Signal regulatory protein α (SIRPα, SHPS-1) is a plasma membrane receptor for CD47 and a key regulator of phagocytosis, growth factor signaling, and migration. Phosphorylation of immunoreceptor tyrosine-based inhibition motifs in its cytoplasmic tail is essential for the functional effects of SIRPα, at least in part, because the phosphorylated immunoreceptor tyrosine-based inhibition motifs recruit Src homology 2 domain-containing tyrosine phosphatases. Ligation by CD47 and integrin engagement both have been thought to regulate SIRPα phosphorylation. However, their distinct contributions have not been distinguished. Here, we show that the importance of CD47 varies with cell type, since ligation of CD47 is not necessary for SIRPα phosphorylation in myeloid cells, whereas it is required in endothelial cells. In contrast, integrin-mediated adhesion is required for SIRPα phosphorylation in both cell types. This shows that SIRPα phosphorylation is dually regulated and demonstrates a new mechanism for functional cooperation between integrins and the integrin-associated protein CD47.

Cell adhesion is an important modulator of phenotype, as contact with extracellular matrix and with adjacent cells modulates responses to proliferative, apoptotic, migratory, phagocytic, and other fundamental cues. Often, these adhesion signals result from ligation of the heterodimeric integrin family of cell surface receptors (1, 2). The integrin contribution to signaling frequently involves activation or amplification of tyrosine kinase cascades that may integrate with other signaling pathways (1, 3, 4). In some cell types, such as endothelial cells, signals from adhesion to extracellular matrix are tightly integrated with cell-cell adhesion signals mediated by members of the cadherin or immunoglobulin superfamilies (5, 6).

Signal regulatory protein α (SIRPα, SHPS-1) is a plasma membrane protein that, like integrins, can modulate cellular responses to growth factors and other soluble signaling molecules (7–10). Its effects on signaling depend on its cytoplasmic tail (11), which contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs).2 When phosphorylated, primarily by Src family kinases (12), the SIRPα ITIMs recruit and activate the Src homology domain 2-containing phosphatases -1 (SHP-1) and -2 (SHP-2) (7, 10, 13, 14). These phosphatases often counteract the effects of tyrosine kinases activated by growth factors, resulting in negative regulation of the proliferative signal (7). Similarly, recruitment of SHP-1 to the SIRPα cytoplasmic tail negatively regulates phagocytosis initiated by Fcγ receptor signaling through the tyrosine kinase Syk in macrophages and other immune cells (15–19). In smooth muscle cells, phosphorylation of SIRPα and subsequent recruitment of SHP-2 in response to IGF-1 is enhanced by its ligand, CD47, but this step promotes rather than dampens IGF-1 signaling (9, 20–22). Thus, the mechanisms for regulation of SIRPα phosphorylation are of considerable interest, since they regulate potent signaling pathways in a variety of cells.

Integrin-mediated adhesion has been shown to promote SIRPα phosphorylation (12, 23, 24), as has transcellular interaction with CD47 (16). One major caveat to these studies is that CD47 and integrins regulate each other’s signaling (25–27). Since CD47 is ubiquitously expressed and was present on all cells used in the previous studies, it is not possible to distinguish the specific contribution of integrin signaling from integrin-CD47 cooperative signaling or even to determine whether integrin ligation simply led to SIRPα-CD47 interactions in these previous reports. Indeed, other studies with antibodies and in mice and cells lacking CD47 have led to the hypothesis that interaction with CD47 is required for SIRPα phosphorylation and recruitment of SHP-1 and SHP-2 phosphatases (9, 16).

In this work, we have used cells genetically deficient in CD47 to dissect the contributions of integrin ligation and interaction with CD47 for SIRPα phosphorylation in leukocytes and endothelial cells. We show that in leukocytes, integrin ligation is necessary and sufficient for SIRPα phosphorylation, which is substantial, even in the complete absence of CD47 expression. CD47 ligation of SIRPα amplifies the signal, but is not required. In endothelial cells, integrin-mediated adhesion also is necessary, but amplification by CD47-SIRPα interaction has a much more dramatic effect on SIRPα phosphorylation and phosphatase recruitment than in leukocytes. Indeed, in these cells, in

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2 The abbreviations used are: ITIM, immunoreceptor tyrosine-based inhibition motif; FN, fibronectin; BMDM, bone marrow derived macrophage(s); MLEC, murine lung endothelial cell(s); AJ, adhesin junction; SHP, Src homology domain 2-containing phosphatase; M-CSF, macrophage colony-stimulating factor; PBS, phosphate-buffered saline; FBS, fetal bovine serum; VE-cadherin, vascular endothelial cadherin.

M. J. dedicates this paper to her beloved B. and S. J. who are greatly missed.

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contrast to leukocytes, SIRPα phosphorylation is almost undetectable without ligation by CD47. Thus, SIRPα phosphorylation requires integrin signaling in all cell types, whereas CD47 appears to be a costimulator of SIRPα phosphorylation whose importance depends upon cell type.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Human fibronectin was obtained from In Vitrogen, human vitronectin was from EMDBiosciences, Inc. (La Jolla, CA), collagen I (“Purecol”) was from Inamed Biomaterials (Fremont, CA), and the poly(RGD) substrate was obtained from BIOSOURCE (Camarillo, CA). (“ProNectin F”) or from Sigma (“fibronectin-like engineered protein polymer”). Fatty-acid free bovine serum albumin and gelatin were also obtained from Sigma.

