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Phosphorylation of Leukocyte PECAM and Its Association with Detergent-Resistant Membranes Regulate Transendothelial Migration

Oliver Florey,*,† Joanne Durgan,‡ and William Muller*

Leukocyte migration across the endothelial lining is a critical step in the body's response to infection and inflammation. The homophilic interaction between endothelial PECAM and leukocyte PECAM is essential for this process. The molecular events that are triggered in the endothelial cell by PECAM engagement have been well characterized; however, the function of leukocyte PECAM remains to be elucidated. To study this, we first blocked leukocyte transmigration using anti-PECAM Ab and then specifically activated leukocyte PECAM. This was sufficient to overcome the block and promote transmigration, suggesting an active signaling role for leukocyte PECAM. Consistent with this, we found that ligation of leukocyte PECAM induces phosphorylation of two tyrosine residues on its cytoplasmic tail. By performing RNA interference-rescue experiments, we demonstrate that these phosphorylation events are indispensable for transendothelial migration. Finally, we show that leukocyte PECAM translocates to a detergent-resistant membrane (DRM) during transmigration. PECAM localized in DRMs displays reduced phosphorylation and does not support transmigration. Together, these data support a model whereby engagement of leukocyte PECAM induces its transient tyrosine phosphorylation and induction of downstream signals that drive transmigration. These signals are then downregulated following PECAM translocation to DRMs.

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The cytoplasmic tail of PECAM contains tyrosine residues at positions 663 and 686, which constitute ITIMs (9, 10). Src kinases have been shown to phosphorylate these residues (11, 12), which then serve as docking sites for Src homology 2 domain-containing proteins such as Src homology region 2 domain-containing phosphatase (SHP) 1, SHP2, phospholipase Cγ, and SHIP (13–15). It is through these phosphorylation events and subsequent protein interactions that PECAM can mediate downstream signaling. Although the requirement for endothelial PECAM phosphorylation has been well studied with respect to endothelial junction function (16, 17) and leukocyte transmigration (6), the role of leukocyte PECAM phosphorylation in the transmigration process remains uncharacterized. In the current study, we generated a leukocyte cell line in which endogenous PECAM was depleted and replaced with either wild-type or nonphosphorylatable Y663F/Y686F mutants. Using this approach, we demonstrate that the phosphorylation of leukocyte PECAM is also required for transmigration.

Several immunoreceptors, including TCR, BCR, and FcγRs, initiate signal transduction by associating with specialized lipid regions in the membrane (18–20), termed detergent-resistant membranes (DRMs) (21). PECAM has previously been shown to associate with DRMs in platelets (22), but this association has not been studied in leukocytes. We present evidence that leukocyte PECAM moves into DRMs during transmigration, where is displays reduced phosphorylation. Forcing PECAM into DRMs decreases transmigration. The data are consistent with a model in which homophilic interaction of PECAM induces signaling through PECAM phosphorylation that is necessary for transmigration. The activation of PECAM is then terminated by movement of PECAM into DRMs.

Materials and Methods

Abs and reagents

Monoclonal mouse anti-human hec7 (anti-PECAM) (23) and hec2 (anti-CD99) (24) were produced from hybridomas. Polyclonal rabbit anti-human PECAM 177 and 301 were generated in-house. The nonblocking mouse

Abbreviations used in this paper: DRM, detergent-resistant membrane; I, insoluble; IB, Western blotting; IP, immunoprecipitated; MJβCDX, methyl-β-cyclodextrin; NSC, nonsilencing control; P-WT, wild-type PECAM; S, soluble; SHP, Src homology region 2 domain-containing phosphatase; shRNA, short hairpin RNA; US, unstimulated; VE, vascular endothelial.

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anti-PECAM mAb P1.1 was a kind gift from Dr. Peter Newman (Blood Center of Wisconsin, Milwaukee, WI). Anti-phosphotyrosine 4G10 was purchased from Millipore (Bedford, MA). F(ab\(^{-}\))\(_2\) goat anti-mouse and goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit anti-mouse IgG-HRP and swine anti-rabbit IgG-HRP were purchased from DakoCytomation (Carpinteria, CA). Src kinase inhibitor PP2 was purchased from Calbiochem (San Diego, CA). Methyl-\(\beta\)-cyclodextrin (MβCDX) was purchased from Sigma-Aldrich (St. Louis, MO).

