Regulation of Integrin Function by CD47 Ligands

DIFFERENTIAL EFFECTS ON \(\alpha_{v}\beta_{3}\) AND \(\alpha_{v}\beta_{1}\) INTEGRIN-MEDIATED ADHESION*

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We examined the regulation of \(\alpha_{v}\beta_{3}\) integrin function in melanoma cells and T cells by ligands of CD47. A CD47 antibody (B6H12) that inhibited \(\alpha_{v}\beta_{3}\)-mediated adhesion of melanoma cells induced by CD47-binding peptides from thrombospondin-1 directly stimulated \(\alpha_{v}\beta_{3}\)-mediated adhesion of the same cells to vascular cell adhesion molecule-1 and N-terminal regions of thrombospondin-1 or thrombospondin-2. B6H12 also stimulated \(\alpha_{v}\beta_{1}\) as well as \(\alpha_{v}\beta_{3}\) and \(\alpha_{v}\beta_{1}\)-mediated adhesion of CD47-expressing T cells but not of CD47-deficient T cells. \(\alpha_{v}\beta_{1}\) and CD47 co-purified as a detergent-stable complex on a CD47 antibody affinity column. CD47-binding peptides based on C-terminal sequences of thrombospondin-1 also specifically enhanced adhesion of melanoma cells and T cells to \(\alpha_{v}\beta_{1}\) ligands. Unexpectedly, activation of \(\alpha_{v}\beta_{1}\) function by the thrombospondin-1 CD47-binding peptides also occurred in CD47-deficient T cells. CD47-independent activation of \(\alpha_{v}\beta_{1}\) required the Val-Val-Met (VVM) motif of the peptides and was sensitive to inhibition by pertussis toxin. These results indicate that activation of \(\alpha_{v}\beta_{1}\) by the CD47 antibody B6H12 and by VVM peptides occurs by different mechanisms. The antibody directly activates a CD47-\(\alpha_{v}\beta_{1}\) complex, whereas VVM peptides may target an unidentified G\(_{i}\)-linked receptor that regulates \(\alpha_{v}\beta_{1}\).

CD47 (integrin-associated protein) is an integral membrane protein that is required for granulocyte and T cell recruitment to sites of infection (1, 2), and its absence on red blood cells leads to their rapid macrophage-mediated clearance (3). CD47 may also function as a costimulator to regulate T cell activation, survival, and Th1 versus Th2 differentiation (4, 5). Endogenous ligands for the extracellular domain of CD47 include the secreted protein thrombospondin-1 (TSP1) and potentially leads to their rapid macrophage-mediated clearance (3). CD47

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1 The abbreviation used are: TSP1, human thrombospondin-1; TSP2, human thrombospondin-2; NoC1, trimeric human thrombospondin-1 residues 1–356; NoC2, thrombospondin-2 residues 1–359; VCAM-1, vascular cell adhesion molecule-1; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CHAPS, 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Cell Culture and Proteins—A2058 melanoma cells and Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. A CD47-deficient T cell line derived from Jurkat cells (JinB8) was graciously provided by Dr. Eric Brown (University of California, San Francisco, CA), and \(\beta_{1}\)

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integrin-deficient cells were provided by Dr. Yoji Shimizu (University of Minnesota Medical School, Minneapolis, MN). Both cell lines were grown at 37 °C with 5% CO\(_2\). All experiments used cells grown from frozen stocks verified by flow cytometry to have the expected levels of \( \beta_1 \) integrin and CD47 expression (19). TSP-1 was purified from the supernatant of thrombin-activated platelet as described (23). Human vitronectin was purchased from Sigma, and fibronectin was purified from human plasma (National Institutes of Health Blood Bank) as described (24). FN33 is a recombinant region of fibronectin containing its \( \alpha\beta_1 \) integrin binding site but not its \( \alpha\beta_2 \) binding sites (25). NoC1 is a recombinant trimeric portion of TSP1 (residues 1–1102 of the mature protein) (26). NoC2 is the corresponding recombinant portion of TSP2 (residues 1–359 of the mature protein). Recombinant soluble 7 domain VCAM-1 (1-STD-VCAM-1, residues 1–674 of the mature protein) was described previously (19). Vitrogen type I collagen was obtained from Cohesion, Palo Alto, CA. The following synthetic peptides derived from TSP1 and their respective inactive controls were prepared as previously described: FIRVVMYEGKK (TN3, residues 1102–1112 of mature TSP1), RFVVMMWK (4N1-1, 1016–1024), KRFYVVMMWK (4N1K, 4N1 flanked with 2 Lys residues), RFYGMWK (4N1GCG, inactive control for 4N1), and FIRVGYEGKQ (p604) and FIRDVYEGKQ (p605), both controls for TN3 (21, 27).

