Endothelial PI 3-kinase activity regulates lymphocyte diapedesis

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Nakhaei-Nejad M, Hussain AM, Zhang Q, Murray AG. Endothelial PI 3-kinase activity regulates lymphocyte diapedesis. Am J Physiol Heart Circ Physiol 293: H3608–H3616, 2007. First published September 21, 2007; doi:10.1152/ajpheart.00321.2007.—Lymphocyte recruitment to sites of inflammation involves a bidirectional series of cues between the endothelial cell (EC) and the leukocyte that culminate in lymphocyte migration into the tissue. Remodeling of the EC F-actin cytoskeleton has been observed after leukocyte adhesion, but the signals to the EC remain poorly defined. We studied the dependence of peripheral blood lymphocyte transendothelial migration (TEM) through an EC monolayer in vitro on EC phosphatidylinositol 3-kinase (PI 3-kinase) activity. Lymphocytes were perfused over cytokine-activated EC using a parallel-plate laminar flow chamber. Inhibition of EC PI 3-kinase activity using LY-294002 or wortmannin decreased lymphocyte TEM (48 ± 6 or 34 ± 7%, respectively, vs. control; mean ± SE; P < 0.05). Similarly, EC knockdown of the p85α regulatory subunit of PI 3-kinase increased lymphocyte transmigration. Treatment of EC with jasplakinolide to inhibit EC F-actin remodeling also decreased lymphocyte TEM to 24 ± 10% vs. control (P < 0.05). EC PI 3-kinase inhibition did not change the strength of lymphocyte adhesion to the EC or formation of the EC “docking structure” after intercellular adhesion molecule-1 ligation, whereas this was inhibited by jasplakinolide treatment. A similar fraction of lymphocytes migrated on control or LY-294002-treated EC and localized to interendothelial junctions. However, lymphocytes failed to extend processes below the level of vascular endothelial (VE)-cadherin on LY-294002-treated EC. Together these observations indicate that EC PI 3-kinase activity and F-actin remodeling are required during lymphocyte diapedesis and identify a PI 3-kinase-dependent step following initial separation of the VE-cadherin barrier.

TRAFFIC OF LYMPHOCYTES from the blood to the tissue compartment underlies the cellular immune responses that mediate allograft rejection and many autoimmune diseases. Vascular endothelial cells (EC) at a site of inflammation provide a series of solid-phase cues to the lymphocyte that prompt tissue localization. Work by numerous investigators over the past decade has identified and characterized the role of various adhesion molecules and chemokines displayed by the EC that provide the cues to promote the initial capture of the lymphocyte from the bloodstream and adhesion to the surface of the vascular endothelium (22). Subsequent lymphocyte migration on the surface of the endothelium followed by diapedesis across the endothelial monolayer at the interendothelial cell junctions may be cued by different or overlapping signals (40, 51).

In addition, evidence has accumulated that leukocyte adhesion signals the EC to actively remodel both its adhesive contacts and cell shape during leukocyte diapedesis. For example, leukocyte adhesion stimulates increased rigidity of the EC cortical F-actin cytoskeleton and the development of F-actin-rich projections to surround the leukocyte in a “docking structure” (3, 46). This docking structure promotes adhesion of the leukocyte to the luminal surface of the endothelium under shear stress conditions (2). In addition, remodeling of the F-actin cytoskeleton and actin-associated interendothelial adhesion junctions has been observed as the leukocyte completes the subsequent diapedesis step (37, 42). The cues to the EC and the signaling events that mediate these late steps of leukocyte migration across the endothelial barrier remain poorly defined.

Phosphatidylinositol 3-kinase (PI 3-kinase)-dependent signal transduction pathways are candidates to mediate some of the events that underlie leukocyte diapedesis. Class I PI 3-kinases are lipid kinases that use phosphatidylinositol-4,5-bisphosphate as substrate and have received particular attention in the context of the immune system. Class I PI 3-kinases are subdivided into classes IA and IB that are coupled downstream exchange factors for Rho family GTP-binding proteins (45). In this way PI 3-kinase activity is able to recruit effector molecules in a spatially restricted fashion.

