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Endothelial CD47 Promotes Vascular Endothelial-Cadherin Tyrosine Phosphorylation and Participates in T Cell Recruitment at Sites of Inflammation In Vivo

Veronica Azcutia,*†,1 Michael Stefanidakis,*†,1 Naotake Tsuboi,*†,3 Tanya Mayadas,*†,7 Kevin J. Croce,†‡ Daiju Fukuda,†‡ Masanori Aikawa,†‡ Gail Newton,* and Francis W. Luscinskas*†

At sites of inflammation, endothelial adhesion molecules bind leukocytes and transmit signals required for transendothelial migration (TEM). We previously reported that adhesive interactions between endothelial cell CD47 and leukocyte signal regulatory protein γ (SIRPγ) regulate human T cell TEM. The role of endothelial CD47 in T cell TEM in vivo, however, has not been explored. In this study, CD47−/− mice showed reduced recruitment of blood T cells as well as neutrophils and monocytes in a dermal air pouch model of TNF-α–induced inflammation. Reconstitution of CD47−/− mice with wild-type bone marrow cells did not restore leukocyte recruitment to the air pouch, indicating a role for endothelial CD47 in the defect in leukocyte TEM in the CD47−/− endothelium. We previously reported that adhesive interactions between endothelial cell CD47 and leukocyte signal regulatory protein γ (SIRPγ) regulate human T cell TEM. The role of endothelial CD47 in T cell TEM in vivo, however, has not been explored. In this study, CD47−/− mice showed reduced recruitment of blood T cells as well as neutrophils and monocytes in a dermal air pouch model of TNF-α–induced inflammation. Reconstitution of CD47−/− mice with wild-type bone marrow cells did not restore leukocyte recruitment to the air pouch, indicating a role for endothelial CD47 in the defect in leukocyte TEM in the CD47−/− endothelium was corroborated by intravital microscopy of inflamed cremaster muscle microcirculation in bone marrow chimera mice. In an in vitro human system, CD47 on both HUVEC and T cells was required for TEM. Although previous studies showed CD47-dependent signaling required Gαi-coupled pathways, this was not the case for endothelial CD47 because pertussis toxin, which inactivates Gαi, had no inhibitory effect, whereas Gαo was required by the T cell for TEM. We next investigated the endothelial CD47-dependent signaling events that accompany leukocyte TEM. Ab-induced cross-linking of CD47 revealed robust actin cytoskeleton reorganization and Src- and Pyk-2–kinase dependent tyrosine phosphorylation of the vascular endothelial-cadherin cytoplasmic tail. This signaling was pertussis toxin insensitive, suggesting that endothelial CD47 signaling is independent of Gαi. These findings suggest that engagement of endothelial CD47 by its ligands triggers outside-in signals in endothelium that facilitate leukocyte TEM. The Journal of Immunology, 2012, 189: 2553–2562.

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not restore leukocyte recruitment to the air pouch, indicating a role for endothelial CD47. The defect in leukocyte TEM in the CD47−/− endothelium was corroborated by intravital microscopy of inflamed cremaster muscle microcirculation in bone marrow chimera mice. In an in vitro human system, CD47 on both HUVEC and T cells was required for TEM. Although previous studies showed CD47-dependent signaling required Gαi-coupled pathways, this was not the case for endothelial CD47 because pertussis toxin, which inactivates Gαi, had no inhibitory effect, whereas Gαo was required by the T cell for TEM. We next investigated the endothelial CD47-dependent signaling events that accompany leukocyte TEM. Ab-induced cross-linking of CD47 revealed robust actin cytoskeleton reorganization and Src- and Pyk-2–kinase dependent tyrosine phosphorylation of the vascular endothelial-cadherin cytoplasmic tail. This signaling was pertussis toxin insensitive, suggesting that endothelial CD47 signaling is independent of Gαi. These findings suggest that engagement of endothelial CD47 by its ligands triggers outside-in signals in endothelium that facilitate leukocyte TEM. The Journal of Immunology, 2012, 189: 2553–2562.

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because displacement of AJ proteins, like vascular endothelial-cadherin (VE-cad), is induced transiently by leukocytes (2–4). Recent studies have provided insight into the underlying mechanisms of TEM at cell–cell junction (paracellular TEM). The engagement of adhesion molecules, such as VCAM-1 and ICAM-1 by their T cell counterreceptors αβ1 and αβ2 integrins, and CD44 interacting with hyaluronan during TEM, triggers outside-in signaling in endothelial cells that results in alterations in proteins localized at cell junctions (reviewed in Refs. 5–7). For example, cross-linking of ICAM-1 induces endothelial actin–cytoskeleton remodeling and phosphorylation of cortactin and VE-cad by Src and Pyk2 protein tyrosine kinases in the endothelium (8, 9). Subsequent studies have implicated tyrosine phosphorylation of the VE-cad cytoplasmic tail as a key event leading to dissociation of the AJs through an incompletely understood mechanism (reviewed in Ref. 6). These latter events, VE-cad phosphorylation, VE-protein tyrosine phosphatase dissociation from VE-cad, and the formation of a VE-cad complex gap, are considered necessary events in leukocyte paracellular TEM (8, 10–13).