Antibodies and Reagents—TSP2/16 (anti-\( \beta_1 \) integrin activating antibody, Hemler 1984) and B6H12 (anti-CD47) were each purified by protein-A affinity chromatography from conditioned media of the respective hybridomas (American Type Culture Collection). Anti-\( \alpha\) integrin antibody (Ab1924) was purchased from Chemicon. A \( \beta_1 \) integrin function-blocking antibody (mAb13) was provided by Dr. Ken Yamada (NIDCR, National Institutes of Health). Anti-CD47 antibody (clone C1Km1) was purchased from ICN Biomedicals. The \( \alpha\beta_1 \) integrin antagonist (4-(2-methylphenyl)aminocarbonylaminomethyl)aminophenylacetyl-LDVP (LDVP) was obtained from Bachem. The \( \alpha_\delta \) integrin antagonist SB223245 was provided by William Miller (Pierce) (29). PP2, 4-amino-5-(4-chlorophenyl)-7-t-butylpyrazolo[3,4-d] pyrimidine, and pertussis toxin were purchased from Calbiochem.

Cell Adhesion Assays—Adhesion was assessed using a microscopic assay. TSP1, recombinant proteins, or STC-1VM-1 diluted in Dulbecco’s PBS or NaHCO\(_3\) buffer were absorbed on bacteriological polystyrene dishes overnight at 4 °C. The dishes were blocked with 1% BSA in PBS overnight at 4 °C and attached cells were quantified by counting using a calibrated haemocytometer (Dade International). Spread of melanoma cells at 37 °C was measured using lodogen (Pierce). Jurkat cells were washed with 4 °C Dulbecco’s PBS, incubated with antibody-coupled matrix overnight at 4 °C, washed again, and attached cells were quantified using a colorimetric assay as previously described (30). TSP1 and the cell suspension was premixed with the indicated concentrations of cell suspension with the indicated treatments was added into each well. The plates were then incubated at 37 °C for 1 hr. An aliquot of 20 μl of each cell suspension was then transferred to a 37 °C water bath for 15 min. The cell suspensions were then transferred to plastic tubes containing 100 μl of Nyosil oil (William F. Nye, Inc.), centrifuged for 1 min, and washed with 200 μl of cell binding buffer. The pellets were collected, and the bound radioactivity was quantified.

Immunoaffinity Purification—Antibody B6H12 or nonspecific mouse IgG (Sigma) were each coupled to Reacti-GelTM HW-65 (Pierce) according to the manufacturer’s recommendations. Jurkat cells were lysed in radioimmune precipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM NaF supplemented with 10 μg/ml each protease inhibitor antipain, pepstatin A, chymostatin, leupeptin, aprotinin, soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride) and preclarified by high speed centrifugation. Equal volumes with equal protein concentration were incubated with antibody-coupled matrix overnight at 4 °C with rocking. The matrix were washed with 10 volumes of Tris-buffered saline (140 mM NaCl, 20 mM Tris, pH 7.5, 0.1% Tween 20), and the matrix were washed with PBS containing 500 μM NaCl, 100 mM glycerol, pH 3.3, 10 mM CHAPS). The eluant was immediately neutralized with 1/10 volume of 1 M Tris, pH 8.