**Cell culture and differentiation**

U937L cells (kindly donated by Dr. F. William Luscinkas, Brigham and Women’s Hospital, Boston, MA) were maintained in RPMI 1640, 10% FBS, t-glutamine, and penicillin/streptomycin and differentiated to a more monocytic lineage with 1 mM dibutyryl-cAMP (Sigma-Aldrich) stimulation for 3 d. These cells have previously been demonstrated to transmigrate HUVEC monolayers in a similar manner to primary monocytes (25).

HUVECs were isolated from fresh umbilical cords, as previously described (23), and grown in medium 199 (M199, Invitrogen, Carlsbad, CA) supplemented with 20% adult human serum and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO\(_2\). Experiments were done routinely with cells at passage two plated on thick hydrated type I collagen gels in 96-well culture plates.

**PBMcs and monocite isolation**

PBMcs were isolated from healthy volunteers by density gradient centrifugation in Ficoll-Paque. Monocytes were isolated from the PBMc fraction using an MACS monocite isolation kit and magnetic depletion columns according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). This yielded >90% monocytes, as determined by flow cytometry following labeling with FITC-labeled anti-CD14.

**Generation of U937L PECAM knockdown cell line**

A GFP-tagged lentiviral short hairpin RNA (shRNA) construct against human PECAM (PEC02) and a nonsilencing control (NSC) construct were generously donated by Dr. Peter Newman and have been previously described (26). U937L cells were infected with lentiviral constructs at a multiplicity of infection of 100 for 5 h at 37°C. Cells, designated U937L PEC02 or U937L NSC were washed and replated in a 12-well plate in 1 ml normal culture media. After 3 d, strongly GFP-positive cells were selected using an FACS-Vantage cell sorter (BD Biosciences, San Jose, CA).

**Lentiviral PECAM rescue construct and transduction of U937L cells**

Full-length human PECAM in the lentiviral plasmid pWPT (kindly donated by Dr. Peter Newman) was used as a template to make PECAM rescue constructs. Six silent mutations within the PECAM small interfering RNA targeting region were introduced using site-directed mutagenesis (Stratagene, La Jolla, CA) along with tyrosine and phenylalanine mutations at positions 663 and 686, as described in the Supplemental Material. pWPT PECAM constructs were mixed with the packaging plasmid pCMVR8.2 and envelope vector pSV-G and cotransfected into 293FT cells. Lentiviral particles were purified, and U937L PEC02 cells were transduced to achieve expression levels close to those of endogenous PECAM as verified by flow cytometry.

**Western blotting**

Cells were lysed for 10 min at 4°C in non-denaturing lysis buffer A, 1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM NaVO\(_4\), 1 mM PMSF, and protease inhibitor mixture (Sigma-Aldrich). Samples were then spun at 14,000 rpm for 10 min at 4°C. Supernatants (Triton soluble fractions) were collected, and the remaining pellets (Triton insoluble) were resuspended in sample buffer and boiled for 5 min, passed through a 26-gauge needle five times, and sonicated for 5 min before storing at −20°C. The samples were run on a 4–12% gradient Tris-Glycine SDS PAGE gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked and then incubated overnight at 4°C with primary. Blots were incubated with HRP-conjugated secondary Abs and proteins were detected using ECL (Amersham Biosciences, Piscataway, NJ).

**Immunoprecipitation**

Protein G Dynabeads (Invitrogen), 20 μl, were first derivatized with 10 μg rabbit polyclonal anti-PECAM, 301Ab, in lysis buffer. After four washes, Triton soluble supernatants were added and rotated for 3 h at 4°C. Samples were washed four times and proteins eluted by addition of sample buffer followed by boiling for 5 min. To immunoprecipitate from Triton insoluble fractions, pellets obtained after Triton lysis were resuspended in a denaturing lysis buffer (1% SDS, 50 mM Tris-HCl, 10 mM NaVO\(_4\)) and then passed through a 26-gauge needle five times, boiled for 5 min, and sonicated for 5 min. Samples were then diluted 1:10 with non-denaturing lysis buffer before being added to Protein G beads as above.

**Isolation of DRMs by sucrose density gradient ultracentrifugation**

Differentiated U937L cells (1 × 10\(^7\)) were stimulated and lysed with 0.5 ml lysis buffer A (see above) on ice for 10 min. Total lysates were passed through a 26-gauge needle five times before being mixed with an equal volume of 80% sucrose in buffer A, containing 0.2% Triton X-100. This was then overlaid with 7 ml 35% sucrose in buffer A and 3 ml 5% sucrose in buffer B. After ultracentrifugation for 20 h at 40,000 rpm in a Sorvall TH-641 rotor, 11 ml fractions were collected starting from the top of the gradient and analyzed by Western blotting.