PI 3-kinase-dependent cell polarization enables directional actin polymerization and leukocyte motility in response to chemotactic stimuli (41). Hence early work identified defective polymorphonuclear leukocyte recruitment to inflammatory sites in mice deficient in the class IA PI 3-kinase and the class IB PI 3-kinase (35, 38). Vascular ECs share expression of PI 3-kinaseδ and PI 3-kinaseγ catalytic isoforms with bone marrow–derived cell lineages (14, 31). Surprisingly, in elegant experiments that studied acute inflammatory responses in PI 3-kinaseδ- or PI 3-kinaseγ-deficient mice reconstituted with wild-type bone marrow, PI 3-kinase activity in EC was found to be required for a robust inflammatory response (31). Intra-vital microscopy revealed a defect in the conversion of selectin-dependent, rolling adhesive interactions to stable, firm adhesion of the leukocyte to the endothelium. Unlike polymorphonuclear leukocytes, lymphocytes exploit immunoglobulin-superfamily adhesion molecules on the endothelium to mediate these events (9, 11, 16). The role of endothelial PI 3-kinase in lymphocyte transmigration is unknown.

In the present series of experiments, we observed that inhibition of EC PI 3-kinase activity decreased lymphocyte transendothelial migration (TEM) through an EC monolayer in vitro. Remodeling of existing endothelial F-actin structures is
required to support lymphocyte transit across an EC monolayer under physiological shear stress. However, PI 3-kinase inhibition did not block endothelial cortical F-actin remodeling or docking structure formation as a consequence of EC transmigration, HUVECs were pretreated with jasplakinolide (300 nM), wortmannin (10 µM), or LY-294002 (30 µM) for 1 h, Akt inhibitor I (as indicated) for 3 h, or Y-27632 (as indicated) for 30 min at 37°C. Next, the EC monolayers were washed three times before SDF-1α was adsorbed to the EC surface. Chemical inhibitors were used at concentrations that maintained HUVEC viability >85% of mock-treated controls as assessed by the XTT assay of mitochondrial activity as described (24). HUVEC expression of ICAM-1, vascular cell adhesion molecule-1, CD31, and JAM-C was monitored using indirect immunofluorescence and quantitated using flow cytometry. No changes were observed with the small molecule inhibitors. We detected no difference in SDF-1α adsorbed to HUVEC pretreated with dimethyl sulfoxide (DMSO) or LY-294002 using cell-surface enzyme-linked immunosorbent assay. The relative cellular G-actin and F-actin in DMSO or jasplakinolide-treated HUVEC were determined by Western blot using an actin quantitation kit according to the manufacturer’s instructions (Cytoskeleton).

**EC transfection.** HUVECs were plated at ~50% confluence in M199 with 2% FBS and ECGS, without antibiotics, 24 h before transfection. Fifty nanomolar control or p85α-specific siRNA was transfected using HiPerFect according to the manufacturer’s instructions. The monolayers were grown to confluence in 35-mm tissue culture plates and used in experiments 72 h after transfection. Endothelial p85α expression was monitored by Western blot in each experiment using p85α-specific mAb.

**Video microscopy imaging.** Laminar flow adhesion assays were done as described previously (18) using 35-mm tissue culture plates as the lower surface of a parallel-plate laminar flow chamber (127 µm gap; Glycotech, Rockville, MD). The chamber was mounted on the stage of an inverted phase-contrast microscope (Leica DM IRB; Leica Microsystems, Richmond Hill, ON), and lymphocyte/EC interactions were observed through a ×20 objective and captured using a charge-coupled device camera (Pixelink; Vitana, Ottawa, ON) at 12 frames/s for a 20-min period.