Western Blotting—Proteins were fractionated on SDS gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS containing 3% BSA and 0.1% Tween 20. The primary antibody was added in the presence of blocking buffer and allowed to incubate while rocking. After repeated washes with PBS containing 0.1% Tween, a horseradish peroxidase-conjugated secondary antibody was added and incubated in blocking buffer. The membranes were washed with PBS-Tween, and antigen antibody complex was visualized using chemiluminescent substrate (Pierce).

RESULTS

A CD47-binding Peptide Stimulates \( \alpha\beta_1 \) Integrin-dependent Adhesion of Melanoma Cells—Ligation of CD47 by TSP1 or by specific TSP1 peptides induces \( \alpha\beta_1 \)-mediated cell spreading via direct association of CD47 with a cell surface complex that contains this integrin and is attenuated by pertussis toxin (14). CD47 also physically associates with the integrins \( \alpha_\delta \beta_3 \) and \( \alpha\beta_1 \), whereas its association with \( \alpha\beta_2 \) integrin has been inferred but not examined directly (2, 19). As previously reported (21), a CD47-binding peptide derived from TSP1, FIRVVMYEGKK, but not the corresponding control peptide, FIRDVGYEGKK, stimulated A2058 melanoma cell spreading mediated by \( \alpha\beta_1 \) integrin on suboptimal concentrations of a vitronectin substrate (Fig. 1A). This enhancement was reversed by the \( \alpha_\delta \) integrin agonist SB223245 but not by the \( \alpha_\delta \) integrin antagonist (4-(2-methylphenyl)aminocarbonylaminomethyl)aminophenylacetyl-LDVP (28) or by a \( \beta_1 \) integrin function-blocking antibody.

Because TSP1 contains an \( \alpha\beta_1 \) recognition sequence in its type 3 repeats and an \( \alpha\beta_1 \) binding site in the N-terminal domain, we used recombinant N-terminal portions of TSP1 and TSP2 that contain only their \( \beta_1 \) integrin binding sites to examine the effect of the CD47-binding peptide on \( \beta_1 \) integrin-mediated melanoma cell adhesion to TSP1 and TSP2 (Fig. 1A). Peptide FIRVVMYEGKK but not the control peptide FIRDVGYEGKK stimulated spreading on both NoC1 and NoC2 to a comparable extent as the \( \beta_1 \) integrin-activating antibody TSP2/16. Melanoma cell spreading on NoC1 and NoC2 was stimulated by peptide FIRVVMYEGKK but not the control peptide FIRVGYEGKK. This enhancement was reversed by the \( \alpha_\delta \) integrin agonist SB223245 but not by the \( \alpha_\delta \) integrin antagonist (4-(2-methylphenyl)aminocarbonylaminomethyl)aminophenylacetyl-LDVP (28) or by a \( \beta_1 \) integrin function-blocking antibody.

Stimulation of \( \alpha\beta_1 \) integrin-mediated spreading of melanoma cells by the TSP1 peptide was verified using the well-defined \( \alpha\beta_1 \) integrin ligand VCAM-1 (Fig. 1B). The CD47-binding TSP1 peptide FIRVVMYEGKK also stimulated spreading on intact TSP1 and on limiting concentrations of type I collagen, an \( \alpha\beta_1 \)-specific substrate for these cells. Therefore, FIRVVMYEGKK increases spreading mediated by several integrins on melanoma cells.

Stimulation of \( \alpha\beta_1 \) integrin-mediated spreading by TSP1 peptides that bind to CD47 was previously shown to be blocked by the CD47 antibody B6H12 (6). We confirmed the inhibitory activity of B6H12 for TSP1 peptide-induced melanoma cell attachment and spreading on limiting concentrations of the \( \alpha\beta_1 \) integrin ligand vitronectin (Fig. 2A). In contrast, the CD47 antibody did not inhibit spreading stimulated by FIRVVMYEGKK on the \( \alpha\beta_1 \) ligands NoC1 or NoC2 and, instead, further stimulated melanoma cell attachment on NoC1 and NoC2 (Fig. 2A).