CD47 (integrin-associated protein) is a 50-kDa transmembrane glycoprotein expressed by most cell types (14, 15). In endothelial cells, CD47 is present on the apical surface and is enriched at endothelial cell–cell junctions (16, 17). CD47 has been shown to interact in cis with αβ1, αβ3, and αβ1 integrins and in trans with members of the signal regulatory protein (SIRP) family and with thrombospondins (reviewed in Ref. 14). SIRPs are a family of regulatory membrane proteins expressed mainly by leukocytes and neurons. SIRPs and SIRPs are ligands for CD47 (18). SIRPs are abundantly expressed on myeloid cells and smooth muscle cells and at low levels by cultured murine and human endothelium (16, 17).
SIRPγ expression is restricted to T cells, NK cells, and some B cells (19, 20). Unlike SIRPα, SIRPγ does not appear to signal to the cytoplasm because its short cytoplasmic tail has no consensus signaling motifs (19).

Previous studies in CD47−/− mice demonstrated that CD47 plays a role in neutrophil emigration in a bacteria-induced murine peritonitis model (21), a LPS-induced acute lung injury and bacterial pneumonia model (22), a TNBS-induced colitis model (23), hapten-stimulated inflammation (24), and in vitro models of neutrophil TEM of endothelium (25) and epithelium (26), and monocyte TEM of endothelium (27). Recently, we reported that human endothelial CD47 interacting with T cell-expressed SIRPγ is required for T cell TEM under flow conditions in vitro (17). The downstream signals mediated by CD47 in the endothelium during T cell TEM and its potential role in leukocyte recruitment in vivo, however, have not been explored.

Based on reports that endothelial cell adhesion molecules involved in leukocyte TEM trigger intracellular signals and elicit downstream effects, we hypothesized that engagement of CD47 triggers alterations in the endothelial cell cytoskeleton and VE-cad phosphorylation, both of which are necessary for TEM. We report that CD47−/− mice have a profound defect in neutrophil, CD3+ T cell, and monocyte recruitment in a dermal air pouch model of TNF-α–induced inflammation that is dependent on CD47 on parenchymal cells, presumably the endothelium, and provide evidence that endothelial CD47 generates intracellular signals that are necessary for leukocyte TEM.

Materials and Methods

Mice

CD47 knockout (CD47−/−) mice (C57BL/6 strain) have been previously described (21) and obtained from E. Brown (Genentech, San Francisco, CA). Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA), and a breeding colony was established in our facility for use as WT control animals. Mice were maintained in a specific pathogen-free barrier unit at our institution. Animal care and experimentation were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Reagents and Abs

Recombinant human TNF-α and CXCL12 were from PeproTech (Rocky Hill, NJ). Recombinant murine TNF-α was purchased from BioLegend (San Diego, CA). The Src kinase inhibitor PP2 and the p38 MAPK inhibitor U0126 were obtained from Calbiochem (San Diego, CA). Pertussis toxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO). The following mAbs were used as purified IgG: mAb B6H12 is a function-blocking mAb to human CD47, and mAb TS1/22 is a function-blocking mAb against α5β1 integrin (American Type Culture Collection, Manassas, VA); mAb 2D3 is a nonblocking anti-CD47 mAb (28) obtained from E. Brown; unlabeled and FITC-labeled rat anti-mouse CD47 function-blocking mAb (miap301) was from BioLegend; anti-VE-cad mAb Hec-1 (29) was from W. Muller (Northwestern University, Chicago, IL); anti-HLA class I (clone 1 MHC) mAb W6/32, anti-ICAM-1 mAb HU5/55, and function-blocking anti-JAM-A mAb HIZA9 were as previously described (30). The blocking mAb to e40HP2 was from Immunotech (Glendale, CA). The anti-phosphotyrosine–specific mAb 4G10 was from Millipore (Billerica, MA). Phospho-specific Abs to VE-cad (pY560 and pY731) and P AK2 (pY402) were from BioSource International (Camarillo, CA). Phosphospecific and polyclonal anti-Src and anti-p38 MAPK Abs were from Cell Signaling Technology (Danvers, MA). FITC–conjugated markers for CD3 T cells (145-2c1), monocytes and macrophages (allophycocyanin–conjugated anti-F4/80), neutrophils (allophycocyanin–conjugated GR1 Ab), and isotype control mAbs were from BD Biosciences (San Jose, CA). Alexa-488– or FITC–conjugated F(ab′)2 goat anti-mouse IgG and phalloidin–Alexa 488 were from Invitrogen (Carlsbad, CA).

In vivo transmigration assay (s.c. air pouch model)

Air pouches were created in the dorsal portion of the back of 8- to 10-wk-old C57BL/6 WT and CD47−/− male mice, as previously described (31). PBS or PBS-containing murine rTNF-α (500 ng) was injected in each air pouch, and 4 or 24 h later cell infiltrates were harvested by lavage with repeated PBS washes. The recovered volume was measured, and the number of recovered cells was determined by hemocytometry. The frequency of CD3+ T cells, neutrophils, and monocytes/macrophages was determined by staining with primary labeled mAb specific for these cell types and acquired on a FACSCalibur flow cytometer (BD Biosciences). FACs data were analyzed using FlowJo software (Treestar, Ashland, OR).