Lymphocytes were perfused over the EC monolayer at low shear flow (0.5 dyn/cm²) and allowed to accumulate on the ECs (accumulation phase). The flow rate was then increased to 1 dyn/cm² and was kept constant throughout the assay by perfusion of fresh binding buffer at 37°C (shear application phase). Analysis of lymphocyte motion was done manually using Quicktime Pro (Apple, Cupertino, CA) on all accumulated cells in video fields selected to contain >50 adherent lymphocytes/field. Lymphocytes entering or leaving the field of view after the initial frame were not included in the analysis. Throughout the analysis period of 20 min, >90% of adherent PBL remained in the field of view. The movement of adherent lymphocytes was categorized into 1) locomotion: lymphocytes that migrate more than one cell body on the surface of the endothelial monolayer or 2) transmigration: lymphocytes that undergo a change from phase-bright to phase-dark appearance as described previously (10). Lymphocyte migration across the EC monolayer was evident when the focal plane of the lymphocyte lay in the gel underlying the EC monolayer. In addition, the track of individual migrating lymphocytes was analyzed to determine if the cell migrated across an interendothelial cell junction. The data are reported as a fraction of the originally accumulated lymphocytes. In aggregate, the fractions of adherent lymphocytes under control conditions showing a motile or transmigration phenotype were 61% ± 3% and 26% ± 4% (mean ± SE), respectively.

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**Fluorescence microscopy imaging.** To characterize docking structure formation, HUVECs were grown to confluence on Matrigel-coated glass cover slips and were treated with TNF-α as above. HUVECs were then pretreated with DMSO carrier or with LY-29402 as indicated. Latex beads (Bang Laboratories, Fishers, IN) were adsorbed with anti-ICAM-1 mAb (clone P2A4) and characterized by 10.220.33.3 on June 11, 2017 http://ajpheart.physiology.org/ Downloaded from
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Using Chi square analysis (SPSS), data from four experiments were pooled and tested for significance to evaluate the position of lymphocytes at the interendothelial junction, by paired Student's t-test using SPSS (SPSS, Chicago, IL). To evaluate the position of lymphocytes at the interendothelial junction, data from four experiments were pooled and tested for significance using Chi square analysis (SPSS).

RESULTS

EC PI 3-kinase activity is required for efficient lymphocyte TEM. Loss-of-function mutations of PI 3-kinase or inhibition of PI 3-kinase activity has been observed to attenuate neutrophil-mediated inflammation in vivo, but the effect on other leukocyte subsets and the mechanism of the defect in recruitment has not been fully characterized (5, 30, 31). In the first series of experiments, we sought to determine if inhibition of EC PI 3-kinase affected lymphocyte TEM. We adopted the technique of Cinamon et al. (10) to study the migration of lymphocytes adherent to a confluent TNF-treated HUVEC monolayer under laminar flow conditions in vitro. Freshly isolated human PBL were perfused over the monolayer and allowed to accumulate on the surface of the EC at a low shear stress. The shear stress was then increased to 1 dyn/cm², and lymphocyte movement was recorded using phase-contrast videomicroscopy. Transmigration across the HUVEC monolayer was evident as a transition in the lymphocyte to a phase-dark appearance and determined at the end of the experiment by confirming that the plane of focus of the lymphocyte was in the Matrigel substratum below the EC monolayer (Fig. 1). HUVEC were pretreated with vehicle or either of the PI 3-kinase inhibitors wortmannin or LY-294002 before to assembly in the laminar flow apparatus. We observed that the fraction of adherent lymphocytes that underwent TEM across wortmannin- or LY-294002-treated EC monolayers was markedly reduced, but the fraction of lymphocytes that moved on the surface of the endothelial monolayer was not inhibited (Fig. 2A). Wortmannin pretreatment of lymphocytes did not inhibit TEM (Fig. 2C).

