antibody

744   mix and incubated for another 2 hours at 4°C with gentle inversion. The protein A

745   agarose was then washed four times with buffer LB1 and bound proteins were eluted

746   with elution buffer (0.2 M glycine pH 2.5) by incubating for 10 minutes shaking at 1100

747   rpm at 4°C. Beads were spun 5000g x 1 min and eluates were collected. The elution

748   was performed twice and pooled together. To neutralize the pH, Tris-Cl pH 8.5 was

749   added to a final concentration of 100 mM, followed by addition of 2X Laemmli sample

750   buffer with 2-mercaptoethanol to prepare samples for SDS-PAGE.

Microscopy and analysis

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

inverted microscope with a Yokogawa spinning disk unit and an Orca Fusion BT scMos

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

The microscope is also equipped with a piezo Z drive and an OkoLabs stage top

incubator for temperature, CO_2 and humidity control. TIRF imaging was performed with

an iLas2 ring TIRF on the same microscope base and same camera. The microscope

was controlled using Nikon Elements.

Quantification and Statistical Analysis

Statistical analysis was performed in Prism 8 (GraphPad). The statistical test used is

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

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

either manual renaming of the files or the Blind-Analalysis-Tools-1.0 ImageJ plug in.

The details of each quantification method and blinding strategy are included in the

Methods section.

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773   **Author contributions**

774   Conceptualization, W.D.M., M.A.M.; Methodology, W.D.M., A.K.M., C.J.S. M.A.M.;

775   Validation, W.D.M.; Formal Analysis W.D.M.; Investigation, W.D.M., A.K.M., C.J.S., AB;

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779   **Declaration of interests**

780   The authors declare no competing interests.

781   **Supplemental Information**

782   Document S1. Figures S1-S3

783   Video S1. Reaching phagocytosis of IgG bead, related to Figure 2

784   Video S2. Sinking phagocytosis of IgG+CD47 bead, related to Figure 2

785   Video S3. Failed reaching phagocytosis, related to Figure 2

786   Video S4: $Rac2^{E62K/+}$ macrophage phagocytoses a wild type L1210 cell, related to

787   Figure 4.

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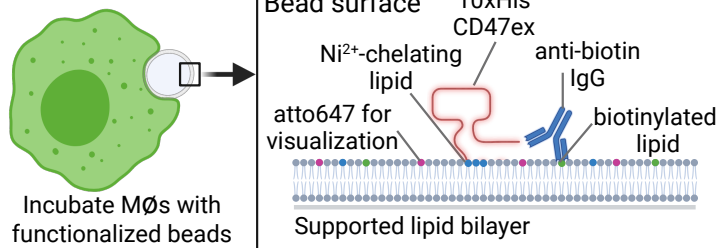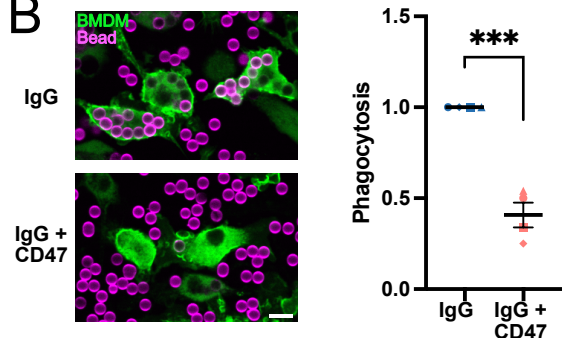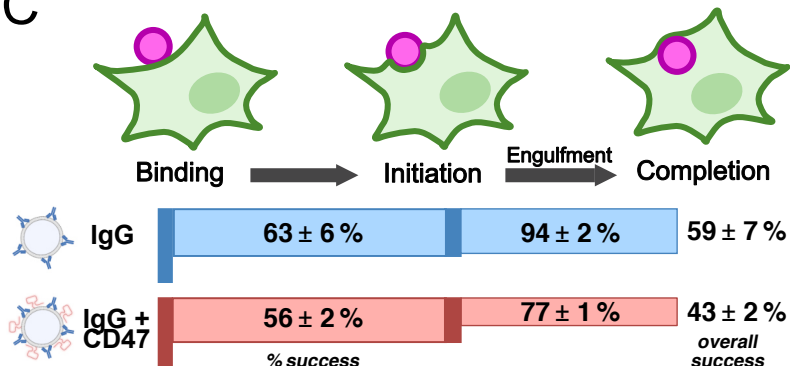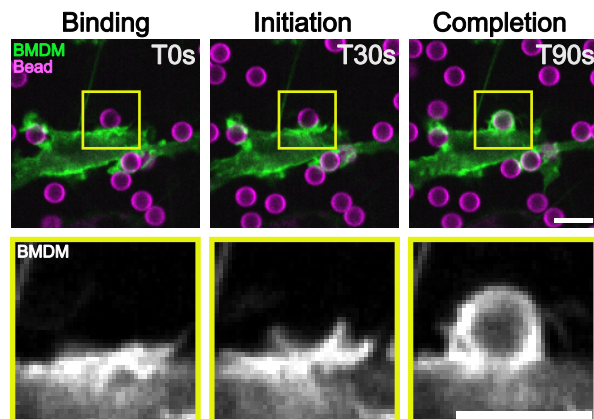948   64. Patel, J. C., Hall, A. & Caron, E. Vav Regulates Activation of Rac but Not Cdc42