Bone marrow transplantation protocol

Donor bone marrow (BM) cells were harvested aseptically from femurs and tibias of 8- to 10-wk-old WT mice.Recipient CD47−/− and WT male mice (8 wk old) received a lethal dose of whole body irradiation with a cesium source (1200 rad in 2 doses, 4 h apart), as described previously (32). Donor BM cells (2 × 106 cells in 0.3 ml 3% FBS in PBS) were transfused by tail vein injected into irradiated WT or CD47−/− recipient mice. Recipient mice received normal chow and water containing antibiotics (Sulfatrarin) 1 wk prior to and 6 wk after transplantation. Air pouch leukocyte recruitment studies were performed 8–9 wk post-BM transplantation. To confirm BM engraftment, leukocytes from chimeric and WT mice were stained with FITC rat anti-mouse CD47. Expression levels were acquired on a FACSCalibur flow cytometer and analyzed using FlowJo software.

Intravital microscopy of leukocyte recruitment during microvascular inflammation in vivo

Intravital microscopy (IVM) of leukocyte transmigration of postcapillary venules of the mouse cromasser muscle was performed at 2 h following intrascrotal injection of mouse TNF-α (500 ng in saline/mouse), as initially described (33). Mice were anesthetized, and surgical exteriorization of the cromasser muscle was performed as we have recently described (34). Microvessel data and physiology indices were obtained using a specialized Olympus FV 1000 intravital microscope (Olympus, Center Valley, PA) fitted with an Olympus 403 water immersion objective (Olympus) (34). Leukocyte transmigration events were recorded from 8 to 12 vessels per mouse using an Olympus DP71 CCD video camera and Olympus Fluoview 1000 software (Olympus Imaging America, Melville, NY). Videos were acquired with the National Institutes of Health software package ImageJ (National Institutes of Health, Bethesda, MD). The number of transmigrated leukocytes per vessel was determined by counting the average number of perivascular cells in a 50 × 100-μm area adjacent to the vessel wall, as previously described (34, 35).

Measurement of leukocytes in murine peripheral blood

Peripheral blood from mice was collected in EDTA-coated vials (Sarstedt) at 4 and 24 h after injections of PBS or PBS–TNF-α. A complete blood count with cellular differential was assessed by automated multispecies hematology instrument (Hemavet 950FS; Drew Scientific, Oxford, CT).

Isolation of human endothelial cells and T cells and TEM under flow conditions

Human CD3 T cells (>95% purity) were isolated by negative selection from anticoagulated whole blood obtained from healthy volunteers, as previously described (17). Blood was obtained from volunteer donors according to Brigham and Women’s Hospital Institutional Review Board–approved protocols for protection of human subjects, and all volunteer subjects gave informed consent, in accordance with the Declaration of Helsinki. Pooled HUVEC were isolated and cultured, as described (17). Confluent HUVEC monolayers were used in all biochemical and TEM studies. Confluent HUVEC monolayers on fibronectin-coated glass coverslips were stimulated with TNF-α (4 h, 25 ng/ml), followed by treatment with CXCL12 (50 ng/ml) for 15 min, a step that promotes T cell TEM, prior to insertion into the flow chamber thermostated to 37°C. T cells (1 × 107 in 0.1 ml) were drawn across HUVEC monolayers at 0.8 dynes/cm2, and the accumulation/mm2 and TEM were measured, as described (17). The percentage of TEM was calculated as follows: total transmigrated T cells/[total adhered + transmigrated T cells] × 100.

RNA interference

Predesigned small interfering RNA (siRNA) against CD47 was obtained from Qiagen (Valencia, CA). The silencing siRNA target sequences (catalog SB0489382 and SB0117401), 5′-CAGCTTTACGTGTT-TACGT-3′ and 5′-TGAGGTTGGTTTCCGCC3′–5′ were analyzed offline and surface-expressed CD47 by 70–80%. A predesigned siRNA oligonucleotide sequence targeting CD47 but with no inhibitory effect on CD47 expression (target sequence, 5′-CACAGTAAGTTTACCCCCCA-3′; catalog
As a control, HUVEC were incubated with secondary mAb only. HUVEC stress fibers were considered positive. Results are representative of four or more separate experiments.

Flow cytometry and analysis

Confluent resting or 4-h TNF-α–stimulated HUVEC monolayers were trypsinized and stained for expression of CD47 and ICAM-1 using a ratio of 10 beads per cell. The expression levels were acquired on a FACScanLibur flow cytometer and analyzed using FlowJo software.