**Transendothelial migration assay**

This assay was performed and quantified as previously described (5). In brief, PBMcs or differentiated U937L cells were preincubated or without 10 μg/ml anti-PECAM Abs on ice for 15 min washed and resuspended in M199 plus 0.1% HSA at 2 × 10\(^9\)/ml. A total of 100 μl cells were added to each replicate well of confluent monolayers of HUVECs grown on hydrated collagen gels and incubated for 1 h at 37°C in a CO\(_2\) incubator. To induce surface leukocyte PECAM cross-linking, F(ab\(^{-}\))\(_2\) secondary Abs, 50 μg/ml final concentration, were added to the desired wells. The percentage of transmigration in 10 different fields of view was calculated by dividing the number of leukocytes below the HUVEC monolayer by the total number of leukocytes in the field.

**Fluorescent microscopy on Triton-extracted samples**

Confluent HUVEC monolayers were grown on fibronectin-coated glass-bottomed dishes and prelabeled with 20 μg/ml Alexa Fluor 488-conjugated anti-vascular endothelial (VE)-cadherin for 20 min at 37°C. PBMcs were prelabeled with Alexa Fluor 546-conjugated nonblocking anti-PECAM (P1.1) on ice before washing and resuspension in M199 plus 0.1% HSA at 2 × 10\(^9\)/ml. A total of 100 μl PBMcs was added to monolayers of HUVECs for 10 min at 37°C. Dishes were either fixed in 2% paraformaldehyde or treated with cold Triton X-100 extraction buffer (0.5% Triton, 138 mM KCl, 3 mM MgCl\(_2\), 2 mM EDTA, 0.32 M sucrose, 10 mM MES, protease inhibitors, 1 mM PMSF [pH 6.1]) for 20 min on ice pret fixation. Dishes were imaged using a Deltavision microscope (Applied Precision, Issaquah, WA). Analysis of fluorescent intensity was carried out using Metamorph software (Analytical Technologies, Sunnyvale, CA).

**Statistics**

The Student t test was used to evaluate statistical significance in the data presented using Prism (GraphPad, San Diego, CA).

**Results**

**Cross-linking monocyte PECAM promotes transendothelial migration**

Leukocyte transendothelial migration can be inhibited using anti-PECAM neutralizing Abs (4, 5) that bind to the homophilic interaction domain, preventing leukocyte PECAM interaction with endothelial cell PECAM. If these neutralizing Abs are subsequently clustered by cross-linking with a secondary Ab, PECAM signaling can be artificially activated. This system allows us to mimic the concentrated PECAM ligation that occurs in time and space on a leukocyte during transmigration and to temporally control PECAM activation in the treated cells.

To analyze the role of leukocyte PECAM, we added primary monocytes, treated with a blocking anti-PECAM IgG Ab, to endothelial monolayers for 30 min. This allowed sufficient time for monocytes to migrate to endothelial junctions, where they are arrested (Fig. 1). Clustering of leukocyte PECAM to induce its activation was then performed by the addition of a F(ab\(^{-}\))\(_2\) secondary cross-linking Ab after 30 min. Fig. 1 shows that blocking monocyte PECAM with mAb hec7 inhibited transmigration. Strikingly, cross-linking PECAM on these arrested cells, after 30 min,
were not inhibited by blocking Fc 
linking (Fig. 2
A
). Using dibuteryl camp-differentiated 
domains. PECAM is not phosphorylated in resting monocytes, but 
these are at positions 663 and 686, which reside within ITIM-like 
become phosphorylated upon activation; the best characterized of 
transduction. PECAM contains several tyrosine residues that may 
promotes transmigration by engaging in intracellular signaling 
Next, we investigated the possibility that leukocyte PECAM 
PECAM is phosphorylated upon cross-linking 
U937L cells as a monocyte model, we were able to see the same 

g(30 min) before PECAM cross-linking for 3 min. Lysates were analyzed by 
Western blotting (performed as in A and B). Results shown are represen-
tive of three separate experiments. IB, Western blotting; IP, immunopre-

depleted endogenous PECAM using a lentiviral shRNA. Levels of 
PECAM knockdown were confirmed by flow cytometry and West-
ern blotting (Fig. 3A, 3B). The stable cell line PEC02 presents a 
robust PECAM knockdown of around 70–80%; a scrambled 
control shRNA was used in a similar way to generate the control cell line NSC. PEC02 cells displayed significantly reduced trans-
migration and showed no further reduction in the presence of anti-
PECAM Abs (Fig. 3C). These data clearly show that U937L cell 
transmigration is dependent upon leukocyte PECAM.