Antibodies to CD47 (clones miap301 and miap420), the antibody to SIRPα (P84), and a control antibody (KLHR2A) were purified from hybridoma supernatants using conventional methods and have been described previously (28–30). For integrin blocking experiments, anti-β1 (clone Ha2/5), anti-αv (clone H9.2B8), and appropriate isotype controls were purchased from BD Biosciences (San Jose, CA). Anti-β2 (clone 5C6) was purchased from AbD Serotec (Raleigh, NC). Antibodies to intracellular cell adhesion molecule 2 (clone 3C4(IC2/4)), platelet-endothelial cell adhesion molecule (clones MEC13.3 and 390), and vascular endothelial cadherin (VE-cadherin) (clone 11D4.1) were purchased from BD Biosciences. Anti-platelet-endothelial cell adhesion molecule clone 390 also was the generous gift of S. M. Albelda (University of Pennsylvania Medical Center). Hybridomas for the antibodies MECA32 and endoglin were purchased from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). The antibody to JAM-A (clone BV11) was the kind gift of E. Dejana (Milan, Italy). Antibodies used for Western blotting included anti-phosphotyrosine (clone 4G10) and a rabbit polyclonal antibody to the cytoplasmic tail of SIRPα, both obtained from Millipore (Billerica, MA), as well as mouse anti-SHP-2 (clone 79) purchased from BD Biosciences. Antibodies to α-, β-, and γ-catenin were purchased from Zymed Laboratories Inc. (South San Francisco, CA), anti-β-actin was from Cell Signaling Technologies (Danvers, MA), and anti-vinculin was from Sigma. Secondary antibodies used for blotting were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and Caltag Laboratories (Burlingame, CA). The appropriate anti-rat, anti-rabbit, and anti-mouse Alexa-488- and Alexa-594-coupled secondary antibodies along with Alexa-647-coupled phalloidin were purchased from Invitrogen for immunofluorescence microscopy experiments. The inhibitory cyclic peptide cyclo(Arg-Gly-Asp-D-Phe-Val) along with a control peptide, cyclo(Arg-Ala-Asp-D-Phe-Val), were purchased from Peptides International (Louisville, KY) and used at 100 μM to assess αv integrin function in some experiments.

Plating onto Mouse CD47-Fc—The mouse CD47-Fc chimera was made using conventional methods by replacing the human IgV domain of CD47 in plasmid pLAP412 (31) with a PCR fragment containing the murine IgV domain (piap369). The resulting construct contains the human leader sequence and the first 13 amino acids of the mature human CD47 protein, the Cys at position 14 mutated to Ser, followed by 102 amino acids in the murine IgV domain and the hinge and constant regions of human IgG1 heavy chain. This soluble protein was purified with Gammabind G-Sepharose (GE Healthcare, Chalfont St. Giles, UK) from the supernatant of 293 cells transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. The concentration of mouse CD47-Fc was determined using a Pierce BCA™ protein assay. Murine CD47-Fc bound to murine SIRPα on BMDM, as demonstrated by flow cytometry and shown previously by others (32).

Tissue culture dishes were incubated with poly-L-lysine (Sigma) and coated with mouse CD47-Fc or human IgG essentially as described (33), following which they were incubated with mouse lung endothelial cells (MLEC) in Iscove’s modified Dulbecco’s medium, 0.1% fatty acid-free bovine serum albumin for various times, and SIRPα phosphorylation was quantitated as described below.

Macrophages—Macrophages were derived from the bone marrow of CD47+/- and CD47-/- mice (31) as well as mice lacking the SIRPα cytoplasmic domain (11) (SIRP CT−/-) as previously described (34). All mice were bred for at least 10 generations onto the C57Fl/6 background. SIRP CT−/- bones were the kind gift of Mary Nakamura (University of California, San Francisco, CA). Macrophages were differentiated and maintained in macrophage complete medium (Dulbecco’s modified medium, 10% fetal bovine serum (FBS, Hyclone, South Logan, UT), 10 mM HEPES with 10% conditioned medium from CMG cells (35) as a source of M-CSF) and grown on bacteriologic plastic (Kord-Valmark, Brampton, Ontario, Canada). For experiments to determine the effect of cell density on SIRPα phosphorylation, macrophages were plated at 3 × 10⁶ cells/10-cm dish (“high density”), 10⁶ cells/10-cm dish (“medium density”), or 0.3 × 10⁶ cells/10-cm dish (“low density”) in complete medium on bacteriologic plastic and allowed to grow for 3 days before doing the experiment. Macrophages were plated at 3 × 10⁶ cells/10-cm dish for all other experiments except those with anti-integrin antibodies, where they were plated at 2 × 10⁶ cells/dish. For some experiments, complete medium was removed, the cells were rinsed with PBS, and serum-free medium (Dulbecco’s modified medium, 10 mM HEPES, 0.11 mg/ml sodium pyruvate, and 0.1% fatty acid-free bovine serum albumin) was added back for the indicated amount of time. For suspension and readhesion experiments, macrophages were harvested with 10 mM EDTA/PBS, washed once with serum-free or complete medium, and then resuspended and replated in the same medium. For experiments to examine integrins, macrophages were starved in serum-free medium overnight. As indicated, cells were harvested, pelleted, and incubated at 37 °C for 1 h 30 min before adding the indicated antibodies at a final concentration of 20 μg/ml, resuspending, and plating onto bacteriologic plastic or onto bacteriologic plastic coated with 10 μg/ml FN. Cells were allowed to adhere for 1 or 2 h, as indicated, at 37 °C before processing for immunoprecipitation. For these experiments, cells treated with anti-β2 (5C6) did not spread on the bacteriologic dishes regardless of genotype, and cells treated with anti-β1 (Ha2/5) did not attach to FN-coated dishes.
Bone Marrow Neutrophils—Neutrophils were isolated from the bone marrow of CD47<sup>+/+</sup> and CD47<sup>-/-</sup> C57Bl/6 mice exactly as described in Ref. 36. The neutrophils were resuspended in HBSS containing 20 mM HEPES, 0.5% FBS, 0.5 mM CaCl2, 1 mM MgCl2, pH 7.4 (“HBSS<sup>+</sup>”) to a concentration of 10 x 10<sup>6</sup> cells/ml. To assess basal levels of SIRPα phosphorylation in nonadherent, nonactivated neutrophils, 1 ml of CD47<sup>+</sup> or CD47<sup>-</sup> neutrophils (10 x 10<sup>6</sup> cells) was rotated at 6 rpm at room temperature for 30 min before processing for immunoprecipitation as described below. For adhesion experiments, 1 ml of each genotype of neutrophils was added to poly(RGD)-coated dishes and allowed to settle onto the surface for 10 min before the addition of 10 μM formylmethionylleucylphenylalanine (Sigma) in HBSS<sup>+</sup>, following which neutrophils were allowed to adhere for an additional 20 min prior to processing for immunoprecipitation. Without additional stimulation, the neutrophils remained round and nonadherent on this surface.