These data suggested that CD47 ligation by B6H12 may directly stimulate \( \alpha\beta_1 \) integrin-mediated adhesion. This was
confirmed by examining the effect of B6H12 in the absence of other CD47 ligands (Fig. 2B). B6H12 markedly enhanced both attachment and spreading of melanoma cells on NoC2, whereas it somewhat inhibited basal attachment and spreading of the melanoma cells on NoC1. Untreated cells (control) attached and spread cells/mm² are presented as mean ± S.D., n = 3. B, a CD47-binding peptide from TSP1 stimulates melanoma cell adhesion on several integrin substrates. Cells were incubated on substrates coated with NoC1 (25 µg/ml, contains αβ1 and αβ1 integrin binding sites (19, 52)), NoC2 (35 µg/ml, contains only an αβ1 binding site (19)), or vitronectin (5 µg/ml, an αβ1-dependent substrate). The cells were either untreated (control), treated with control peptide FIRVVAIYEGKK (p604), or treated with a TSP1 peptide containing a CD47-binding sequence FIRVVMYEGKK (p7N3) alone or in combination with a β1 integrin blocking antibody (mAb13, 5 µg/ml) or the αβ1 antagonist SB223245 (1 µM). Melanoma cells were also treated with a β1-activating antibody alone (TS2/16, 5 µg/ml). The numbers of attached and spread cells/mm² are presented as mean ± S.D., n = 3. B, a CD47-binding peptide from TSP1 stimulates melanoma cell adhesion on several integrin substrates. Untreated cells (control) or cells treated with peptide FIRVVMYEGKK (p7N3, 10 µM) were allowed to adhere onto substrates coated with 25 µg/ml TSP1, 5 µg/ml vitronectin, 5 µg/ml VCAM-1, or 0.5 µg/ml type I collagen. The numbers of attached and spread cells/mm² are presented as mean ± S.D., n = 3.

**Fig. 1.** CD47-binding peptide from TSP1 stimulates melanoma cell adhesion on αβ1 and αvβ1 ligands. A, melanoma cell adhesion on recombinant portions of TSP1 and TSP2. N-terminal regions of TSP1 (NoC1) and TSP1 (NoC2) were used to eliminate the αβ1 integrin binding sites of these proteins. Cells were incubated on substrates coated with NoC1 (25 µg/ml) contains αβ1 and αβ1 integrin binding sites (19, 52), NoC2 (35 µg/ml, contains only an αβ1 binding site (19)), or vitronectin (5 µg/ml, an αβ1-dependent substrate). The cells were either untreated (Control), treated with control peptide FIRVVAIYEGKK (p604), or treated with a TSP1 peptide containing a CD47-binding sequence FIRVVMYEGKK (p7N3) alone or in combination with β1 integrin antagonist SB223245 (1 µM). Melanoma cells were allowed to adhere onto NoC1 (25 µg/ml), NoC2 (35 µg/ml), or vitronectin (5 µg/ml) substrates. The cells were either untreated (Control), treated with control peptide FIRVVAIYEGKK (p605) or the CD47-binding peptide from TSP1, FIRVVMYEGKK (p7N3) alone at 10 µM or in combination with the indicated concentrations of anti-CD47 antibody B6H12. Attached (left panel) and spread cells (right panel) are presented as the mean ± S.D., n = 3. B, anti-CD47 antibody B6H12 specifically stimulates αβ1 integrin-mediated spreading in the absence of TSP1 peptide. Melanoma cells were allowed to adhere on substrates coated with suboptimal concentrations of an αβ1 integrin ligand, NoC2 (20 µg/ml), or an αβ1 ligand, vitronectin (3 µg/ml). The cells were either untreated (control) or treated with the indicated concentrations of anti-CD47 antibody B6H12, a control anti-CD47 antibody (C1Km1, 20 µg/ml), or the CD47-binding peptide FIRVVMYEGKK (p7N3, 10 µM). Attached (left panel) and spread cells (right panel) are presented as mean ± S.D., n = 3.