Furthermore, we used RNAi to the p85α regulatory subunit, common to the α, β, and δ catalytic isoforms of class IA PI 3-kinases, to confirm the results obtained using the pharmacological inhibitors of PI 3-kinase activity. We observed marked inhibition of endothelial p85 expression (Fig. 2B) after treatment with specific siRNA and tested confluent p85-deficient EC monolayers for the efficiency of lymphocyte transmigration (Figs. 1D and 2A). Lymphocyte transmigration across p85-deficient monolayers, but not migration on the surface of the monolayer, was inhibited. These observations indicate that endothelial PI 3-kinase activity is required during the process of TEM and is partly dependent on p85 regulation.

EC F-actin remodeling facilitates lymphocyte TEM. PI 3-kinase-mediated generation of PIP₃ locally regulates the activity of a variety of actin-associated proteins to indirectly regulate F-actin turnover (52). F-actin polymerization is thought to be important in the development of a docking structure that is associated with stable adhesion and leukocyte TEM (2, 6, 7). Therefore, we sought to determine if inhibition of EC F-actin remodeling had a similar effect on lymphocyte TEM as inhibition of endothelial PI 3-kinase activity. ECs were pretreated with vehicle or jasplakinolide before assembly in the parallel-plate laminar flow apparatus to inhibit remodeling of F-actin structures in the living EC. We observed that jasplakinolide pretreatment significantly reduced the fraction of lymphocytes...
able to transmigrate across the EC monolayer (Fig. 3). However, there was no difference in lymphocyte migration on the surface of the EC monolayer.

Jasplakinolide is a macrolide marine toxin that specifically binds and inhibits the remodeling of established F-actin microfilaments (43). To confirm that jasplakinolide pretreatment of the HUVEC at the concentrations used in these experiments was sufficient to stabilize EC F-actin, we tested the ability of cytochalasin D to depolymerize F-actin in EC lysates in vitro. Figure 3B shows that HUVEC pretreated with jasplakinolide have a greater fraction of actin incorporated in F-actin structures than carrier-treated HUVEC monolayers. Moreover F-actin from the jasplakinolide-treated HUVEC was resistant to cytochalasin D-mediated depolymerization. Taken together, then, these results indicate that stabilization of the HUVEC F-actin cytoskeleton impairs lymphocyte TEM.

Strong adhesion of lymphocytes to EC requires EC cytoskeletal remodeling but is independent of PI 3-kinase activity. Previous work has identified an association between the formation of a docking structure by the endothelium at sites of mononuclear leukocyte adhesion and subsequent leukocyte TEM (7). In the next series of experiments, we sought to determine if EC PI 3-kinase activity or remodeling of the endothelial F-actin cytoskeleton was required to stabilize lymphocyte adhesion and to form the docking structure. Freshly isolated human PBL were allowed to adhere to TNF-pretreated HUVEC, and then lymphocyte adhesion to the EC monolayer was determined under conditions of increasing shear stress. Figure 4A demonstrates that inhibition of endothelial PI 3-kinase activity does not change the adhesiveness of PBL to TNF-pretreated EC, but that jasplakinolide pretreatment of EC inhibits stable lymphocyte adhesion under high shear stress conditions.

To directly confirm that inhibition of endothelial PI 3-kinase activity does not disturb F-actin remodeling required for docking structure formation, we examined the formation of F-actin rings after adhesion of latex beads to the EC surface (Fig. 4B). These data indicate that inhibition of EC PI 3-kinase does not affect formation of the docking structure or reorganization of endothelial F-actin at the site of lymphocyte adhesion. Taken together, these data indicate that inhibition of endothelial PI 3-kinase impairs lymphocyte TEM.