Murine Brain Endothelial Cells—For some experiments, murine brain-derived, polyoma middle T antigen-transformed endothelial cells, bEND.3 (37), were used. These cells, the kind gift of W. A. Frazier (Washington University, St. Louis, MO), were grown in Dulbecco’s modified medium (Invitrogen), 10 mM HEPES, 10% FBS, and 50 μg/ml gentamicin in a humidified 37 °C tissue culture incubator with 95% air, 5% CO2. For experiments to examine cell density effects on SIRPα phosphorylation, bEND.3 cells were plated at 0.1 x 10<sup>6</sup> cells/10-cm dish (low density), 0.3 x 10<sup>6</sup> cells/dish (medium density), or 10<sup>6</sup> cells/dish (high density).

MLEC Line Isolation and Culture—An immortalizing transgene carried by the ImmortoMouse® (Charles River Laboratories, Wilmington, MA) was bred into Sv129 CD47<sup>+/+</sup> and CD47<sup>-/-</sup> mice to facilitate establishment of endothelial cell lines. This immortalizing transgene consists of a temperature-sensitive SV40 large T antigen under the control of the major histocompatibility complex class I H-2K<sup>+</sup> promoter (38). Endothelial cells were isolated from the lungs of transgene-positive CD47<sup>+</sup> and CD47<sup>-</sup> mice using a protocol generously provided by J. Lively and R. O. Hynes (Massachusetts Institute of Technology, Cambridge, MA) (39). Polyclonal cultures of CD47<sup>+</sup> and CD47<sup>-</sup> MLEC were sorted for high and equivalent expression of intercellular cell adhesion molecule 2. Cell lines were maintained at 32.5 °C in “Lively medium” composed of Dulbecco’s modified medium/Ham’s F-12 medium (Invitrogen), 20% heat-inactivated FBS, 50 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA), 0.1 mg/ml heparin (Sigma), 20 units/ml interferon-γ (R&D Systems, Minneapolis, MN), and 50 μg/ml gentamicin (Sigma). Cells were passaged every 2–3 days using trypsin/EDTA and always plated onto tissue culture plastic that had been coated with 0.1% gelatin, collagen I at 20 μg/ml, or vitronectin at 10 μg/ml, as indicated. For experiments to examine the effects of cell-cell contact in the absence of adherence to a substrate by pelleting the cells, MLEC were plated at low density, harvested, and combined as described above. Some cells were then replated at higher density, whereas others were pelleted (233 x g, 5 min, room temperature). As indicated, some pelleted cells were lysed immediately, whereas others were incubated for an additional 60 min at 32.5 °C in the presence or absence of EDTA or MnCl2 before lysis and processing for immunoprecipitation. For experiments in which CD47<sup>-</sup> MLEC were mixed with SIRP<sup>CT</sup><sup>-/-</sup> BMDM, CD47<sup>-</sup> MLEC were initially plated at low density and subsequently incubated with SIRP<sup>CT</sup><sup>-/-</sup> BMDM to achieve a total cell density equal to the MLEC plated at high density, as described above. As controls, MLEC of both genotypes were harvested and replated at high density, as described above. Cells were allowed to readhere for 2 h before lysis and processing for immunoprecipitation. To examine components of the adherens junction (AJ), cells were plated at the indicated densities. Twenty minutes prior to lysis, cells were treated with 0.5 μM pervanadate to preserve phosphorylation levels of VE-cadherin and the α, β, and γ-catenins (40).

Flow Cytometry—The expression of a variety of cell surface molecules on CD47<sup>+</sup> and CD47<sup>-</sup> MLEC, BMDM, or polymorphonuclear leukocytes was determined using a Coulter Epics XL flow cytometer (Coulter Corp., Hialeah, FL). For each marker assessed, 2 x 10<sup>5</sup> MLEC or 1 x 10<sup>5</sup> BMDM were incu-
bated with primary antibody at a final concentration of 10 μg/ml (for purified antibodies) or in hybridoma supernatant for 30–60 min on ice, washed, and analyzed. For BMDM, 1 mg/ml human IgG was included in the incubation buffer to block FcγR binding of the monoclonal antibodies. Incubation with a rat monoclonal antibody anti-keyhole limpet hemocyanin, KLHR2A, was used to assess nonspecific binding.

Binding of mouse CD47-Fc to BMDM was assessed by flow cytometry as described above. 20 μg/ml anti-FcγRII/III 2.4G2 was used to inhibit FcγR binding of the construct, and nonspecific binding was assessed using human IgG rather than CD47-Fc.