CD47 Ab cross-linking studies and Western blotting

Twenty-four–hour TNF-α–stimulated HUVEC in 35-mm diameter wells (6-well culture plate; Corning Life Sciences, Lowell, MA) were serum starved for 2 h in OPTI-MEM I (Life Technologies/Invitrogen) and then incubated for 10 min at 37°C with Dynabeads (Invitrogen Dynal AS, Oslo, Norway) prebound to mAb to CD47 (B6H12). ICAM-1 (Hu5/3), class I MHC, or murine IgG Ab (10 μg/ml) using a ratio of 10 beads per cell. The HUVEC were washed twice in ice-cold PBS to remove nonadherent beads and immediately lysed in 250 μl boiling sample buffer under reducing conditions. Cell lysates were diluted to allow immunoprecipitation with anti-phosphotyrosine mAb 4G10, as described previously (8, 11). Recovered proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and Western blotted for phospho-Src (Tyr416), phospho-Pyk2 (Tyr405), phosphor-VE-cad (Tyr573), and phosphor-VE-cad (Tyr368) and detected by an ECL (Pierce Biotechnology, Rockford, IL) following incubation with appropriate HRP-conjugated secondary Abs. Band intensity was quantified by NIH Image J software.

Statistical analysis

Data are expressed as mean ± SEM and were compared using Student’s t test for experiments with two groups or one-way ANOVA with Tukey multiple comparison posttest for experiments with three or more groups by Prism software v5.00 for Windows (GraphPad, San Diego, CA). Data were considered statistically significant at p < 0.05.

Results

CD47−/− mice exhibit defective leukocyte recruitment to TNF-α in a dermal air pouch model of inflammation

CD47 has been shown previously to play a role in T cell recruitment in vitro under physiologically relevant shear flow conditions (17, 37). In this study, the involvement of CD47 in regulating TNF-α–induced leukocyte recruitment was investigated using a skin air pouch model used by other investigators to examine the contribution of adhesion molecules and chemokines to leukocyte recruitment (31, 38). In WT mice, TNF-α triggered increased leukocyte accumulation in air pouches at 4 and 24 h (Fig. 1A). Flow cytometric analysis showed that recruited leukocytes consisted of neutrophils, T cells, and monocytes/macrophages (Fig. 1B–D). In contrast to WT mice, recruitment of each leukocyte type in CD47−/− mice was dramatically reduced at both 4 and 24 h. The peripheral blood leukocyte counts at 0, 4, and 24 h were similar in both lines of mice (Fig. 1E). Consistent with a previous study, the percentage of lymphocytes in blood was reduced by 22% in CD47−/− compared with WT, as shown in Table I (21, 39). We conclude that CD47 plays an important role in the recruitment of blood T cells, neutrophils, and monocytes in this murine model of inflammation.

We next addressed the role of BM and host-expressed CD47 in leukocyte recruitment in the air pouch model by comparing CD47−/− and WT mice reconstituted with WT BM (WT BM→CD47−/−; WT BM→WT mice). The reciprocal reconstitution of CD47−/− BM→WT is not feasible because transfused CD47−/− BM cells are cleared rapidly from the circulation by splenic macrophages in WT animals, and these mice do not survive (40). Reconstituted WT BM→CD47−/− mice had comparable levels of CD47 expression on leukocytes as WT BM→WT animals. Analysis of TNF-α–induced leukocyte accumulation in these animals showed that reconstitution of CD47−/− mice with WT BM (WT BM→CD47−/−) does not rescue the defect in recruitment of neutrophils, T cells, or monocytes in response to TNF-α at both 4 and 24 h (Fig. 2A–D). Because WT BM→CD47−/− chimeric animals have CD47+ hematopoietic cells, but lack CD47 in parenchymal cells, these data indicate host cell CD47, presumably the vascular endothelium, is necessary for leukocyte recruitment in vivo.

IVM studies in CD47−/− BM chimeras mice support a role for endothelial CD47 in leukocyte TEM

To directly visualize leukocyte TEM in vivo, we performed IVM experiments in 2-h TNF-α–activated cremaster muscle microcirculation of WT BM→CD47−/−/− chimeric mice. Hemodynamic parameters and estimated wall shear rates were similar among the animals after TNF-α treatment (data not shown). As shown in Fig. 2E, the number of emigrated leukocytes in WT BM→CD47−/−/− chimeric animals was significantly less as compared with the WT BM→WT chimeric animals. Representative images of a segment of venules in WT and CD47−/−/− animals depicting transmigrated leukocytes are shown (Fig. 2F, 2G). The results in the chimeras will indicate that endothelial cell CD47 contributes directly to leukocyte TEM in vivo.