Fig. 2. Inhibition of endothelial phosphatidylinositol 3-kinase (PI 3-kinase) inhibits lymphocyte transendothelial migration. A: quantitation of the fraction of adherent lymphocytes that locomote on the luminal surface of the endothelial cell monolayer (filled bars) or transmigrate across the monolayer (open bars). Lymphocyte migration across HUVEC monolayers pretreated with wortmannin (10 μM) or LY-294002 (30 μM) was performed as in MATERIALS AND METHODS. The p85α regulatory subunit of class IA PI 3-kinases was knocked down by RNAi as indicated in MATERIALS AND METHODS (mean ± SE, n = 4 experiments; *P < 0.05 vs. control). B: p85α-specific siRNA decreases HUVEC p85α expression by Western blot as described in MATERIALS AND METHODS. C: inhibition of lymphocyte PI 3-kinase activity with wortmannin does not decrease either lymphocyte locomotion or transendothelial migration (mean ± SE, n = 3 experiments).

Fig. 3. Endothelial F-actin remodeling is required for lymphocyte transendothelial migration. A: fraction of adherent lymphocytes migrating on the surface (filled bars) or transmigrating (open bars) across carrier-, cytochalasin D-, or jasplakinolide-pretreated HUVEC monolayers under 1 dyn/cm² laminar shear stress was determined as in MATERIALS AND METHODS (mean ± SE, n = 5 experiments; *P < 0.05 vs. control). B: jasplakinolide stabilizes the endothelial F-actin cytoskeleton. HUVEC were treated with dimethyl sulfoxide (DMSO) carrier or 300 nM jasplakinolide for 1 h, then the cells were lysed, and an aliquot of each lysate was treated with cytochalasin D to depolymerize F-actin. Globular (G) vs. filamentous (F) actin was resolved as indicated in MATERIALS AND METHODS. Data are representative of 3 experiments.
3-kinase does not significantly alter functional or structural characteristics of lymphocyte adhesion to TNF-pretreated EC.

**EC Rho but not protein kinase B is required for efficient lymphocyte TEM.** PI 3-kinase activity to generate PIP3-enriched domains of plasma membrane can serve to create a docking site for the subcellular localization of effector molecules, such as protein kinase B/Akt and Rho GTP-binding protein exchange factors via interaction with PH domains of the protein (32). PI 3-kinase activation of Akt has been implicated in regulation of cortical actin remodeling acting upstream of the Rac GTP-binding protein (36). However, we observed that inhibition of EC Akt had no effect on either lymphocyte surface migration or diapedesis (Fig. 5). Therefore, the effects of endothelial PI 3-kinase inhibition on lymphocyte TEM appear to be independent of signaling through the Akt pathway.

The RhoA GTP-binding protein is variably reported to regulate docking structure formation to promote leukocyte adhesion, or stress fiber formation and myosin contractility to generate centripetal tension that may contribute to the release of interendothelial cell adhesion during leukocyte diapedesis. Because LY-294002-mediated inhibition of PI 3-kinase inhibited lymphocyte TEM, we next tested the hypothesis that Rho GTP-binding protein signaling was involved. We observed that inhibition of EC Rho kinase with Y-27632 had a modest inhibitory effect on lymphocyte TEM under shear stress conditions (Fig. 5), but the magnitude of the inhibition was consistently less than that seen with PI 3-kinase inhibition. Nevertheless, this is consistent with the model that Rho kinase-dependent signaling occurs downstream of PI 3-kinase activity to facilitate lymphocyte TEM.

**Lymphocyte penetration of endothelial intercellular junctions requires endothelial PI 3-kinase activity and F-actin remodeling.** Leukocytes preferentially transit an endothelial monolayer at interendothelial cell junctions (4, 7, 47, 51). Because endothelial PI 3-kinase inhibition did not block docking structure formation, we determined the fraction of motile and stationary adherent lymphocytes that contacted the interendothelial cell margins of control, jasplakinolide-, and LY-294002-treated EC monolayers. As shown in Fig. 6A, analysis of the videomicrographs indicates a similar fraction of lymphocytes localized to the interendothelial cell margins among vehicle- and inhibitor-treated endothelial monolayers. Furthermore, a similar number of lymphocytes migrated along interendothelial cell margins among each group (data not shown).