**Immunoprecipitations**—For all immunoprecipitations, antibodies (5–10 μg/sample) were precoupled to resin (20–30 μl/sample of 50% slurry) by incubating with Gammainbend G-Sepharose (GE Healthcare) overnight at 4 °C with rotation in 20 mM HEPES, pH 7.4, 150 mM NaCl, followed by cross-linking with 10 mM dimethyl pimelimidate (Pierce). For all endothelial cell experiments, MLEC were transferred to medium lacking serum and added growth factors for 48 h prior to lysis. Cells were solubilized in ice-cold lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40 (Pierce), 1 mM NaF, 10 μg/ml leupeptin (Roche Applied Science), 10 μg/ml aprotinin (Roche Applied Science), 2 mM diisopropylfluorophosphate (Sigma) and 0.5 μM pervanadate). After removing nuclei and debris by centrifugation, the protein concentration of each sample was assessed using a Pierce BCA™ protein assay. For each experiment, equal amounts of protein in each lysate were used for each immunoprecipitation. After incubating at 4 °C with rotation overnight, the immunoprecipitates were washed three times with 0.75 ml of wash buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1% Nonidet P-40, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.5 μM pervanadate) before adding reducing sample buffer and boiling for 5 min.

SDS-PAGE and Western blotting were performed according to standard procedures. Western blot band densities were quantitated using CCD detection of emitted light during blot development with chemiluminescent reagents (Pierce) using the Fluoroochem8000 (Alpha Innotech, San Leandro, CA). The development with chemiluminescent reagents (Pierce) using Prolong (Invitrogen) and inspected.

**RESULTS**

**Macrophage SIRPα Phosphorylation Requires Adhesion and Is Enhanced by CD47**—Previous work has demonstrated that macrophage SIRPα phosphorylation increases upon exposure to CD47+ erythrocytes of the same species, inhibiting phagocytosis of these red blood cells (16). However, regulation of basal SIRPα phosphorylation by CD47 was not investigated. CD47+ and CD47– BMDM express equal amounts of SIRPα (supplemental Fig. 2), and there is substantial SIRPα phosphorylation in adherent CD47+ BMDM, whether or not they are in medium containing M-CSF and serum (Fig. 1A). SIRPα phosphorylation was greater in CD47+ BMDM; the extent of SIRPα phosphorylation in confluent CD47+ BMDM cultured in complete medium was 61 ± 9% of that in confluent CD47+ BMDM (quantitated as described under “Experimental Procedures”) (Fig. 1A). In the absence of M-CSF and serum, SIRPα phosphorylation in CD47+ decreased by about 15%, whereas it remained virtually unchanged in CD47+ BMDM, suggesting that CD47 expression enhances the effects of M-CSF and/or serum-derived growth factors that lead to SIRPα phosphorylation.

When BMDM were put into suspension for 1 h, SIRPα phosphorylation decreased dramatically, regardless of whether the macrophages expressed CD47 or were exposed to M-CSF and serum (Fig. 1A). After 1 h in suspension, the amount of phosphorylated SIRPα in both CD47+ and CD47– BMDM decreased more than 80% compared with adherent cells in both complete and serum-free medium. These data indicated that adhesion was a major stimulus for SIRPα phosphorylation. CD47+ expression enhanced adhesion-dependent phosphorylation by about 1.6-fold in macrophages in complete medium and by about 1.3-fold in the absence of serum or additional growth factors. The addition of M-CSF and other growth factors had only a small effect on the extent of SIRPα phosphorylation in either adherent or nonadherent BMDM.

Unlike BMDM, neutrophils are not spontaneously adherent, but they do express high levels of SIRPα. In both CD47+ and CD47– bone marrow-derived neutrophils, SIRPα phosphorylation in suspended cells was not detectable (Fig. 1B). Significant SIRPα phosphorylation was induced when formylmethylleucylphenylalanine-stimulated neutrophils were allowed to adhere to a surface coated with the synthetic integrin ligand poly(RGD) (Fig. 1B). As in macrophages, SIRPα phosphorylation did not require, but was enhanced by, CD47 expression.
To examine the role of CD47 further, CD47⁺ and CD47⁻ BMDM were plated at different densities. Cells plated at low density grew as single cells without touching other cells. Cells plated at medium density were ~50% confluent. Cells at high density appeared as a confluent layer of macrophages with all cells in contact with their neighbors. As shown in Fig. 2A, significant levels of phospho-SIRPα were found in both CD47⁺ and CD47⁻ macrophages at all three cell densities. SIRPα phosphorylation was enhanced with increasing cell density in CD47⁺ but not CD47⁻ cells, suggesting that CD47 interactions with SIRPα on adjacent cells increased SIRPα phosphorylation, as suggested by previous work with macrophage-erythrocyte interactions (16, 17). However, cell-cell contact alone was insufficient to induce SIRPα phosphorylation, because when either CD47⁺ or CD47⁻ BMDM were centrifuged and incubated in a pellet for 1.5 h, no increase in SIRPα phosphorylation was observed (Fig. 2B). In fact, SIRPα phosphorylation decreased by about 50% in each cell type without adhesion, despite the close cell-cell contact induced by centrifugation. Together, these data show that BMDM SIRPα phosphorylation requires adhesion and can be enhanced by CD47, probably because of intercellular interactions between CD47 and SIRPα.