**Fig. 2.** A CD47 function blocking antibody inhibits αβ1-mediated spreading but enhances αβ1 integrin-mediated spreading in the absence of a CD47-binding TSP1 peptide. Melanoma cells were allowed to adhere onto NoC1 (25 µg/ml), NoC2 (35 µg/ml), or vitronectin (5 µg/ml) substrates. The cells were either untreated (Control), treated with control peptide FIRVVAIYEGKK (p605) or the CD47-binding peptide from TSP1, FIRVVMYEGKK (p7N3) alone at 10 µM or in combination with the indicated concentrations of anti-CD47 antibody B6H12. Attached (left panel) and spread cells (right panel) are presented as the mean ± S.D., n = 3. B, anti-CD47 antibody B6H12 specifically stimulates αβ1 integrin-mediated spreading in the absence of TSP1 peptide. Melanoma cells were allowed to adhere on substrates coated with suboptimal concentrations of an αβ1 integrin ligand, NoC2 (20 µg/ml), or an αβ1 ligand, vitronectin (3 µg/ml). The cells were either untreated (control) or treated with the indicated concentrations of anti-CD47 antibody B6H12, a control anti-CD47 antibody (C1Km1, 20 µg/ml), or the CD47-binding peptide FIRVVMYEGKK (p7N3, 10 µM). Attached (left panel) and spread cells (right panel) are presented as mean ± S.D., n = 3.
from an enhancement of $\alpha_\beta_1$ integrin affinity by this antibody.

Consistent with the activity of the B6H12 on Jurkat cells, FIRVVMYEGKK stimulated $\alpha_\beta_1$ integrin-mediated adhesion on $\alpha_\beta_1$-dependent substrates for either unstimulated or TS2/16-stimulated Jurkat cells (Fig. 6A). The response to FIRVVMYEGKK on a TSP1 substrate was stronger than on the NoC2 fragment containing only the $\alpha_\beta_1$ integrin binding site (Fig. 6A), suggesting that interaction of the TSP1 type 3 repeats with $\alpha_\beta_1$ integrin may also be stimulated. To detect changes in $\alpha_\beta_1$-mediated adhesion specifically, we used a 33-kDa recombinant cell binding portion of fibronectin containing only this integrin recognition site (25). Adhesion on FN33 was stimulated by either FIRVVMYEGKK (Fig. 6A) or B6H12 (Fig. 6B). In contrast, FIRVVMYEGKK only weakly stimulated T cell adhesion on type I collagen. However, after activation of the cells using the $\beta_1$ integrin antibody, the peptide further stimulated adhesion on type I collagen (Fig. 6A). In contrast, the CD47 antibody B6H12 stimulated spreading of unstimulated T cells on collagen (Fig. 6B). As was found in melanoma cells, the B6H12 antibody did not inhibit stimulation by the TSP1 peptide, although significant additivity was not detected for any of the ligands tested.

$\alpha_\beta_1$ Integrin Is Physically Associated with CD47—CD47 was previously shown to physically associate with $\alpha_\beta_3$, $\alpha_\beta_2$, and $\alpha_\beta_1$ integrins (6, 8, 9, 17). Similarly, we found that $\alpha_\beta_1$ integrin associates with CD47 (Fig. 7). A detergent-solubilized CD47 complex immunoaffinity purified on immobilized B6H12 contained the characteristic unReduced 70- and 80-kDa $\alpha$ in
tegrin chains (37) as well as the 150-kDa unReduced $\beta_1$ chain. The integrins were not detected in the eluant from a control IgG column. These results suggest that CD47 may modulate $\alpha_\beta_1$ integrin function through a physical association.