Both human endothelial and leukocyte CD47 are required for T cell TEM

Human CD3+ T cells (95 ± 11% purity) arrest uniformly on 4- or 18-h TNF-α– or IL-1β–activated HUVEC and transmigrate at cell junctions in the presence of exogenously added apical chemokine CXCL12 under shear flow conditions (17, 41). Pretreatment of TNF-activated HUVEC with blocking mAb to CD47 had no significant effect on T cell accumulation (Fig. 3A), but significantly reduced T cell TEM (Fig. 3B) as compared with control mAb to MHC class I. Function blocking mAb to ICAM-1 in HUVEC (Fig. 3B), or to αβ integrin, its ligand on T cells, also significantly reduced T cell TEM, whereas anti-α4 integrin-blocking mAb did not (Supplemental Fig. 1), which is consistent with a previous report (41). Next, the effect of CD47 blockade in T cells was examined in parallel. Treatment of T cells with anti-CD47 mAb significantly reduced T cell transmigration without altering T cell adhesion. mAb blocking of CD47 in both HUVEC and T cell was no more effective than blocking either cell type alone. These data demonstrate that both endothelial and T cell CD47 play an important role in T cell TEM in vitro.

siRNA knockdown of CD47 in HUVEC reduces T cell TEM under flow

To corroborate CD47 mAb blocking of TEM in Fig. 3, siRNA silencing of CD47 in HUVEC was performed. Silencing of CD47 by two different siRNA caused a 70–80% reduction (n = 4) in surface CD47 staining in resting (data not shown) and 4-h TNF-α–
activated HUVEC (Fig. 4A, 4B) as compared with control (non-silencing) siRNA targeting of CD47. As observed with CD47 mAb blocking, CD47 siRNA significantly reduced T cell TEM and also did not alter T cell accumulation (Fig. 4C, 4D). However, HUVEC transfected with control targeting CD47 siRNA, but with no inhibitory effects, supported T cell adhesion and robust TEM (Fig. 4C, 4D). Live cell differential interference contrast (DIC) imaging of TEM showed that adherent T cells polarize and transmigrate in control siRNA-treated TNF-activated HUVEC, whereas treatment of HUVEC with CD47-specific siRNA inhibited T cell TEM (Fig. 4E, arrowheads identify nontransmigrated T-cells; arrows, transmigrated T cells). To control for off-target effects of siRNA, we observed that silencing CD47 siRNA and nonsilencing control CD47 siRNA-treated HUVEC showed no difference in the expression of ICAM-1 (Fig. 4F), or in surface expression of VE-cad, VCAM-1, or E-selectin in 4-h TNF-α–activated HUVEC (data not shown).

PTX treatment of endothelial cells does not inhibit T cell TEM under shear flow conditions

CD47 has been reported to signal through a heterotrimeric Gi-coupled receptor complex in a variety of cell types (14, 15, 42). To examine whether CD47 signals through the Gi pathway in endothelium during TEM, we preincubated HUVEC with different concentrations of PTX for 5 h to inactivate Gi subunits and assessed T cell adhesion and TEM (Fig. 5A, 5B). PTX pretreatments of HUVEC had no inhibitory effect on T cell adhesion or TEM, whereas PTX treatment of T cells significantly blocked transmigration (Fig. 5C), consistent with earlier reports (41, 43, 44). Videomicroscopy of the TEM assay revealed that adherent T cells flatten, polarize, and transmigrate in PTX-treated HUVEC (Fig. 5D, left panel; arrowheads). In contrast, pretreatment of T cells with PTX abrogated T cell TEM, and most adherent T cells did not flatten or migrate on the HUVEC monolayer (Fig. 5D, right panel; arrowheads). Because PTX treatment of rodent endothelium has variable inhibitory effects, ranging from no effect in T cell TEM of rat HEV (43) to 80% inhibition of T cell TEM of murine brain endothelial cells in vitro (45) or neutrophil and eosinophil TEM of endothelial monolayers isolated from Gi2/2 mice (46), we tested the effects of PTX in endothelium derived from human dermal microvessels and saphenous vein. Consistent with the results in HUVEC, T cell adhesion or TEM across either human dermal microvessels or human saphenous vein was not inhibited by PTX pretreatment (Supplemental Fig. 2A, 2B).

### Table I. Blood neutrophil and lymphocyte differential and total numbers in WT and CD47−/− mice

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<thead>
<tr>
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<th>% NE</th>
<th>% LY</th>
<th>WBC (×10^3/ml)</th>
<th>NE</th>
<th>LY</th>
<th>n</th>
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<tr>
<td>WT</td>
<td>28.11 ± 2.42</td>
<td>64.17 ± 2.94</td>
<td>5.02 ± 2.07</td>
<td>1.47 ± 0.30</td>
<td>3.16 ± 0.40</td>
<td>10</td>
</tr>
<tr>
<td>CD47−/−</td>
<td>41.19 ± 3.57**</td>
<td>49.83 ± 3.10**</td>
<td>4.95 ± 0.56</td>
<td>2.06 ± 0.27</td>
<td>2.46 ± 0.32</td>
<td>9</td>
</tr>
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A complete blood count with cellular differential was determined by automated multispecies hematology instrument (Hemavet 950FS; Drew Scientific, Oxford, CT). CD47−/− % NE and % LY are significantly different from WT. **p < 0.01 (Student t test).

LY, Lymphocyte; NE, neutrophil.
Treatment of HUVEC with PTX had no effect on the TNF-α-induced increase in ICAM-1, E-selectin, or VCAM-1 (data not shown), and the treated and control monolayers maintained the same normal cobblestone morphology throughout the study. In addition, PTX treatment of HUVEC had no effect on neutrophil TEM under flow conditions (data not shown). These results suggest a CD47-dependent mechanism that is independent of Gαi protein signaling in human endothelium during TEM.