To determine the kinetics of SIRPα phosphorylation in response to adhesion, CD47⁺ and CD47⁻ macrophages were plated into suspension for 1 h and replated, and SIRPα phosphorylation was quantified at various times. At 30 min after replating, most cells were round and loosely tethered to the dish, and very few had spread; by 60 min, cells were more tightly tethered and had begun to spread. These kinetics of adhesion and spreading correlated with SIRPα phosphorylation, with detectably increased SIRPα phosphorylation by 30 min and maximal phosphorylation in both CD47⁺ and CD47⁻ BMDM by 60 min (Fig. 3A). CD47 expression did not affect the kinetics of SIRPα phosphorylation in response to adhesion.

To characterize the role of integrins in the induction of SIRPα phosphorylation in BMDM, CD47⁺ and CD47⁻ BMDM from serum-starved cultures were harvested and incubated in a pellet for 1.5 h before replating onto bacteriologic plastic for 2 h in the presence of antibodies to several different integrins. Adhesion and spreading on bacteriologic plastic is known to require β2 integrins (41). Anti-β2 antibodies inhibited adhesion-dependent induction of SIRPα phosphorylation in both CD47⁺ and CD47⁻ BMDM (Fig. 3B). Antibodies to the αv and β1 inte-
Regulation of SIRPα Phosphorylation

**A.**

![Graph](image)

**B.**

![Graph](image)

**C.**

![Graph](image)

**FIGURE 3.** BMDM SIRPα phosphorylation requires integrin-mediated adhesion. A, top, SIRPα phosphorylation was quantitated in CD47+ (solid bars) and CD47− (checkered bars) BMDM continuously adherent (Adh), after 1 h in suspension (Sus, 60'), and after various times of readhesion. Phosphorylation in continuously adherent CD47+ BMDM was set to 100%. Data were obtained from 3–5 independent experiments (*, p < 0.05; **, p < 0.001). Bottom, representative Western blots (WB) from SIRPα immunoprecipitations (IP). The position of a 116-kDa molecular mass marker is indicated. B, SIRPα phosphorylation was quantitated in CD47+ (solid bars) and CD47− (checkered bars) BMDM that were continuously adherent (Adh), pelleted to maintain cell-cell contact (pellet), or removed from the pellet and allowed to readhere to bacteriologic plastic for 2 h with antibodies to the indicated integrin chains or control antibody. Statistical comparisons were made between BMDM allowed to readhere with control antibodies or anti-integrin antibodies for each genotype (*, p < 0.05; #, p < 0.01). C, SIRPα phosphorylation was quantitated in CD47+ (solid bars) and CD47− (checkered bars) BMDM that were continuously adherent (Adh), pelleted to maintain cell-cell contact (pellet), or removed from the pellet and allowed to readhere to bacteriologic plastic (bact. plastic) or to FN-coated plastic for 1 h with a control or anti-β1 integrin antibody. Statistical comparisons were made between BMDM allowed to readhere with control antibody or anti-β1 for each genotype (*, p < 0.05). For B and C, data shown are the summary of 3–6 independent experiments. pY, phosphotyrosine.

Also led to adhesion-dependent induction of SIRPα phosphorylation in both cell types (Fig. 3C). Induction of SIRPα phosphorylation on FN was inhibited in both genotypes when BMDM were plated in the presence of antibodies to the β1 integrin, demonstrating the importance of integrin-mediated adhesion independent of the expression of CD47. The addition of anti-β2 or cyclic RGD peptides did not have any additional inhibitory effect when combined with anti-β1, probably because they did not have any further inhibitory effects on adhesion to the FN-coated surface under the conditions used (data not shown). Thus, induction of SIRPα phosphorylation in BMDM requires integrin-mediated adhesion but not CD47.

**Endothelial SIRPα Phosphorylation Requires CD47**—Although SIRPα expression and function has been studied primarily in myeloid cells and neurons, it also is expressed in endothelial cells. CD47+ and CD47− murine lung endothelial cell lines expressed similar amounts of SIRPα on their surfaces (supplemental Fig. 3). Although both CD47+ and CD47− MLEC grew as adherent cell lines, phosphorylated SIRPα was detected only in the CD47+ MLEC (Fig. 4A). In contrast to BMDM, where CD47 expression increased SIRPα phosphorylation by less than 2-fold (Fig. 1A), CD47 expression increased SIRPα phosphorylation by more than 30-fold. SHP-2, a cytosolic phosphatase known to be recruited in a phosphorylation-dependent manner to the cytoplasmic ITIM motifs of SIRPα (12), was detected in anti-SIRPα immunoprecipitates only from CD47+ lysates (Fig. 4A). SHP-1, also known to interact with the SIRPα ITIMs, was not detected in MLEC (data not shown). To determine whether CD47-SIRPα interaction was required for MLEC SIRPα phosphorylation, CD47+ and CD47− MLEC were grown in the presence of antibodies that block CD47-SIRPα interaction (30). Four days after plating, the amount of phosphorylated SIRPα was assessed. As shown in Fig. 4B, the anti-CD47 antibodies miap301 and miap420 significantly inhibited SIRPα phos-
phosphorylation in the CD47+ MLEC. Thus, in contrast to BMDM and neutrophils, MLEC SIRPα phosphorylation requires ligation by CD47.

To examine this requirement further, CD47+ and CD47− MLEC were plated at three different densities. For these experiments, cells plated at low density were just reaching confluence on the day of the experiment, cells plated at medium density reached confluence 1–2 days prior to the day of the experiment, and cells plated at high density were confluent for 3–4 days prior to the day of the experiment. Identical amounts of lysate protein were used for each immunoprecipitation. Although plating density did not alter the surface expression levels of SIRPα (data not shown), it had a significant effect on the extent of phosphorylated SIRPα detected in the CD47+ cells (Fig. 4C). Phosphorylated SIRPα was not detected in CD47− MLEC at low or medium density and was minimally detectable in high density CD47− MLEC (Fig. 4C). Increasing cell density also increased the extent of SIRPα phosphorylation in the brain-derived CD47+ endothelial cell line bEND.3 (Fig. 4D). Because increased phosphorylation occurred with increased cell density, it is likely that these CD47-SIRPα interactions occurred at intercellular contacts.