T Cell Responses to Some CD47 Ligands Are CD47-independent—CD47 binding antibodies have been shown to act as both agonists and antagonists of specific CD47 responses (4, 38). Therefore, the differences in responses to integrin ligands induced by B6H12 and FIRVVMYEGKK in Fig. 6B could be explained by their acting as selective agonists of CD47. However, the recent evidence that a related CD47-binding peptide from TSP1 modulates platelet aggregation independent of CD47 (22) suggested an alternate explanation for these results. To determine whether modulation of T cell adhesion by the CD47-binding peptides required CD47 expression, we compared responses in wild type and CD47-deficient Jurkat cells (Fig. 8). In contrast to B6H12 (see Fig. 4D), peptides containing either of the known CD47-binding sequences from TSP1 had equivalent stimulatory activities for adhesion of Jurkat cells expressing or lacking CD47 (Fig. 8A). However, activities of peptides derived from both VVM motif regions in TSP1 in the CD47-deficient mutant were specific because control peptides with amino acid substitutions shown previously to ablate CD47 binding were inactive.

Notably, FIRVVMYEGKK containing the second VVM motif of TSP1 activated T cell adhesion to TSP1 to a greater extent than an optimal concentration of the $\beta_1$ integrin-activating antibody TS2/16 (Fig. 8A), suggesting that this stimulatory activity might also be $\beta_1$ integrin-independent. However, stimulation of adhesion by FIRVVMYEGKK was markedly diminished in $\beta_1$-deficient Jurkat cells (Fig. 8B). Similarly reduced stimulation of adhesion was observed using NoC2 (results not shown), but the peptide did not increase adhesion on fibronectin (Fig. 8B). The $\beta_1$-deficient clone lacks any detectable $\beta_1$ integrin by flow cytometry and is unresponsive to a $\beta_1$ function stimulating antibody but has normal levels of cell surface CD47 (19). Therefore, stimulation of T cell adhesion on fibronectin by the peptide is entirely $\beta_1$ integrin-dependent, but an alternate
adhesion receptor may also contribute to the TSP1 peptide enhancement of adhesion on TSP1 and NoC2. 1 integrins are required, however, for maximal responses on TSP1 and NoC2 substrates. CD47-mediated responses to the TSP1 peptides were shown previously to require pertussis toxin-sensitive G proteins (6, 9, 14, 17), whereas the CD47-independent activity of these peptides reported in platelets was sensitive to Src inhibitors (22). Remarkably, the stimulation by FIRVVMYEGKK of adhesion on limiting concentrations of TSP1 or NoC2 was equally sensitive to pertussis toxin in both the wild type and CD47-deficient Jurkat cells (Fig. 9), but the Src inhibitor PP2 had no effect on either response. Therefore, the adhesion responses to VVM peptides in both T cell lines differ from the aggregation response of platelets to the same peptides and involve G protein signaling that is independent of CD47.

**DISCUSSION**

We have identified two additional integrins that are functionally regulated by CD47, αβ1, and αβ3. Two CD47 ligands, VVM-containing peptides and the anti-CD47 antibody B6H12, stimulated cell attachment or spreading mediated by these integrins. Although activity of the CD47 antibody is clearly dependent on CD47 and we show that CD47 physically associates with αβ1 integrin, at least part of the activities of the VVM-containing peptides to stimulate αβ1-dependent adhesion are independent of CD47. The stimulatory activity of B6H12 for αβ1 integrin responses contrasts with the inhibitory activities of B6H12 for αβ3 (6) and αβ1,β3 integrin functions (9). Ligation of CD47 by B6H12, therefore, can selectively enhance and inhibit the function of different integrins. This dif-
CD47 ligands differentially regulate function of several integrins in T cells. A. A CD47-binding peptide activates \( \alpha_\beta_1 \) integrin in Jurkat cells. Jurkat cells were allowed to adhere onto TSP1 (16 \( \mu g/ml \)), a recombinant portion of TSP2 containing its \( \alpha_\beta_1 \) binding site (NoC2, 12 \( \mu g/ml \)), or a recombinant portion of fibronectin containing its \( \alpha_\beta_1 \) binding site (FN33, 25 \( \mu g/ml \)). Cells were either untreated (control) or treated with CD47-binding peptide FIRVVMYEGKK (p7N3, 10 \( \mu g/ml \)), control peptide FGMYEGKK (p604, 10 \( \mu g/ml \)), or the \( \beta_1 \)-activating antibody (TS2/16, 4 \( \mu g/ml \)) alone or in combination with p7N3 or p604. Attached cells are presented as mean \( \pm \) S.D., \( n = 3 \). B. anti-CD47 antibody B6H12 activates \( \alpha_\beta_1 \) integrin in Jurkat cells. Jurkat cells were allowed to adhere onto TSP1 (16 \( \mu g/ml \)), NoC2 (12 \( \mu g/ml \)), FN33 (25 \( \mu g/ml \)), and type I collagen (25 \( \mu g/ml \)). Cells were either untreated (control) or treated with CD47-binding peptide FIRVVMYEGKK (p7N3, 10 \( \mu g/ml \)), control peptide FGMYEGKK (p604, 10 \( \mu g/ml \)), or the \( \beta_1 \)-activating antibody (TS2/16, 4 \( \mu g/ml \)) alone or in combination with p7N3 or p604. Attached cells are presented as mean \( \pm \) S.D., \( n = 3 \).

