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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,*,†,‡ Michael Stefanidakis,*,†,‡ Naotake Tsuboi,*,†,‡ Tanya Mayadas,*,† 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 T cell TEM. 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 Gαi 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.

L eukocyte recruitment from the peripheral blood to sites of inflammation involves the well-established multistep adhesion cascade (1). In most models of inflammation, adherens junctions (AJs) play an important role in regulating leukocyte transendothelial migration (TEM) at cell–cell junctions 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 αβ3, 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 α3β1, αβ3, and α5β1 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. SIRPα and SIRPγ are ligands for CD47 (18). SIRPα is 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 were 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 SB203580 were 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 against CD47; mAb 12G10 is a function-blocking mAb against SIRPα; and mAb TS1/22 is a function-blocking mAb against SIRPγ. Recombinant murine TNF-α (R&D Systems) and CXCL12 were from PeproTech (Rocky Hill, NJ). mAbs were used as purified IgG: mAb B6H12 is a function-blocking mAb against CD47; mAb 12G10 is a function-blocking mAb against SIRPα; and mAb TS1/22 is a function-blocking mAb against SIRPγ.

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 previously (34, 35).

Isolation of endothelial cells from 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).

In vivo microscopical study of leukocyte recruitment during microvascular inflammation in vivo

Intravital microscopy (IVM) of leukocyte transmigration of postcapillary venules of the mouse cremaster 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 cremaster 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 imaging software (Olympus). Videos were analyzed online 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).

RNA interference

Pre-designed small interfering RNA (siRNA) against CD47 was obtained from Qiagen (Valencia, CA). The silencing siRNA target sequences (catolog SI04894582 and SI03117401) were 5′-ACUGCAGTACTGCGATCTCCA-3′. Both reduced surface-expressed CD47 by 70–80%. A pre-designed siRNA oligonucleotide sequence targeting CD47 but with no inhibitory effect on CD47 expression (target sequence, 5′-CAGCATGTTTCTCCCGCCA-3′; catalog SI04894582 and SI03117401) were 5′-ACUGCAGTACTGCGATCTCCA-3′. Both reduced surface-expressed CD47 by 70–80%. A pre-designed siRNA oligonucleotide sequence targeting CD47 but with no inhibitory effect on CD47 expression (target sequence, 5′-CAGCATGTTTCTCCCGCCA-3′; catalog SI04894582 and SI03117401) were 5′-ACUGCAGTACTGCGATCTCCA-3′. Both reduced surface-expressed CD47 by 70–80%.
SB0023168) was used as a negative control. HUVEC were transfected with siRNAs (100 nM) using Lipofectamine in Optimem sera-free culture medium (Invitrogen). After 92 h, HUVEC were stimulated with TNF-α (25 ng/ml, 4 h) and used in studies.

**Immunofluorescence microscopy of CD47 expression and phalloidin staining**

HUVEC monolayers transfected with different siRNAs were fixed and stained with B6H12 anti-CD47 mAb, and primary mAb was detected by staining with Alexa-488-conjugated goat anti-mouse IgG, as previously described (36). Confluent resting or 4-h TNF-α-activated HUVEC on fibronectin-coated coverslips were treated with anti-CD47 mAb (B6H12) (30 μg/ml, 30 min) and washed, and then goat anti-mouse secondary mAb (10 μg/ml) was used to cross-link CD47 mAb for 0, 5, 10, 20, and 30 min. As a control, HUVEC were incubated with secondary mAb only. HUVEC were fixed and permeabilized, and endogenous F-actin was detected by staining with Alexa-568–phalloidin, according to the manufacturer’s protocol (Invitrogen). Actin stress fiber formation was quantified in >100 cells in multiple fields at the 30-min time point. Cells with five or more 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 FITC-tagged secondary B6H12 and Hu5/3 mAbs, respectively, followed by FITC-tagged secondary F(ab′)2 mAb, as previously described (17). Expression levels were acquired by two different siRNA caused a 70–80% reduction (Fig. 3B–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 chimera 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 cremaster model 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 α1β2 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 Gαi–coupled receptor complex in a variety of cell types (14, 15, 42). To examine whether CD47 signals through the Gαi pathway in endothelium during TEM, we preincubated HUVEC with different concentrations of PTX for 5 h to inactivate Gαi 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 Gαi2/2 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**

<table>
<thead>
<tr>
<th></th>
<th>% NE</th>
<th>% LY</th>
<th>WBC (×10⁷/ml)</th>
<th>NE</th>
<th>LY</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>28.11 ± 2.42</td>
<td>64.17 ± 2.94</td>
<td>5.02 ± 0.07</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>
</tbody>
</table>

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.

**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 (8, 10, 11). Our laboratory has previously reported (9) that mAb cross-linking of ICAM-1 leads to a rapid increase in actin cytoskeletal remodeling in HUVEC. To determine whether engaging endothelial CD47 triggers remodeling of the actin cytoskeleton, we preincubated HUVEC with an anti-CD47 mAb for 10 min, followed by a secondary mAb for different periods of time (Fig. 6A). Cross-linking with anti-CD47 mAb triggers robust stress fiber formation, as assessed by phalloidin staining, as early as 5 min with most cells responding by 30 min (Fig. 6A, right panel). In the absence of CD47 cross-linking, or cross-linking of endothelial class I MHC mAb (control), HUVEC showed few stress fibers (data not shown).

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 phosphotyrosine 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-1– 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 SIRPα 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-
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 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 recruitment 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 SIRPα-expressing 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...
system. In \( G_{\alpha i}^{-/} \) animals, leukocyte recruitment in an allergen-induced lung model was significantly impaired and was dependent on endothelial cell \( G_{\alpha i} \)-dependent signaling (46). In these models and in models of leukocyte chemotaxis (41), PTX pretreatment essentially blocked >80% of leukocyte migration, indicating a critical role of T cell \( G_{\alpha i} \) proteins. However, the role of \( G_{\alpha i} \) proteins in TEM of human endothelial cells has not been reported. In this study, we observed that PTX pretreatment of endothelium isolated from three different human vascular beds had no effect on T cell adhesion or TEM, indicating that \( G_{\alpha i} \) proteins in human endothelial cells are not involved in TEM.

**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 PTK or by a p38 MAPK inhibitor. Taken together, Src can act as the main regulator of VE-cad phosphorylation in T cell transmigration and in recruitment of T cells, neutrophils, and monocytes to extravasative 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
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References