Cross-linking of endothelial cell CD47 triggers robust cytoskeletal remodeling and tyrosine phosphorylation of VE-cad

Rapid remodeling of the endothelial actin cytoskeleton and increased phosphorylation of tyrosine residues 658 and 731 in the cytoplasmic domain of VE-cad are necessary events in T cell and neutrophil TEM under flow conditions (data not shown). These results suggest a CD47-dependent mechanism that is independent of Gαi protein signaling in human endothelium during TEM.

ICAM-1 cross-linking by anti–ICAM-1 mAb or mAb-coated polystyrene beads are well-characterized approaches that mimic ICAM-1 binding by leukocytes. We next assessed the effects of incubating 24-h TNF-α–activated HUVEC with 3 μm Dynal beads coated with either anti-CD47 or anti–ICAM-1 mAbs and found a 2- to 3-fold increase in phosphorylation of tyrosine 658 and 731 in the VE-cad cytoplasmic tail (Fig. 6B, 6C) as compared with medium or anti-class I MHC mAb–coated beads in both cases. Class I mAb–coated beads bind equally as well as CD47 mAb-coated beads to HUVEC monolayers, but did not trigger increased phosphorylation of VE-cad tyrosine 658 or 731. Previous studies have shown that engagement of ICAM-1 activates Src and Pyk2 family kinases, which lead to tyrosine phosphorylation
of several endothelial cell proteins, including VE-cad (8, 10, 36). To determine whether engaging CD47 also induced Src and Pyk2 kinase activation, we challenged CD47 and ICAM-1 or as control class I MHC with appropriate mAb-coated beads. Western blot analysis of cell lysates with phospho-specific Abs directed against activation-induced phosphorytrosine py416 of Src or pY402 of Pyk2 was performed. Both anti-CD47 and anti–ICAM-1 mAb-coated beads triggered a 2-fold increase in phosphorylation of both Src and Pyk2 (Fig. 7A). In contrast, class I mAb beads did not alter phosphorylation of either Src or Pyk2.

We next used a pharmacological approach to corroborate involvement of Src and Pyk2 family kinases or PTX to block Gαi-coupled signaling. Because ICAM-1 cross-linking by mAbs is known to induce MAPK activation (reviewed in Ref. 47), we also used a p38 MAPK inhibitor to test its effects on ICAM– and CD47-induced VE-cad phosphorylation. Interestingly, ICAM–1– and CD47-induced VE-cad phosphorylation was not prevented by pretreatment of HUVEC with either PTX or the p38 MAPK inhibitor, SB203580 (Fig. 7B). However, pretreatment of cells with Src kinase inhibitor, PP2, significantly inhibited both ICAM–1– and CD47-induced VE-cad phosphorylation at tyrosines 658 and 731 (Fig. 7B).

Discussion

A number of endothelial-expressed adhesion molecules, including ICAM-1, VCAM-1, ESAM, CD99, and PECAM-1, are involved in adhesive and/or intracellular signaling events required for leukocyte transmigration (5–7). In addition, endothelial presented chemokines (e.g., IL-8, MCP-1) and chemoattractants (e.g., LeukotrieneB4) promote leukocyte arrest and polarized migration on and across the endothelium and into tissues (48, 49). CD47 is involved in a broad range of cell adhesion processes, including neutrophil transepithelial and transendothelial migration (25, 26), monocyte transendothelial migration (27), T cell transendothelial migration (17), human platelet adhesion to vascular endothelium (50), dendritic cell migration (51, 52), as well as regulation of innate and adaptive immune responses (reviewed in Refs. 14, 53). In human endothelial cells, we recently reported that CD47 was localized to the apical surface and was enriched at cell–cell junctions, and that adhesive interactions between endothelial cell CD47 and human T cell-expressed SIRPγ played an important role in T cell TEM under flow conditions in vitro (17). In this study, we examined the contribution of endothelial CD47 in leukocyte recruitment in an experimental animal model of inflammation and sought to gain insight into signals elicited by CD47 engagement in endothelium that might contribute to TEM events.

CD47−/− animals have defects in leukocyte recruitment

A murine dermal air pouch was employed as a model of TNF-α–induced skin inflammation to study the role of CD47 in leukocyte extravasation (31, 38). The data show that injection of TNF-α into the dorsal site of WT mice induced a time-dependent accumula-

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**FIGURE 3.** Both endothelial and T cell-expressed CD47 are required for TEM. (A and B) Four-hour or 18-h TNF-α (10 or 25 ng/ml)–activated HUVEC monolayers were preincubated with function-blocking mAb to CD47 (B6H12), ICAM-1 (Hu5/3), or a control binding mAb to class I MHC (W6/32) (each mAb used at 30 μg/ml, 30-min preincubation). Where noted, human T cells were also preincubated with function-blocking mAb to CD47 (Tc). The total number of adherent T cells (T-cells/mm²) and the percentage of adherent T cells that transmigrated after 10 min were determined by live cell imaging, as described earlier (17). Data are mean ± SEM, n = 3 independent experiments. *p < 0.003 versus medium or class I for CD47 (EC + Tc), and p < 0.002 versus medium or class I for CD47 (Tc), p < 0.01 versus medium or class I for CD47 (EC + Tc), and p < 0.02 versus medium or class I for ICAM-1 (one-way ANOVA, followed by the Tukey test). Medium versus class I was not statistically different (p = 0.34).

