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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 Abs and then specifically activated leukocyte PECAM. This was sufficient to overcome the block and promote transmigration, suggesting an active 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.

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
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-β-cyclodextrin (MβCDX) was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and differentiation
U937L cells (kindly donated by Dr. F. William Luscinaskis, Brigham and Women’s Hospital, Boston, MA) were maintained in RPMI 1640, 10% FBS, t-glutamine, and penicillin-streptomycin and differentiated to a more monocyte 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% CO2. Experiments were done routinely with cells at passage two plated on thick hydrated type I collagen gels in 96-well culture plates.

PBMCs and monocyte isolation
PBMCs were isolated from healthy volunteers by density gradient centrifugation in Ficoll-Paque. Monocytes were isolated from the PBMC fraction using an MACS monocyte 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 domain 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 nondenaturing lysis buffer A, 1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM NaVO4, 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 fraction) 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 ul, 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, peltes obtained after Triton lysis were resuspended in a denaturing lysis buffer (1% SDS, 50 mM Tris-HCl, 10 mM NaVO4) 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 nondenaturing lysis buffer before being added to Protein G beads as above.

Isolation of DRM by sucrose density gradient ultracentrifugation
Differenitated U937L cells (1×107) 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 A. After ultracentrifugation for 20 h at 40,000 rpm in a Sorvall TH-641 rotor, 11 1-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 2×106/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 CO2 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 2×106/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 MgCl2, 2 mM EGTA, 0.32 M sucrose, 10 mM MES, protease inhibitors, 1 mM PMSF (pH 6.1)) for 20 min on ice preactivation. 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,
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, demonstrating specificity. In our hands, this method of Ab cross-linking does not result in nonspecific activation of FcγRs, as we see similar results using F(ab')2 Abs, and effects were not inhibited by blocking FcγRs (data not shown). These data suggest that the role of leukocyte PECAM extends beyond its function as an adhesion receptor and that activation of this molecule may be required during transmigration. We found no evidence to suggest that PECAM activation mediates the release of soluble factors that aide the transmigration event (Supplemental Fig. 1), although we cannot formally exclude the possibility of extremely localized short-lived factors.

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 lacking the 663 and 686 tyrosine phosphorylation sites. To make sure that the cells expressed the same levels of total PECAM, we first reversed this blockade and significantly promoted transmigration. Similar results were seen using the blocking polyclonal anti-PECAM Ab, 177. [The partial nature of the recovery is explained by the biology of the system, as elucidated by the experiments that follow (see Discussion).] However, Abs directed against CD99, a protein important in transmigration at a step distal to that regulated by PECAM and expressed on both leukocytes and endothelial cells (24), failed to promote transmigration after cross-linking, demonstrating specificity. In our hands, this method of Ab cross-linking does not result in nonspecific activation of FcγRs, as we see similar results using F(ab')2 Abs, and effects were not inhibited by blocking FcγRs (data not shown). These data suggest that the role of leukocyte PECAM extends beyond its function as an adhesion receptor and that activation of this molecule may be required during transmigration. We found no evidence to suggest that PECAM activation mediates the release of soluble factors that aide the transmigration event (Supplemental Fig. 1), although we cannot formally exclude the possibility of extremely localized short-lived factors.

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U937L cells re-expressing P-WT regained the ability to transmigrate to levels similar to those of control cells and displayed sensitivity to a PECAM-blocking Ab (Fig. 3F). Strikingly, however, re-expression of the Y663/686F mutant was unable to rescue the defect in transmigration caused by depletion of the endogenous protein (Fig. 3F). We found no difference in the level of adhesion or ability to polarize between the different cell lines (data not shown). From these results, we conclude that the tyrosine phosphorylation within the ITIM domains of leukocyte PECAM plays a critical role in transendothelial migration.

**Leukocyte PECAM resists detergent extraction after cross-linking**

Coupled with the observation of leukocyte PECAM phosphorylation after 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, ~50% of PECAM is found in the insoluble pellet. This movement is specific to PECAM, as other receptors, CD64 and CD32a, which are known to associate with Triton X-100-insoluble membranes upon activation, remain in the soluble fraction after PECAM cross-linking (Fig. 4A).