To determine whether intercellular CD47-SIRPα interactions were required for SIRPα phosphorylation in adherent MLEC, CD47− MLEC were cultured for 2 h with BMDM from CD47+, SIRP CT−/− macrophages. Because these BMDM express SIRPα lacking its cytoplasmic tyrosine phosphorylation sites, SIRPα immunoprecipitated from these cells does not contribute to the phosphorylation signal. CD47− MLEC cultured at either low or high density had barely detectable phospho-SIRPα, whereas there was density-dependent SIRPα phosphorylation in CD47+ MLEC (Fig. 4E). When CD47− MLEC were cocultured with SIRP CT−/− BMDM, the extent of SIRPα phosphorylation was equivalent to that in CD47+ MLEC (Fig. 4E). Since CD47 was expressed only on BMDM and SIRPα could only be phosphorylated on MLEC, this experiment demonstrates that intercellular interactions between CD47 and SIRPα induce SIRPα phosphorylation in adherent endothelial cells. Consistent with a primary role for intercellular interaction in MLEC SIRPα
Regulation of SIRPα Phosphorylation

phosphorylation, both CD47 and SIRPα concentrated at cell-cell junctions in nonpermeabilized MLEC (supplemental Fig. 4). Thus, intercellular SIRPα-CD47 interactions have a much more significant role in regulation of SIRPα phosphorylation in MLEC than in BMDM or neutrophils.

Kinetics of SIRPα Phosphorylation in MLEC—Because the requirements for CD47 in SIRPα phosphorylation in MLEC and leukocytes were distinct, we examined the kinetics of SIRPα phosphorylation in MLEC. MLEC plated at low density, which show minimal SIRPα phosphorylation, were harvested and then replated at higher density for various times. When replated onto surfaces coated with fibronectin and gelatin or gelatin alone (data not shown), robust SIRPα phosphorylation was detected by 60 min in CD47+/MLEC (Fig. 5A). At this time, about 90% of both the CD47+/ and CD47− MLEC had attached and spread (data not shown). This rapid density-dependent augmentation of SIRPα phosphorylation was inhibited by antibody to CD47 both for MLEC (Fig. 5B) and bEND.3 cells (Fig. 5C). There was a less robust increase in SIRPα phosphorylation in bEND.3 cells than MLEC after 1 h at high density (Fig. 5C), probably because bEND.3 did not attach or spread as quickly as MLEC.

Adhesion to a CD47-coated Surface Is Not Sufficient for Endothelial SIRPα Phosphorylation—Since SIRPα phosphorylation in endothelial cells, unlike BMDM or polymorphonuclear leukocytes, required CD47, we tested whether MLEC adhesion to CD47 was sufficient to induce this signaling. MLEC, grown at low density to minimize SIRPα phosphorylation, were replated at higher density onto tissue culture dishes coated with mouse CD47-Fc or human IgG as a control (Fig. 6A). Murine CD47-Fc is a soluble ligand competent to bind cell-expressed SIRPα, as shown previously by others (32). At no time did surface-bound mouse CD47-Fc induce SIRPα phosphorylation in CD47+/MLEC (Fig. 6A). Increasing SIRPα phosphorylation occurred over time in CD47+/MLEC but was equivalent in cells plated onto mouse CD47-Fc and nonspecific human IgG. Over this extended time course, the MLEC adhered and spread on the protein-coated surface. Since SIRPα phosphorylation occurred only in CD47+ MLEC and was equivalent on mouse CD47-Fc and control surfaces, these data suggest that adhesion to the tissue culture plate together with intercellular interactions was responsible for SIRPα phosphorylation rather than direct ligation of MLEC SIRPα by the surface-bound CD47-Fc. As a further test for the sufficiency of CD47-SIRPα interaction to induce SIRPα phosphorylation, CD47+/MLEC grown at low density were suspended and then pelleted by centrifugation to maximize cell-cell contact (Fig. 6B). Without cell adhesion to a substratum, SIRPα phosphorylation was not activated in the pelleted cells. Equivalent data were obtained with bEND.3 cells (Fig. 6C). Together, these experiments demonstrate that CD47-SIRPα interaction is not sufficient to induce SIRPα phosphorylation.

Integrin Ligation Is Required for Induction of SIRPα Phosphorylation in MLEC—Several experiments suggested a role for integrins in the induction of MLEC SIRPα phosphorylation. The addition of EDTA, which blocks integrin recognition of ligands, prevented induction of SIRPα phosphorylation in MLEC (Fig. 5B). Plating MLEC, even at high density, on surfaces lacking integrin ligands did not induce SIRPα phosphorylation (Fig. 6, A and B), and pelleting MLEC in a buffer containing Mn2+, which increases integrin affinity, enhanced SIRPα phosphorylation compared with cells in buffer lacking divalent cations (Fig. 6B).