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**FIGURE 4.** CD47 siRNA-treated HUVEC show reduced T cell transmigration. (A) Four-hour TNF-α–stimulated HUVEC previously transfected with CD47-targeting siRNA, control (nonsilencing CD47-targeted) siRNA, or sham-treated (transfection reagent without siRNA) were assessed for CD47 expression by flow cytometry, as described in Materials and Methods (data are representative of n = 5; control = nonbinding mouse IgG). (B) Fluorescent micrographs of CD47 surface staining in HUVEC transfected with control or CD47-silencing siRNA. Scale bar, 20 μm. Total fluorescence of CD47 signal was reduced by 75%, as assessed by ImageJ software. (C and D) TNF-α–stimulated HUVEC were transfected with CD47-targeted siRNA, a control, nonsilencing CD47-targeted siRNA or left untreated, or HUVEC were pretreated with function-blocking mAb to CD47 (B6H12), CD47 nonblocking (2D3) or control class I MHC mAb, and adhesion and TEM, assessed as described in Materials and Methods. Data are the mean ± SEM of duplicate coverslips for each condition, three independent experiments. *p < 0.05 for indicated comparisons of CD47 siRNA versus control siRNA and blocking versus nonblocking CD47 mAb (Student t test). (E) The morphology of T cells on CD7-targeted siRNA-treated (arrowheads) and control, nonsilencing CD7-targeted siRNA (arrows) HUVEC is shown as DIC images of live cells at the conclusion of the TEM assay (∼20 objective). Arrowheads identify T cells that have defects in polarization, and arrows identify T cells that completed TEM. (F) HUVEC transfected with CD47-targeting or CD47-targeted, nonsilencing (control) siRNA stimulated with TNF-α for 4 h show no difference in ICAM-1 expression (representative histogram from n = 3).
tion of neutrophils, T cells, and monocytes in the air pouch exudates, whereas recruitment of leukocytes in CD47−/− mice was strikingly reduced and nearly absent. Analysis of BM chimera mice indicates that endothelial cell CD47 plays a significant role in leukocyte recruitment. In addition, the analysis of inflamed cremaster muscle microcirculation in WT and CD47−/− BM chimera animals by IVM revealed an important role of endothelial cell CD47 in TEM. These in vivo observations are consistent with our in vitro human T cell TEM results that show function-blocking mAb to CD47 on T cells or human endothelial monolayers significantly reduced T cell TEM (Figs. 3, 4, Supplemental Figs. 2A, 2B). Silencing of CD47 by siRNA in HUVEC strongly reduced T cell TEM, but not adhesion under flow conditions in vitro, and thus corroborates the function-blocking mAb-blocking results. We conclude that endothelial CD47 plays a key role in the transmigration of multiple leukocyte types to sites of TNF-α–induced skin inflammation and in vitro models of TEM. CD47 on T cells appears to also contribute to TEM in vitro, but the in vivo relevance of this could not be addressed because the reciprocal BM reconstitution of WT mice with BM from CD47−/− animals is not feasible due to clearance of these cells by splenic macrophages (40).

Our data demonstrate an important role for CD47 in TEM by both the T cell and endothelium. In T cells, CD47 provides several stimulatory signals (reviewed in Ref. 14). In particular, CD47 was reported to participate in TCR-mediated Jurkat T cell spreading, and in Jurkat T cell adhesion to an endothelial cell line (EA.hy926) or VCAM-1 under shear flow or static assay conditions through regulation of VLA-4 integrins (37, 54). The precise role that T cell CD47 plays in adhesion and TEM in vivo or in vitro, however, is as yet unexplored and, hence, will require future in-depth studies. Interestingly, the combined mAb blocking of T cell and HUVEC CD47 was not additive, and this was unexpected. The reason for this result is not immediately apparent, but may be due to the complexity of CD47 interactions with its endothelial and leukocyte ligands thrombospondins and SIRPs and with its well-characterized regulation of multiple integrins. Future studies will be required to address these questions.

Two other studies have evaluated the contribution of CD47 to T cell TEM in a dermal contact hypersensitivity (CHS) model of inflammation. The level of inflammation in CD47−/− animals as compared with WT mice was similar (no reduction) in one CHS study (55) and significantly elevated and also protracted in a second study (24). The CHS, however, is complex, and neutrophils and NK cells as well as T cells are present (56). Although this might seem at odds with our air pouch data, one explanation is that the mechanism(s) of leukocyte recruitment depends on the nature of the inflammatory stimulus (i.e., induction of different chemokines or cytokines) and/or that recruited T cells may be different in the CHS site versus the dermal air pouch, and one subset is more dependent on CD47 than the other.