Although ligation of CD47 is now known to stimulate functions of three \( \beta_1 \) integrins, this response is not universal in that \( \alpha_\beta_1 \) integrin function was not stimulated by a CD47-binding peptide (39). As previously demonstrated for \( \beta_1 \) integrins and \( \alpha_\beta_1 \), CD47 is physically associated with \( \alpha_\beta_1 \) in cells, where it regulates function of this \( \beta_1 \) integrin. Both CD47 (40, 41) and \( \alpha_\beta_1 \) integrin (42) associate with lipid rafts in Jurkat cells, suggesting that their interaction may involve these membrane microdomains. Notably, \( \alpha_\beta_1 \) activity is increased when CD47 translocates out of raft domains (43).

Function of the \( \alpha_\beta_3 \) integrin is modulated by CD47, and this integrin can in turn regulate \( \alpha_\beta_1 \) integrin (36). In this study, however, we show that regulation of \( \alpha_\beta_1 \) integrin by CD47 is independent of \( \alpha_\beta_1 \) integrin expression. Instead, CD47 may modulate \( \alpha_\beta_1 \) integrin function by physically associating with the integrin. Association of integrins with other extracellular proteins has been generally mapped to the extracellular domains of both the integrin and the associating membrane protein (44-46). Consistent with this finding, regulation of \( \alpha_\beta_1 \) integrin function on T cells appears to be distinct from the transmembrane domain (2).

Although some activities of the CD47-binding peptide from TSP1 are clearly mediated by CD47, the peptides must also interact with a different receptor. Activity of these TSP1 peptides in CD47-deficient platelets was partially dependent on FeR \( \gamma \) expression (22), but Jurkat cells do not express this receptor (47) and do not show the same sensitivity to Src inhibition as was reported in platelets (22). Understanding the role of CD47 in response to the TSP1 peptide is further complicated by our observation that the CD47-independent pathway shares the sensitivity to pertussis toxin that characterizes CD47 signaling. Until the second receptor for the TSP1 peptide is identified and its ability to recognize intact TSP1 is assessed, activities of these peptides should be interpreted with caution. Furthermore, recognizing that the CD47-binding peptides from TSP1 can, in at least two cell types, act independent of CD47 may necessitate a reexamination of the conclusion that CD47 “function-blocking” antibodies block a CD47-signaling pathway. In almost all such cases, the reported blocking of CD47 responses represents antagonism of TSP1 peptide responses (6, 17, 32). An equally plausible hypothesis is that the CD47 antibody indirectly antagonizes signaling stimulated by binding of the TSP1 peptides to a receptor other than CD47. In this case, the observed antagonism could be a negative cross-talk between two agonist pathways.