To test formally the role for integrins in induction of SIRPα phosphorylation in CD47+ MLEC, MLEC were plated onto various surfaces. First, cells grown at low density to minimize SIRPα phosphorylation were replated on gelatin in the presence of antibodies to β1 integrins with and without αv integrin antibodies. Antibodies to β1 integrins completely inhibited the increased SIRPα phosphorylation resulting from replating...
CD47+ MLEC at high density (Fig. 7A). On their own, antibodies to the αv integrin slightly decreased the amount of induced SIRPα phosphorylation (data not shown) but did not augment the effect of the anti-β1 antibodies when combined with anti-β1. To test the requirement for integrins further, CD47+ MLEC were plated onto mouse CD47-Fc- or human IgG-coated surfaces, and SIRPα phosphorylation was detected as described in previous figure legends. B, CD47+ MLEC plated at low density were left adherent or harvested for replating at high density on FN or uncoated tissue culture plastic or were pelleted after deadhesion. Some MLEC were pelleted in the presence of 3 mM EDTA or 1 mM MnCl2. SIRPα phosphorylation was quantitated as previously described, and phosphorylation in MLEC kept at low density was set at 100%. Statistical comparisons were made between other groups and MLEC at low density (*, p < 0.05). Data were from three independent experiments. C, CD47 bEND.3 plated at low density were left adherent or harvested and pelleted as described above for MLEC. SIRPα phosphorylation was quantitated, and the phosphorylation of low density bEND.3 was set at 100%. Statistical comparisons of SIRPα phosphorylation in adherent cultures and pelleted cells were performed (**, p < 0.01). Data were from three independent experiments. FN/gel, fibronectin/gelatin-coated tissue culture plastic; TC, uncoated tissue culture plastic; pellet 0, pelleted cells lysed immediately after centrifugation; pellet 60, pelleted cells lysed after 60 min; pellet + EDTA, 60, cells pelleted and maintained in the presence of 1 mM MnCl2 for 60 min before lysis; IP, immunoprecipitation; WB, Western blot; pY, phosphotyrosine.
Regulation of SIRPα Phosphorylation

MLEC were replated at high density onto collagen I and vitronectin, two well defined integrin ligands. Replating CD47+ MLEC at higher density onto these defined integrin ligands also induced SIRPα phosphorylation (Fig. 7B). SIRPα phosphorylation was inhibited by antibody to β1 integrin when cells were plated on collagen and by a cyclic RGD peptide when cells were plated on vitronectin but not by control antibodies or peptides (Fig. 7B). We conclude that integrin ligation and CD47-SIRPα interactions are both required for induction of SIRPα phosphorylation in MLEC and that both αv and β1 integrins can provide the required adhesive signal.

Adherens Junction Components Are More Highly Phosphorylated in CD47− MLEC—Since CD47-SIRPα interactions appeared to occur at cell-cell junctions in MLEC and also led to SHP-2 recruitment, we tested whether tyrosine phosphorylation of other junctional components was altered in CD47-deficient MLEC. Indeed, tyrosine phosphorylation of VE-cadherin and associated α-, β-, and γ-catenins increased with increasing cell density in CD47− MLEC but not in CD47+ cells (Fig. 7C). Thus, it is possible that CD47-SIRPα interactions at cell-cell borders in endothelial cells contribute to the recruitment of tyrosine phosphatase activity required to regulate endothelial adherens junctions. Although phosphorylation levels of these junctional components were different, CD47+ and CD47− MLEC monolayers appear virtually identical to one another with no apparent differences in morphology or monolayer integrity (data not shown) under basal conditions.

SIRPα Localization Differs in BMDM and MLEC—Because the dependence on CD47 for induction of SIRPα phosphorylation differed between BMDM and MLEC, we examined SIRPα localization in the two cell types. The predominant location for both CD47 and SIRPα in intact CD47+ MLEC was at cell-cell junctions, and SIRPα localized to cell-cell junctions less well in CD47− MLEC (supplemental Fig. 4). In contrast, SIRPα significantly localized to the adhesive surface of BMDM and was prominent at the membrane periphery in both CD47+ and CD47− cells (Fig. 8 and supplemental Fig. 5). To investigate whether SIRPα localization correlated with known cytoskeletal structures, CD47+ and CD47− BMDM and MLEC were stained for vinculin, a marker of focal adhesions, focal complexes, and podosomes, and for polymerized actin structures. As determined by confocal microscopy, SIRPα showed considerable colocalization with polymerized actin and vinculin in both CD47+ and CD47− BMDM (Fig. 8 and supplemental Fig. 5). The colocalization of SIRPα, vinculin, and phalloidin typically appeared strongest at the periphery of both CD47+ and CD47− BMDM, coinciding with the greatest concentrations of SIRPα, although more internal adhesive structures also showed some SIRPα colocalization (Fig. 8). Phalloidin staining showed a similar pattern of colocalization with SIRPα, indicating a close association between SIRPα and the actin cytoskeleton in these cells. In contrast, SIRPα staining showed little colocalization with either vinculin or actin in permeabilized CD47+ and CD47− MLEC (Fig. 8 and supplemental Fig. 5). There was no colocalization of SIRPα staining with focal adhesions demonstrated by vinculin staining or with phalloidin staining (Fig. 8). These data demonstrate that there is more SIRPα association with sites of substrate adhesion in BMDM than in MLEC. This association with sites of integrin-mediated adhesion may account for SIRPα phosphorylation independent of CD47 in this cell type.
**DISCUSSION**