Human CD47 binds to thrombospondins, SIRPα and SIRPγ. The interaction of human endothelial CD47 with SIRPγ expressed on monocytes, and this pathway was critical for monocyte adhesion and TEM. Currently, a rat or mouse SIRPγ ortholog has not yet been described or predicted from searches of genomic or EST sequence databases. However, there is no evidence that rodent T cells lack receptors for CD47. To the contrary, functional assays, as those described in this study, indicate the presence of a CD47 ligand on T cells whose identity remains to be determined.

**Endothelial CD47 signaling in leukocyte TEM is Gαi independent**

CD47 has been shown to participate in the regulation of cell–cell adhesion and cell migration through reorganization of the actin cytoskeleton in epithelial cells, a process mediated by activation of the MAPK pathway (57). Several studies reported that cis interactions between CD47 and integrins recruit a PTX-sensitive Gαi protein-thrombospondin complex (reviewed in Ref. 14). Of relevance to our study, treatment of rodent endothelium with PTX resulted in reduced T cell transmigration; the level of inhibition was variable and ranged from no effect in a rat HEV system (43) to an 80% reduction in murine brain endothelial cell migration.
indicating a critical role of T cell G proteins in TEM. In these models and in models of leukocyte chemotaxis (41), PTX was dependent on endothelial cell G proteins. Allergen-induced lung model was significantly impaired and had no effect on T cell adhesion or TEM, indicating that G proteins in endothelium isolated from three different human vascular beds (58) reported. In this study, we observed that PTX pretreatment of G protein-restricted experiments; phospho-Y658 graph, *p < 0.0004 versus medium and anti-class I (one-way ANOVA, followed by Tukey test).

CD47 engagement induces actin stress fibers and activates Src and Pyk2 to phosphorylate VE-cad

Previous studies have shown that ICAM-1 cross-linking triggers robust actin stress fiber formation in HUVEC by activating Rho A (58) and induces tyrosine phosphorylation of cortactin (9, 36, 59, 60) and VE-cad (8, 10, 11), and inhibition of these events significantly reduces leukocyte TEM (reviewed in Refs. 6, 7). Engagement of ICAM-1 also activates Pyk2, which collaborates with Src protein kinases to phosphorylate tyrosine 658 and 731 in the cytoplasmic tail of VE-cad (8). Tyrosine phosphorylation of VE-cad is widely thought to facilitate dissociation of junctional proteins during paracellular migration of leukocytes. Previous studies by our laboratory showed that inhibition of Src leads to reduced leukocyte TEM (36). Our results show that, like ICAM-1, CD47 ligation triggers actin cytoskeletal remodeling and Src- and Pyk2-mediated VE-cad tyrosine phosphorylation in HUVEC. CD47-
induced VE-cad phosphorylation was significantly inhibited by a Src inhibitor, but was not affected by PTX or by a p38 MAPK inhibitor. Taken together, Src can act as the main regulator of CD47- and ICAM-1–induced phosphorylation events required for TEM in HUVEC.

In conclusion, our in vivo and in vitro studies show a crucial role for endothelial CD47 in T cell transmigration and in recruitment of T cells, neutrophils, and monocytes to extravascular sites of inflammation. We also identify signaling pathways mediated by CD47 that contribute to leukocyte TEM. Uncontrolled recruitment of leukocytes and increased endothelial cell permeability are observed in various pathologies, such as psoriasis, autoimmune diseases, multiple sclerosis, and rheumatoid arthritis. We propose that CD47 and/or the CD47 pathway downstream could serve as potential future targets for the treatment of such inflammatory conditions.

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Disclosures
The authors have no financial conflicts of interest.

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Supplemental Figure Legends

**Figure 1. T cell TEM.** T-cells were preincubated with function blocking mAb to $\alpha_4\beta_1$ (HP2/1) or $\alpha_L\beta_2$ (TS1/22) integrin, or both mAb to $\alpha_4\beta_1 + \alpha_L\beta_2$, or control non-blocking JAM-A mAb (all mAb used at 30μg/ml, 30 min preincubation) or medium alone and assessed for TEM as described (17), * $P < 0.001$ vs. medium (one-way ANOVA, followed by the Tukey test).

**Figure 2. Pertussis toxin (PTX) pretreatment of HDMVEC or HSVEC does not block T-cell transmigration.**

A, Human dermal microvascular endothelial cell (HDMEC) monolayers were pretreated with PTX (200ng/ml, 5 h) or medium alone and T-cells were pretreated with PTX (200 ng/ml) for 2 h exactly as described above in panel A for HUVEC. Treatment of HDMVEC had no effect on TEM whereas PTX treatment of T-cells blocked TEM. CD47 mAb blocked TEM while a control Class I MHC mAb had no effect. Data are mean ± SEM of 3 experiments performed in duplicates. * $P < 0.001$ vs medium for PTX; CD47 mAb vs Class I MHC mAb (Student $t$ test).

B, Human saphenous vein endothelial cell (HSVEC) monolayers and T-cells were treated exactly as described for HDMVEC in D. Data are mean ± SEM, n=3 independent experiments. * $P < 0.001$ versus medium for PTX; CD47 mAb vs Class I MHC).