![Figure 4](http://ajpheart.physiology.org/) Endothelial cell F-actin stabilization but not PI 3-kinase inhibition impairs docking structure development. 

![Figure 5](http://ajpheart.physiology.org/) Endothelial Rho kinase but not protein kinase B (Akt) activity is required for lymphocyte transendothelial migration. Lymphocyte transendothelial migration through HUVEC monolayers pretreated as in MATERIALS AND METHODS with Akt inhibitor I (A) or Y-27632 (B) was analyzed as in Fig. 2. The locomotion fraction (filled bars) and transmigration fraction (open bars) were determined (mean ± SE, n = 3 experiments; *P < 0.05 vs. control).
To characterize the point that diapedesis is impeded during lymphocyte transit of the interendothelial junction, we used confocal microscopy to study the junction-associated lymphocytes, fixed after 10 min of interaction with EC monolayers under shear stress. We grouped the lymphocytes into those that were located in the z-axis above the level of the endothelial VE-cadherin, completely below the endothelial VE-cadherin, or those that extended both above and below the adherens junction (Fig. 7). Results from four independent experiments were pooled for analysis. We observed that 43 vs. 18% (*P < 0.001) of lymphocytes migrating across control vs. LY-294002-pretreated monolayers had completed diapedesis and were completely below the level of VE-cadherin staining. A similar fraction (10 vs. 11%) of lymphocytes were seen in the migration channel between adjacent EC in control and LY-294002-pretreated monolayers. In contrast, 71% of lymphocytes associated with interendothelial cell junctions among LY-294002-pretreated EC monolayers were localized above the level of VE-cadherin vs. 48% among control monolayers (*P < 0.001). Of those lymphocytes adherent over endothelial adherens junctions, 22% traversing LY-294002-treated monolayers were associated with a >2-μm gap in the VE-cadherin barrier vs. 9% crossing control monolayers (*P = 0.003). Because VE-cadherin has been shown to be excluded from the developing migration pore as the leukocyte begins diapedesis (42), this suggests that the lymphocytes are able to initiate separation of the interendothelial junctions despite EC PI 3-kinase inhibition but are inefficient in extending processes to interpenetrate adjacent EC.

Finally, as a measure of the ability of the EC to accommodate lymphocyte interposition between adjacent EC, the time taken by a lymphocyte to complete transit of the EC monolayer from the point of initial interposition between adjacent LY-294002- or jasplakinolide-treated ECs was determined from the videomicrographs. As shown in Fig. 6B, lymphocytes that successfully completed diapedesis across EC monolayers treated to inhibit PI 3-kinase activity or F-actin remodeling transited the monolayer slower than lymphocytes transmigrating across control monolayers.

Taken together, these data indicate that neither inhibition of EC PI 3-kinase activity nor F-actin remodeling affected the ability of the lymphocytes to migrate on the surface of the endothelium to the favored site of most TEM events at the interendothelial cell junction. However, lymphocyte interposition between the adjacent EC to complete the diapedesis step was impaired. These observations suggest that endothelial PI

Fig. 6. Inhibition of endothelial PI 3-kinase or F-actin remodeling does not impair lymphocyte contact with the interendothelial cell margins but prolongs transit time through the migration channel. A: HUVEC monolayers were treated with carrier, jasplakinolide, LY-294002, or Y-27632 as in MATERIALS AND METHODS and then the fraction of adherent lymphocytes overlying or migrating on the luminal surface across interendothelial junctions under 1 dyn/cm² shear stress was determined by analysis of videomicroscopy images (mean ± SE, n = at least 3 experiments; *P = NS). B: time to complete transit across HUVEC monolayers was determined by analysis of videomicroscopy images of control, LY-294002-, or jasplakinolide-treated HUVEC monolayers (mean ± SE, n = at least 3 experiments; *P < 0.05 vs. control).