U937L cell lines were generated re-expressing either wild-type 
PECAM (P-WT) or double Y663F and Y686F (P-663/686) mutations. Fig. 3D shows Western blot data confirming the re-expression of 
PECAM at close to endogenous levels. The phosphorylation status of re-expressed PECAM was tested by immunoprecipitation and 
anti-phosphotyrosine Western blots. Fig. 3E demonstrates that the 
double mutant has reduced PECAM phosphorylation after cross-
linking compared with control U937L or P-WT cells, indicating that 
these represent major phosphorylation sites following leukocyte 
PECAM activation. 

Leukocyte PECAM is phosphorylated upon cross-linking 
Next, we investigated the possibility that leukocyte PECAM 
promotes transmigration by engaging in intracellular signaling 
transduction. PECAM contains several tyrosine residues that may 
become phosphorylated upon activation; the best characterized of 
these are at positions 663 and 686, which reside within ITIM-like 
domains. PECAM is not phosphorylated in resting monocytes, but 
becomes rapidly and transiently tyrosine phosphorylated upon 
cross-linking (Fig. 2A). Using dibuteryl camp-differentiated 
U937L cells as a monocyte model, we were able to see the same 
pattern of transient PECAM phosphorylation after cross-linking 
(Fig. 2B). The phosphorylation of PECAM was inhibited using the 
Src kinase inhibitor PP2 (Fig. 2C). We next set out to determine 
whether the phosphorylation events triggered by leukocyte 
PECAM clustering were involved in the transmigration process.

Phosphorylation of tyrosine 663/686 on leukocyte PECAM is 
required for transendothelial migration 
To test whether leukocyte PECAM phosphorylation is involved in 
transendothelial migration, we generated monocytic U937L cell 
lines expressing either wild-type or mutant forms of PECAM lack-
ing the 663 and 686 tyrosine phosphorylation sites. To make sure 
that the cells expressed the same levels of total PECAM, we first 

FIGURE 1. Cross-linking leukocyte PECAM reverses an anti-PECAM 
block in transmigration. Human monocyte transmigration through unsti-

tulated HUVEC monolayers grown on collagen gels. Monocytes were 
prelabeled on ice with blocking Abs against PECAM (hec7, 177) and 
CD99 (hec2) at 20 µg/ml before being washed free of unbound Ab and 
added to HUVEC monolayers for 60 min. PECAM was activated by the 
addition of cross-linking secondary Abs (50 µg/ml) 30 min into the assay 
where indicated. Data represent mean ± SEM from seven separate experi-

ments. ***p < 0.003; **p < 0.001.

FIGURE 2. Leukocyte PECAM becomes tyrosine-phosphorylated after 
cross-linking. Human monocytes (A) or differentiated U937L cells (B) were stimulated by PECAM cross-linking for the indicated times and 
lysates were immunoprecipitated with anti-PECAM Abs. Samples were 
resolved on SDS-PAGE gels and analyzed by Western blotting (IB) using 
anti-phosphotyrosine and PECAM Abs. Band intensities were measured 
via densitometry, and the ratio of phosphorylated PECAM to total PECAM 
is shown below the Western blots. C. Differentiated U937L were left either 
untreated or pretreated with the Src kinase inhibitor PP2 (20 or 100 µM, 
30 min) before PECAM cross-linking for 3 min. Lysates were analyzed by 
Western blotting (performed as in A and B). Results shown are represen-
tive of three separate experiments. IB, Western blotting; IP, immunopre-
cipitated; US, unstimulated (no PECAM cross-linking).
PECAM cross-linking (Fig. 4) membranes upon activation, remain in the soluble fraction after which are known to associate with Triton X-100–insoluble is specific to PECAM, as other receptors, CD64 and CD32a, ∼50% of PECAM is found in the insoluble pellet. This movement conditions. However, following 3 min of PECAM cross-linking, solubilized in 1% cold Triton X-100 lysis buffer under resting conditions. However, following 3 min of PECAM cross-linking, a change in its detergent solubility was also detected. Fig. 4A shows that U937L PECAM is completely solubilized in 1% cold Triton X-100 lysis buffer under resting conditions. However, following 3 min of PECAM cross-linking, a portion of PECAM movement to DRMs (data not shown). These results are therefore consistent with PECAM partitioning into DRMs. Furthermore, we found that the movement to an insoluble fraction is both rapid and prolonged. PECAM translocated to DRMs within 3 min and remained there for up to 60 min after cross-linking (Fig. 4B).