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A Incubate MØs with functionalized beads

Bead surface

10xHis CD47ex

Ni²⁺-chelating lipid

anti-biotin IgG

atto647 for visualization

biotinylated lipid

Supported lipid bilayer

B IgG / IgG + CD47 — BMDM (green) / Bead (magenta); Phagocytosis ***

C Binding → Initiation → (Engulfment) → Completion

IgG: 63 ± 6 % | 94 ± 2 % | 59 ± 7 %

IgG + CD47: 56 ± 2 % | 77 ± 1 % | 43 ± 2 %

% success | overall success

D Binding T0s / Initiation T30s / Completion T90s — BMDM (green) / Bead (magenta)

E Binding probability — Fraction bound — ns

F Initiation time — Time (s) — ns

G Initiation probability — Fraction initiated from bound — ns
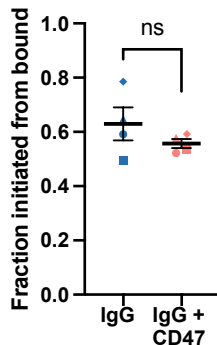
H Engulfment time — Time (s) — **
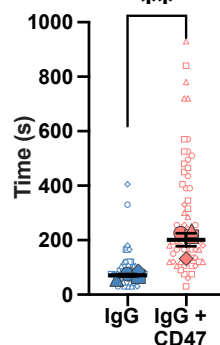
I Completion probability — Fraction completed from initiated — ***

A IgG

BMDM
Bead

T0s | T15s | T30s | T60s | T135s

B IgG+CD47

T0s | T60s | T225s | T450s | T700s

C

Frequency of sinking phagocytosis

***

IgG   IgG + CD47

D

Membrane
Target (centroid)
Cell cortex
Relative position to cortex

Relative position to cortex at completion (um)

****

IgG   IgG + CD47

E

Retracted reaching cups / total reaching cups

**

IgG   IgG+ CD47

F IgG+CD47

T0s | T90s | T120s | T330s | T360s

A

F-actin rim / F-actin cortex

IgG | IgG+CD47

F-actin

B

MFI at rim/cortex

IgG | IgG + CD47

*

C

Bead Phagocytosis

Phagocytosis

Bead target:	IgG	IgG+ CD47	--	IgG	IgG+ CD47	--	IgG	IgG+ CD47	--	IgG	IgG+ CD47	--
BMDM treatment:	*Untreated*			*NSC23766*			*MBQ-167*			*C3 Transferase*		
				Rac inhibitors						Rho inhibitor		

*** *** *** **** ****

D

L1210 Cell Phagocytosis

Phagocytosis

L1210 target:	CD47^{KO}	WT	CD47^{KO}	WT	CD47^{KO}	WT	CD47^{KO}	WT
BMDM treatment:	*Untreated*		*NSC23766*		*MBQ-167*		*C3 Transferase*	
			Rac inhibitors				Rho inhibitor	

*** *** **** **** *

A WT BMDMs + CD47^KO L1210s
BMDM / L1210
0 min   3 min   6 min   12 min   60 min

B Rac2^E62K/+ BMDMs + CD47^KO L1210s
BMDM / L1210
0 min   3 min   6 min   12 min   60 min

C WT BMDMs + WT L1210s
BMDM / L1210
0 min   18 min   36 min   60 min   120 min

D Rac2^E62K/+ BMDMs + WT L1210s
BMDM / L1210
0 min   6 min   12 min   21 min   60 min

E

Phagocytosis

L1210: CD47^KO   WT   CD47^KO   WT
BMDM: WT   Rac2^E62K/+

ns
**   **
**

A

Whole cell lysates

| | -- | IgG | IgG+CD47 |

α-pSyk
α-Syk
α-pVav1
α-Vav1

B

Phospho-Syk / Total Syk

C

Phospho-Vav1 / Total Vav1

D

Input

α-Vav1

IP: anti-Vav1

α-Vav1

α-pTyr

A TIRF

IgG bilayer: IgG (green), Vav (magenta), merge with inset

Linescan: IgG intensity (green), Vav intensity (magenta)

IgG + CD47 bilayer: IgG (green), Vav (magenta), merge with inset

Linescan: IgG intensity (green), Vav intensity (magenta)

B

Vav recruitment to IgG clusters — Vav MFI at IgG clusters (au); IgG vs IgG+CD47; ns

IgG cluster sizes — Area of clustered IgG (µm²); IgG vs IgG+CD47; ns

C TIRF

IgG bilayer; IgG+CD47 bilayer; 40s, 80s, 120s, 160s

Area of cell-bilayer contact (um) vs seconds; IgG; IgG+CD47

D

Colocalization of Vav with IgG — Pearson's Correlation Coefficient of IgG:Vav; IgG vs IgG+CD47; ***

E Hyperactive Vav phagocytosis

Phagocytosis; ns; **; ns

Beads: IgG, IgG+CD47, --; BMDMs: mCherry, Vav3F

F

CD47 → SIRPα → SHP1 ⊣ Vav

IgG → FcR → Syk

Syk → Vav → Rac → Reaching phagocytosis

Syk → GEF? → Rho → Sinking phagocytosis