Fig. 7. Inhibition of endothelial PI 3-kinase does not prevent focal disassembly of the endothelial adherens junction. A: lymphocyte (cytoplasm stained with CellTracker Red) migrating across a control HUVEC monolayer is seen to extend between and beneath EC adherens junctions (vascular endothelial (VE)-cadherin stained green). The panel in center shows the x-y dimension, the panel on top show the x-z dimension, and the panel on the left shows the y-z dimension along a line passing through the migration channel. The basal surface of the EC monolayer in the z dimension is to the right or bottom of the y-z or x-z orthogonal projections, respectively. B: lymphocyte adherent to a monolayer treated with LY-294002 is associated with a gap in the adherens junction but fails to extend beneath the level of endothelial VE-cadherin. Quantitation of 526 adherent lymphocytes grouped by position in the migration channel is described in the text.
3-kinase activity is required during lymphocyte diapedesis at the interendothelial cell junction.

DISCUSSION

Molecules that mediate leukocyte movement from the blood to the tissue compartment have been identified as potential targets for therapeutic intervention in inflammation. Our observations describe the requirement for vascular EC PI 3-kinase activity, one such molecular target, to support lymphocyte diapedesis across human vascular ECs. Furthermore, we demonstrate that remodeling of the existing endothelial F-actin structures is also required. We observe that inhibition of either endothelial phosphoinositide generation or F-actin remodeling impairs lymphocyte TEM without blocking surface migration toward interendothelial cell junctions. Nevertheless, careful analysis of the developing interendothelial migration channel indicates that endothelial PI 3-kinase inhibition does not impair the development of a gap in the endothelial adherens junction, an early event associated with migration channel formation. Taken together, these observations indicate that endothelial PI 3-kinase activity and remodeling of endothelial cortical F-actin structures is necessary at the interendothelial cell junction site of diapedesis to facilitate TEM.

We inhibited endothelial PI 3-kinase activity using two pharmacological inhibitors of all class I PI 3-kinase catalytic isoforms. In addition, we used RNAi to knock down expression of the p85α regulatory subunit in EC, commonly used by the class IA PI 3-kinase isoforms. Although both the pharmacological and RNAi approaches inhibited lymphocyte TEM, the magnitude of the decrease was modest after p85α inhibition. This indicates that endothelial PI 3-kinase activity during lymphocyte transmigration is regulated through the p85α subunit and implicates endothelial class IA PI 3-kinase activity in remodeling of the interendothelial cell junction during lymphocyte transmigration. However, redundancy with class IB PI 3-kinase activity, or among the alternate p55, p55, or p50 regulatory subunits of class IA PI 3-kinases as described earlier (12, 44), may have salvaged activity through the pathway in the absence of p85α expression.

In contrast to the effect of inhibition of endothelial PI 3-kinase activity on lymphocyte transmigration, we saw little effect of lymphocyte PI 3-kinase inhibition. Although earlier work identified reduced migration of lymphocyte lines in Boyden chamber assays after PI 3-kinase inhibition (20, 28), under the short physiological time frames of transmigration under shear stress, lymphocyte PI 3-kinase activity appears to be dispensable (10). Recent work identifies an alternate signal pathway to polarize lymphocytes through the atypical exchange factor Dock2 to Rho GTP-binding protein activation in response to chemotactic chemokine stimulation (13, 25). Chemokines stimulate the class IB PI 3-kinase isoform in lymphocytes (33), but deficiency of PI 3-kinase activity in vivo has minimal effect on T cell movement (1) or homing to lymphoid organs (27, 38).

Recent work in vivo has identified an important role for class IB PI 3-kinase and class IA PI 3-kinaseδ activity in the development of neutrophil-dependent inflammation (17, 30). Intravital microscopy identifies impaired conversion from a loose adhesive interaction to stable adhesion between wild-type neutrophils and vascular ECs deficient in PI 3-kinase-δ or γ activity in the murine cremasteric vein model (31). This suggests that ECs use PI 3-kinase to stabilize the interaction between the leukocyte and endothelium in the presence of shear forces.