With a few exceptions (4), the CD47 antibody B6H12 has been generally found to block responses mediated by other CD47 ligands (7, 17, 31-35). Our data confirm that B6H12 reverses the activation of \( \alpha_\beta_1 \) integrin function stimulated by a CD47-binding peptide from TSP1, but we also show that the antibody has a direct stimulating function for at least two \( \beta_1 \) integrins in the same cells. This stimulating function was confirmed in both melanoma cells and T cells. Therefore, it may be proper to reclassify B6H12 as a function-modifying antibody for CD47. A function blocking antibody recognizing \( \beta_1 \) integrins was similarly shown to allosterically modulate ligand binding (48). Because B6H12 has opposing effects on \( \beta_1 \) and \( \beta_2 \) integrin function in melanoma cells, both the positive and negative effects of B6H12 on integrin function may result from direct or allosteric regulation of CD47 association with or signaling to integrins rather than direct inhibition of ligand binding to CD47.

In T cells, peptide FIRVVMYEGKK and a \( \beta_1 \) integrin-activating antibody had additive effects on adhesion to TSP1 and
collagen. However, the peptide had minimal activity in the absence of the β1 integrin-activating antibody, suggesting that TSP1 signaling through CD47 is insufficient to activate αβ1 integrin in these cells. The additivity also suggests that the effect of this signal is to increase integrin avidity rather than affinity. Likewise, the maximal dose of the TSP1 peptide only stimulated ~50% of T cells to attach on an αβ1 integrin ligand, whereas the addition of the β1-activating antibody further stimulated αβ1-dependent adhesion at saturating doses of the TSP1 peptide. This differs from the strong stimulation of αβ1-mediated adhesion by the CD47-binding peptide. Thus, the TSP1 peptide differentially affects β1 integrins in the T cells. The mechanisms for differential cross-talk between this signal and each integrin remain to be defined.

B6H12 was previously reported to delay neutrophil transmigration toward formylmethionyleucylphenylalanine (49). B6H12 also inhibited chemotaxis of endothelial cells stimulated by TSP1 and its CD47-binding peptide (32). We found that B6H12 inhibited T cell chemotaxis to TSP1, NoC1, and NoC2 (19). Because NoC1 and NoC2 do not contain the CD47-binding sites, we concluded that CD47 ligation must indirectly inhibit migration (19). This inhibition was interpreted as indicating a positive requirement for CD47 in αβ1-mediated chemotaxis, consistent with the absence of motility in CD47-deficient cells (19), but the same results could also be obtained if B6H12 binding to CD47 increased the avidity of αβ1. Inhibition of cell motility has been previously observed as a result of activation as well as inhibition of integrins (50, 51). B6H12 may, therefore, inhibit migration by activating integrins.

CD47 and αβ1 integrin are two signaling receptors that sense the extracellular environment. Because TSP1 is a ligand for both receptors, we examined the roles of each in mediating responses of T cells to TSP1 (19). For mediating adhesion on TSP1, αβ1 was necessary and sufficient (19). However, the present results show that ligation of CD47 can modulate this response by activating αβ1. For stimulating chemotaxis to TSP1, the integrin and CD47 are both necessary, but direct interaction of TSP1 with the integrin is sufficient to stimulate chemotaxis. The same mechanism applies to regulation of matrix metalloproteinase gene expression; CD47 and αβ1 are both necessary, but the role of CD47 is indirect. Conversely, for inhibiting T cell receptor signaling or T cell proliferation, CD47 is necessary, but αβ1 integrin is not. Therefore, we concluded that CD47 and αβ1 integrin are each functional signaling receptors for TSP1 in T cells that elicit distinct signaling pathways (19). In some cases, these pathways engage in cross-talk. In addition to potential cross-talk between their downstream effectors, the present data demonstrate that CD47 may modulate αβ1 integrin function through lateral interactions in the plasma membrane. We found that ligation of CD47 can increase αβ1 integrin activity to mediate cell adhesion, but these lateral interactions may also modulate outside-in signaling through αβ1 integrin. The mechanism of this regulation and the possible requirement for other membrane proteins to mediate CD47-αβ1 cross-talk remains to be defined.

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Regulation of Integrin Function by CD47 Ligands: DIFFERENTIAL EFFECTS ON ανβ3 AND α4β1 INTEGRIN-MEDIATED ADHESION

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