SIRPα phosphorylation is a critical regulatory step in a variety of signaling pathways and cell types, including macrophages, neutrophils, dendritic cells, smooth muscle cells, and fibroblasts. Although growth factors, cell adhesion, and CD47 ligation are known to modulate SIRPα phosphorylation in fibroblasts, macrophages, and smooth muscle cells (7, 9, 12, 14, 16, 23), all of these signals interact, and the specific requirements for SIRPα phosphorylation have not been carefully dissected. In this work, a genetic approach was taken in three different cell types to isolate the effects of cell adhesion and intercellular interactions in regulating SIRPα phosphorylation. In macrophages and neutrophils, SIRPα phosphorylation was highly dependent on integrin-mediated adhesion. Expression of CD47 enhanced this phosphorylation but clearly was not required, since abundant phospho-SIRPα was detected in adherent CD47−/− BMDM and neutrophils. In contrast, basal SIRPα phosphorylation in endothelial cells required both expression of CD47 and integrin-mediated adhesion. Increased phosphorylation of SIRPα was detected in CD47+ MLEC and bEND.3 cells plated at increasing cell density, consistent with an intercellular interaction between CD47 and SIRPα. Antibodies to CD47 inhibited SIRPα phosphorylation, confirming the requirement of CD47-SIRPα interactions in this phosphorylation. Furthermore, SIRPα expressed by CD47− MLEC mixed with CD47+, SIRP CT−/− BMDM was phosphorylated, demonstrating the importance of CD47 ligation in *trans* in MLEC. As in BMDM and neutrophils, phosphorylation of SIRPα in endothelial cells required adhesion, since CD47+ MLEC incubated in a pellet, in which cell-cell contact was maximized, but not adherent to a surface, had reduced levels of SIRPα phosphorylation, and even contact with CD47 on a surface could not induce SIRPα phosphorylation in the absence of integrin-mediated adhesion. Although previous reports have shown that integrin-mediated adhesion could induce SIRPα phosphorylation, these earlier studies all utilized CD47− cells. Thus, these earlier reports could not differentiate between the roles of CD47 in modulating integrin activity, in CD47-integrin cooperation, or in direct CD47-SIRPα interactions. Our genetic data demonstrate that in both myeloid and endothelial cells, integrin-mediated adhesion is a requirement for SIRPα phosphorylation and also illuminate a fundamental distinction in regulation of this phosphorylation. In myeloid cells, CD47 expression increased levels of SIRPα phosphorylation by ~1.5-fold, with abundant phospho-SIRPα detected in CD47− cells, whereas in endothelial cells, CD47 expression increased SIRPα phosphorylation more than 30-fold, and phospho-SIRPα was essentially undetectable in CD47− MLEC. Thus, adhesion is sufficient in the myeloid cells, whereas adhesion and ligation of SIRPα with CD47 both are required in endothelia.

The nature of the adhesive signal required for SIRPα phosphorylation in leukocytes and endothelial cells is consistent with activation of Src family kinases by integrin signaling, since this family has been shown to phosphorylate SIRPα ITIMs (12, 13, 42). Indeed, the Src family kinase inhibitors PP1 and PP2 blocked adhesion-dependent SIRPα phosphorylation in both fibroblasts (12) and endothelial cells.3 Our data suggest that the difference in requirement for CD47 for SIRPα phosphorylation in leukocytes and endothelial cells is due to a difference in the association of SIRPα with the actin cytoskeleton in the two cell types. In BMDM, SIRPα is tightly associated with actin, whereas in MLEC, we found no such colocalization. These data suggest that the close proximity of SIRPα in myeloid cells to focal complexes and podosomes where integrin ligation recruits activated Src family kinases presumably enhances the kinetics and extent of phosphorylation. In endothelial cells, the majority of integrin-containing focal adhesions (and associated Src family kinase activity) are located on the basal surface of the cells, and actin fibers are organized around the perimeter of the cells, where they connect to junctional complexes between adjacent cells. Src family kinases are recruited to these intercellular contacts, where they are involved in the regulation of VE-cadherin phosphorylation and endothelial permeability (6, 43–47). We hypothesize that ligation of SIRPα with CD47 at cell-cell junctions in endothelial cells facilitates its phosphorylation by Src family kinases recruited to nearby adherens junctions.

Several previous studies have suggested that macrophage SIRPα phosphorylation induced by interaction with target CD47 negatively regulates FcγR- and CR3-mediated phagocytosis (15–18, 48). The presence of considerable basal, CD47-independent SIRPα phosphorylation in macrophages potentially complicates these models. In order for CD47-mediated SIRPα phosphorylation to inhibit phagocytosis in the presence of a considerable background of CD47-independent phosphorylation, the cell probably must be able to decipher spatial cues as well. In other words, the adherent macrophages would have to distinguish phosphorylated SIRPα associated with adhesion from those molecules phosphorylated in response to CD47 on a phagocytic target. One possibility is that the macrophage is able to differentiate between SIRPα phosphorylation occurring at its apical surface from SIRPα phosphorylation occurring at its lateral edges or basal surface. Another possibility is that other receptor-ligand interactions, in addition to CD47-SIRPα, are required for the macrophage to see SIRPα phosphorylation as a “don’t eat me signal” as opposed to a steady state adherence signal.

Our experiments suggest that in endothelial cells, CD47 and SIRPα interactions occur at cell-cell contacts. The increasing amounts of phosphorylated SIRPα that occur with increasing cell density in endothelia are reminiscent, although opposite in intensity, of phosphorylation changes that occur with the adherens junction components VE-cadherin and associated catenins in maturing endothelial monolayers (40, 47). It has been suggested that decreased cadherin and catenin phosphorylation results from recruitment of SHP-2 to the adherens junction, leading to strengthening of the association of the adherens junction with the actin cytoskeleton (40, 47, 49). We detected increased phosphorylation of VE-cadherin and associated catenins in high density CD47− MLEC monolayers relative to CD47+ monolayers (Fig. 7B), although there appear to

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3. M. L. Johansen and E. J. Brown, unpublished data.
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be similar amounts of α-catenin associated with VE-cadherin in
both the CD47+ and CD47− MLEC.3 Thus, CD47-SIRPα interac-
tions in endothelial cells may be a mechanism for recruit-
ment of SHP-2 to the adherens junction with consequences for
junctival maturation.

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