PECAM is recruited to low-density DMGs after its cross-linking

To further test the hypothesis that PECAM was recruited to DMGs after cross-linking, we employed a more definitive technique. DMGs can be purified by density ultracentrifugation due to their high lipid content. After cross-linking PECAM, differentiated U937L cells were subjected to sucrose gradient centrifugation. Fig. 4C shows that, in unstimulated cells, PECAM was found in the high-density fractions of the gradient representing the soluble protein. After its cross-linking, movement of a portion of PECAM to lower density fractions was observed. A known DMG component, lyn kinase (27), was also enriched in the same light-density fraction and remained the same before and after PECAM cross-linking.

Depletion of membrane cholesterol by MβCDX has previously been shown to disrupt DMGs (28). Pretreating cells with 20 mM MβCDX for 45 min inhibited PECAM translocation to DMGs upon cross-linking (Fig. 4C). Lyn kinase also moved to a cold Triton-soluble fraction after MβCDX treatment, verifying the disruption of DMGs. Similar results of cholesterol-dependent movement of PECAM to a cold Triton-insoluble fraction were seen using purified human monocytes (Fig. 4D). These cells remained

**FIGURE 3.** Tyrosine phosphorylation of leukocyte PECAM is required for transendothelial migration. Control or stable U937L cells expressing either a control shRNA or a shRNA targeting PECAM (named NSC or PEC02, respectively) were analyzed for PECAM expression by flow cytometry (A) and Western blotting (B). C, Differentiated control and NSC U937L cells transmigrate across IL-1β–treated HUVEC monolayers (200 pg/ml, 4 h) to similar levels that are blocked in the presence of anti-PECAM Abs. PEC02 cells show a reduced level of transmigration, which is not further inhibited by anti-PECAM Abs. Data shown are mean ± SEM from three experiments. *p < 0.01. D, Western blot analysis of lysates from NSC, PEC02, and PEC02 cells transduced to re-express either P-WT or Y663/686F (P-663/686) forms of PECAM. Immunoblotting was performed against PECAM and actin. E, Mutating tyrosine sites 663 and 686 to phenylalanine reduces PECAM phosphorylation after cross-linking. Following PECAM cross-linking for 3 min, lysates from all the U937L cell lines were immunoprecipitated with anti-PECAM and probed for anti-phosphotyrosine. Ratios of phospho-PECAM in the cell lines are presented below the blots. F, Differentiated U937L cell lines were allowed to transmigrate across IL-1β–treated HUVEC monolayers for 1 h in the presence or absence of anti-PECAM Abs. PEC02 cells re-expressing P-WT showed rescue of transmigration to levels similar to those on control U937L cells. Experiments show the mean ± SEM from three experiments. Cells expressing the 663 and 686 mutant form of PECAM (P-663/686) remained inhibited in their trans-migration. **⁎p < 0.03; † †p < 0.02.
viable after MβCDX treatment and showed clear signs of cholesterol depletion, including cell rounding and loss of polarized morphology (data not shown). The movement of PECAM to DRMs is independent of tyrosine phosphorylation, as PP2 did not stop PECAM movement to DRMs after cross-linking. PECAM from the P-663/686 cell line also translocates to DRMs after cross-linking (Supplemental Fig. 2).

**Leukocyte PECAM is found in DRMs during transendothelial migration**

We next asked whether movement of PECAM into DRMs actually occurs during leukocyte transmigration. To do this, we designed an assay to detect Triton X-100–insoluble proteins using detergent extraction and fluorescence microscopy. In this assay, monocytes were prelabeled with an Alexa Fluor 546-conjugated anti-PECAM Ab (P1.1), which does not block transmigration. Endothelial cells were preincubated with a nonblocking Alexa Fluor 488-conjugated anti–VE-cadherin to mark junctions. Fig. 5A shows that PECAM of unstimulated monocytes adhered to glass is completely removed from cells by cold Triton X-100 extraction, whereas VE-cadherin remains visible at endothelial cell junctions. Some monocytes present at endothelial junctions exhibited PECAM staining (asterisk) away from the junction showed no PECAM staining (Fig. 5B). The maximum fluorescent intensity of PECAM was quantified for >50 monocytes for which the position was classed as junctional or nonjunctional. A greater proportion of monocytes found at endothelial junctions displayed significantly greater PECAM staining (Fig. 5C). These data suggest that leukocyte PECAM can move to DRMs upon homophilic ligation with endothelial PECAM.