Resistance to cold Triton extraction could be due to partitioning into DRMs or association with the cytoskeleton. We found that U937L PECAM could be efficiently solubilized using warm 1% Triton X-100 lysis buffer even after cross-linking, a property that would be inconsistent with cytoskeletal association (data not shown). Similarly, PECAM insolubility was not due to cytoskeletal association, as cytochalasin D had no effect on cross-linking-induced 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 DRMs after its cross-linking**

To further test the hypothesis that PECAM was recruited to DRMs after cross-linking, we employed a more definitive technique. DRMs 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 DRM component, lyn kinase (27), was also enriched in the same light-density fractions and remained the same before and after PECAM cross-linking.

Depletion of membrane cholesterol by MβCDX has previously been shown to disrupt DRMs (28). Pretreating cells with 20 mM MβCDX for 45 min inhibited PECAM translocation to DRMs upon cross-linking (Fig. 4C). Lyn kinase also moved to a cold Triton-soluble fraction after MβCDX treatment, verifying the disruption of DRMs. 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...
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 (due to its association with the actin cytoskeleton). Consistent with the biochemical approaches described above, leukocyte PECAM became resistant to cold Triton extraction and was still visible following Ab-mediated cross-linking (Fig. 5A).

Using a similar approach, prelabeled monocytes were added onto prelabeled HUVEC monolayers for 10 min at 37°C followed by cold Triton extraction and fixation to analyze leukocyte PECAM behavior during transmigration. We then compared the levels of PECAM on monocytes in the process of transmigration, as assessed by their progression through VE-cadherin–stained junctions. Some monocytes present at endothelial junctions exhibited PECAM staining even after Triton X-100 extraction (arrow), whereas cells away from the junction showed no PECAM staining (asterisk) (Fig. 5B). The maximum fluorescent intensity of PECAM was quantified for >50 monocytes for which the position was classified 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...
a nonblocking anti-PECAM Ab (P1.1), which does not inhibit transmigration, but when cross-linked translocates PECAM to DRMs and retains it there (Fig. 7A) without effecting its homophilic adhesion properties (Supplemental Fig. 3). Forcing leukocyte PECAMs into DRMs through P1.1 cross-linking prior to their addition to HUVEC (t = 0) resulted in profound inhibition of transmigration. Meanwhile, P1.1 on its own or cross-linked after 30 min (t = 30) had little effect (Fig. 7B). Similarly, cross-linking PECAM with the blocking Ab hec7 at t = 0 inhibited transmigration, whereas cross-linking at t = 30 overcomes the block (Figs. 1, 7C). These data suggest that the timing of leukocyte PECAM activation and subsequent translocation to DRMs is important for transmigration. Forcing PECAM into DRMs before monocytes are able to get to endothelial junctions (i.e., at t = 0) inhibits transendothelial migration. Together, these data suggest that the retention of leukocyte PECAM in DRMs leads to a decrease in its phosphorylation and a subsequent inhibition of transmigration.

**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 transmigntion 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 dibutryl cAMP-differentiated U937L cells in which we depleted endogenous PECAM and re-expressed phspho-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-phenylalanine mutations at positions 663 and 686 displayed a sustained inhibition 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 and activation 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 (38–0), but SHP2 is capable of transmitting both stimulatory and inhibitory signals (41, 42). The fact that we see an increase in transmigration upon PECAM ligation suggests that in our system, PECAM acts as a stimulatory molecule. Consistent with this possibility, we and other groups have reported integrin activation downstream of leukocyte PECAM ligation (34, 43, 44). The identification and characterization of signals initiated downstream of leukocyte PECAM phosphorylation is currently under investigation. It is tempting to speculate that phosphorylation of leukocyte PECAM at endothelial junctions may activate a subpopulation of integrins to provide an anchor point while the cell passes through the endothelial monolayer. Integrin affinity changes have been monitored during leukocyte migration over endothelial substrates (45), but as yet there are no studies looking at their activation status during transmigntion.

The association of immune receptors with specialized domains within the plasma membrane has been implicated in their ability to transduce signals (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 death 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
The authors have no financial conflicts of interest.

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.