**Tyrosine-phosphorylated leukocyte PECAM is enriched in non-DRM regions**

As the phosphorylation state of PECAM was not important in its movement to DRMs (Supplemental Fig. 2), we investigated whether the inclusion of PECAM in DRMs had an effect on PECAM phosphorylation. Unexpectedly, we found that phosphorylated PECAM in U937L cells was more abundant in the Triton-soluble fraction than the insoluble fraction (Fig. 6). The levels of PECAM protein appear lower in insoluble fractions; however, this is a reflection of the efficiency of immunoprecipitation from insoluble fractions compared with soluble fractions rather than a difference in total protein. Thus, the PECAM to phospho-PECAM ratio was used to compare the fractions. Consistent with this result, disruption of DRMs by MβCDX treatment resulted in a hyperphosphorylation of PECAM. These data suggest that PECAM is phosphorylated in non-DRM regions, whereas PECAM in DRMs either undergoes less phosphorylation or is actively dephosphorylated.

**Leukocyte PECAM movement to DRMs negatively regulates transendothelial migration**

We have demonstrated that leukocyte PECAM phosphorylation is important for transmigration and also that leukocyte PECAM in DRMs is less phosphorylated after cross-linking. We next sought to examine whether movement of leukocyte PECAM to DRMs affected transendothelial migration. Transmigration experiments using MβCDX to disrupt DRMs could not be performed, as cholesterol depletion affects the ability of leukocytes to adhere, polarize, and migrate, which would all impair the assay (29–31). Instead of inhibiting PECAM movement, we thus chose to force and lock PECAM into DRMs. To achieve this without affecting PECAM’s ability to undergo homophilic interactions, we exploited
FIGURE 6. Leukocyte PECAM moves to a Triton-insoluble fraction during transmigration. A, HUVEC monolayers labeled with Alexa Fluor 488-conjugated anti–VE-cadherin and human monocytes adhered to glass labeled with nonblocking Alexa Fluor 546 anti-PECAM with or without addition of secondary cross-linking Abs. Samples were fixed in 2% paraformaldehyde or incubated with a Triton X-100 extraction buffer at 4°C for 30 min prefixation and imaged by wide-field microscopy (original magnification ×63). B, Cells were labeled as in A, and monocytes were added to endothelial monolayers for 10 min at 37°C. Cells were then fixed and processed as above. Leukocytes were seen adhered away from endothelial junctions (asterisks) and directly over junctions (arrows). Representative images are shown. C, The maximal fluorescence intensity of PECAM found on leukocytes at junction and nonjunction positions was analyzed for 40 cells. Graphs show individual data points with mean fluorescence intensity. **p < 0.007.

Discussion

PECAM has previously been implicated in signal transduction mediated by the phosphorylation of its cytoplasmic tail and association with other signaling complexes (32). Because PECAM is so fundamental to the process of leukocyte transmigration, it would seem likely that it acts as a signaling molecule in this setting, too. Although much work has been undertaken to elucidate the role of endothelial PECAM, far less has been done to explore the function of leukocyte PECAM during transmigration. Our present study reveals a key role for the tyrosine phosphorylation of leukocyte PECAM in transmigration. Furthermore, we describe a novel mechanism regulating the phosphorylation of leukocyte PECAM through its association with specialized lipid domains (DRMs).

Many immune receptors have been shown to cluster on the cell surface after ligand binding, and this clustering plays an important role in initiating downstream signaling events (33). There is a high local enrichment of endothelial PECAM at junctions, and it seems likely that this may induce leukocyte PECAM to cluster during transmigration. We hypothesized that such a clustering of leukocyte PECAM might transmit signals required for the continuation and completion of transmigration. Ab mediated cross-linking of surface receptors initiates signal transduction, although the actual mechanism of how this works remains unclear. In the current study, we used cross-linking of leukocyte PECAM in a novel manner to mimic its natural clustering and thus precisely control its activation during transmigration.

Blocking leukocytes with anti-PECAM Abs inhibited homophilic engagement with endothelial cell PECAM and thus arrested cells at endothelial junctions, unable to transmigrate through the monolayer (5, 34). Importantly, we found that the subsequent activation of leukocyte PECAM by the addition of secondary cross-linking Abs was sufficient to promote transmigration in a subpopulation of the arrested cells. We hypothesize that the partial nature of the recovery may reflect the fact that only leukocytes located at permissible sites (e.g., endothelial junctions) at the time of cross-linking would be prompted to transmigrate. For those cells not engaging endothelial junctions at the time, PECAM cross-linking would drive PECAM into DRMs, where it would be held in a state of deactivation that would actually inhibit transmigration. Indeed, cross-linking PECAM on leukocytes before they get to endothelial junctions inhibits their transmigration (Fig. 7). These data confirm that leukocyte PECAM...
is critical during transmigration and suggest that, in addition to its accepted role as an adhesion molecule, it plays an active signaling function.

As phosphorylation was a consequence of PECAM ligation, we examined its role in transmigration using dibutyl camp-differentiated U937L cells in which we depleted endogenous PECAM and re-expressed phospho-mutant constructs. We found that depletion of endogenous PECAM led to a block in transmigration that could be rescued by re-expression of the wild-type protein. Strikingly however, U937L cells re-expressing PECAM with tyrosine-phosphorylalanine mutations at positions 663 and 686 displayed a suppression of transmigration. Further work will be required to determine the relative contributions of phosphorylation at the Y663 and Y686 sites during transmigration; however, our data provide clear evidence for the importance of phosphorylation within the ITIM domains of leukocyte PECAM.

Previous studies suggest that a downstream consequence of PECAM phosphorylation at sites 663 and 686 is the recruitment of Src homology 2 domain-containing phosphatases SHP1 and SHP2 (13–15). Other signaling proteins, such as phospholipase Cγ and SHIP, have also been proposed to complex with phosphorylated PECAM, although this remains controversial (35, 36), and indirect association of PI3K and Grb2 with PECAM has been reported (15, 37). PECAM is often cited as a negative regulator of signaling (33). The clustering of receptors into these specialized lipid domains brings them into close proximity with and exposes them to other proteins, such as kinases, phosphatases, and scaffolding proteins that act to initiate and amplify signal transduction. These domains are often rich in cholesterol and sphingolipids, which reduces their solubility in some nonionic detergents (21, 46), and are often termed DRMs. We provide evidence that in resting monocytes, PECAM is found almost exclusively in a Triton X-100–soluble fraction, but that a portion of it becomes associated with DRMs after cross-linking. We examined PECAM movement to DRMs both by analyzing its solubility in different lysis buffers and by sucrose gradient analysis. Using both techniques, we obtained clear evidence for this activation-induced translocation, although the percentage of PECAM shifted differed. This disparity is most likely due to differences in the methods used to obtain samples and the efficiency of solubilization during the procedures. Although we cannot accurately define the exact amount of PECAM that moves to the DRM, we were able to efficiently manipulate the translocation and analyze its functional implications.

Most important, we found that leukocyte PECAM associates with DRMs during actual transmigration (Fig. 5). PECAM on leukocytes adherent to endothelial monolayers demonstrated greater detergent
resistance when located at endothelial junctions compared with positions away from junctions. PECAM ligation and clustering would likely occur at junctions due to the local enrichment of endothelial PECAM. Amplification of leukocyte PECAM clustering in this region could be achieved by the active enrichment of endothelial PECAM from the lateral border-recycling compartment that occurs in transmigration (7, 47). Ours is the first direct evidence to suggest that leukocyte proteins partition into DRMs during the transmigration process. It would be of interest to see if other leukocyte proteins known to be involved in transmigration also partition into DRMs.

For most transmembrane receptors, movement into DRMs is associated with an increase in their signaling potential. This can be due to the exclusion of negative regulators from the DRM, such as phosphatases (48), or the enrichment of activating kinases (49, 50). Unexpectedly, we found that PECAM association with DRMs had a negative effect on its activation and signaling capacity; PECAM was less phosphorylated in the Triton-insoluble fraction as compared with soluble fractions. Consistent with this, we saw that disruption of DRMs by cholesterol depletion led to hyperphosphorylation of PECAM phosphorylation within the Triton-soluble fraction. As the kinase and/or phosphatase involved in controlling leukocyte PECAM phosphorylation are not known, it is difficult to understand by what mechanism PECAM is negatively regulated in DRMs.

Because PECAM association with DRMs decreases its phosphorylation (Fig. 6), and our data (Fig. 3) define a key role for leukocyte PECAM phosphorylation in transmigration, we sought to determine if the movement of PECAM to DRMs affected transmigration. Experiments to disrupt DRMs via cholesterol depletion could not be carried out in the context of transmigration, as this disrupts many crucial upstream processes (i.e., adhesion, polarization, migration). Instead, we chose to force PECAM into DRMs by cross-linking with nonblocking anti-PECAM Abs prior to incubation on the endothelial monolayer. This does not affect its ability to homophilically bind PECAM but could potentially affect its ability to initiate further signal transduction. Cross-linking PECAM on leukocytes this way, before they have adhered and migrated to endothelial junctions, significantly inhibits their ability to transmigrate (Fig. 7). We propose that this is due to cross-linked PECAM being shifted to DRMs, where its phosphorylation capacity is lower. These data may seem at odds with that of Fig. 1, in which cross-linking PECAM on blocked leukocytes promoted transmigration. However, a critically important distinction is that PECAM activation was induced at different times in these experiments. When leukocyte PECAM is activated at the start of the assay, before leukocytes adhere or move to the junctions, there is no transmigration because PECAM has been sequestered in DRMs in a long-term, dephosphorylated, signaling-incompetent state. However, when cross-linking is performed when cells are poised at junctions ready to transmigrate, transmigration is promoted because the transient activation of PECAM can drive this process. Our current working model proposes that during normal transmigration, leukocyte PECAM is subject to transient activation, stimulated by engagement with locally enriched endothelial PECAM at the junction, which is critical to drive transmigration. This may be followed shortly afterward by downregulation of PECAM signaling by sequestration and dephosphorylation in DRMs (Fig. 7D). This may turn off the local signaling within the leukocyte at that point, allowing leukocyte deathdegeneration and progression of that portion of the leukocyte across the endothelial cell border. All of this is likely to work in concert with PECAM-mediated events triggered in the endothelial cell.

In summary, we provide evidence that the precise spatial and temporal control of leukocyte PECAM signaling is required for transmigration. Deregulation of leukocyte PECAM activation disrupts transmigration. Further work will be required to define the signaling events downstream of leukocyte PECAM and to establish whether dysregulation of this pathway may contribute to disease.

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Disclosures
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References
Figure S1.
A) Representative confocal z-stack of transmigrated monocytes labeled with or without CSFE. All cells are stained with rhodamine phalloidin. Bar = 10μM. (B) Monocytes were pre-labeled with CSFE and mouse anti-PECAM antibody hec7 (Monocyte B) or rabbit anti-PECAM 177 alone (Monocyte A) before being added to HUVEC monolayers. Secondary cross-linking antibodies against mouse IgG were added after 30 minutes specifically cross-link Monocyte B, and samples left for another 30 minutes. Samples were then washed three times with PBS and fixed at room temperature in 2% paraformaldehyde with 0.1% Triton X100 for 15 minutes. After two washes in PBS 20mM glycine, cells were stained with AlexaFluor 546 phalloidin (Molecular Probes) for 35 minutes, washed again and analyzed using a LSM 510 confocal microscope (Carl Zeiss). The percentages of monocytes from both pools were calculated for their position on top or below the endothelial monolayer. Data represent mean ± SEM from 2 separate experiments. Those monocytes that underwent PECAM cross-linking showed a reversal of anti-PECAM blockade and increased transmigration, while monocytes in close proximity that did not receive PECAM activation remained blocked, Figure S1B. Thus, we found no evidence to suggest that PECAM activation mediates the release of soluble factors that aide the transmigration event, although we cannot formally exclude the possibility of extremely localized short-lived factors.
Figure S2

(A) Differentiated U937L were left either untreated or pretreated with the src kinase inhibitor PP2 (20 or 100mM, 30 minutes) before PECAM cross-linking. Triton X100 Soluble and insoluble fractions were analysed by western blotting. (B) Soluble (S) and insoluble (I) fractions of U937L NSC and P-663/686 were analysed for PECAM before and after PECAM cross-linking. These data show that the phosphorylation state of PECAM has no effect on its partitioning into DRMs.
Figure S3.
L-cells stably expressing human PECAM were non-enzymatically removed from flasks washed then resuspended to 1 x 10^6 cells/ml in HBSS with 1 mM Ca2+. PECAM expressing L-cells were allowed to aggregate for the indicated times in the presence of blocking (177) and non-blocking (P1.1) anti-PECAM antibodies, or with the cross-linking of P1.1 by the addition of F(ab')2 rabbit anti-mouse IgG, on pre-blocked 24-well non-tissue culture plates (Costar Corp., Cambridge, MA) which were then rotated on a gyratory platform (90 rpm) at 37°C for 45 minutes. The numbers of cells in aggregates of greater than three cells, as well as the total number cells, were counted, with at least 600 cells counted from each sample. Data were expressed as the percent of total cells present in aggregates.