F-actin-rich projections of the EC develop around adherent leukocytes in vitro and have been proposed to serve this function (2). However, we observed no effect of endothelial PI 3-kinase inhibition on either the strength of the adhesive interaction between the adherent lymphocyte and the endothelial surface under flow conditions or on F-actin polymerization surrounding beads that cross-link the endothelial adhesion molecule CD54. Whereas neutrophils use adhesion molecules of the selectin class and subsequently engage CD54, lymphocytes engage both CD106 and CD54 on the EC to mediate rolling and tight adhesion (11, 16). The difference in the adhesion molecules employed by the two types of leukocytes may account for the apparent difference in the requirement for endothelial PI 3-kinase activity for tight adhesion and warrants further investigation.

Leukocyte TEM across the interendothelial junctions is associated with gaps in VE-cadherin (4). Indeed, VE-cadherin is dynamically excluded from the interendothelial cell junction underlying an adherent leukocyte as the migration channel develops (42). We observe that lymphocytes accumulate on the surface of LY-294002-treated endothelial monolayers and are associated with gaps in VE-cadherin of comparable dimensions to gaps we have observed associated with lymphocyte migration. Interestingly, the lymphocytes are not seen to extend processes below the level of the adherens junction marker, suggesting that a LY-294002-sensitive barrier exists subsequent to and independent of VE-cadherin gap formation. Our observation that the transit time to cross the EC monolayer is prolonged among lymphocytes that successfully transmigrate LY-294002- or jasplakinolide-pretreated EC is consistent with a model indicating that PI 3-kinase activity and F-actin remodeling are linked.

A similar defect in transmigration, with leukocytes delayed above the interendothelial cell junction, has been reported under conditions of blockade of endothelial intercellular junction proteins JAM-C (19), CD31 (23) or the poliovirus receptor (34), whereas CD99 blockade inhibits mononuclear cell transmigration at a somewhat later step, and monocytes are observed to be trapped in the migration channel (39). PI 3-kinase activity has been indirectly linked to engagement of CD31 in ECs, but truncation of the cytoplasmic domain of CD31 does not block leukocyte transmigration (21, 26). Our observations lend support to the idea that discrete steps can be identified during leukocyte diapedesis. Further work is required to determine if engagement of endothelial junction molecules promotes PI 3-kinase activity in EC.

Activation of PH domain-containing proteins such as Akt or Rho GDP/GTP exchange factors is linked to PI 3-kinase-dependent PIP3 display on the plasma membrane. Activated Akt associates with the actin cytoskeleton (8) and participates in F-actin remodeling (32). However, we observe no effect of endothelial Akt inhibition on lymphocyte TEM. In contrast, inhibition of Rho kinase, a downstream effector molecule of the monomeric GTP-binding protein RhoA, modestly inhibited lymphocyte TEM. RhoA activity is regulated indirectly by PI 3-kinase activity through membrane localization of Rho-specific GTP/GDP exchange factors to membrane sites enriched in...
PI(3)K (48). The coordinated activity of several Rho family members is implicated in adhesion junction remodeling (49). The effect of PI3 kinase inhibition to block lymphocyte transmigration is consistently greater than the effect of RhoA or Rho kinase inhibition, suggesting other Rho family members may also participate in remodeling the interendothelial cell junction.

In summary, endothelial PI 3-kinase activity is required for efficient lymphocyte TEM. The defect in lymphocyte TEM created by inhibition of endothelial PI 3-kinase is not at the formation of the docking structure. Lymphocytes migrate on the surface of the endothelium, contact interendothelial cell borders, are associated with gaps in VE-cadherin in the absence of endothelial PI 3-kinase activity, but fail to complete diapedesis across the interendothelial cell junctions. This suggests that, in addition to gap formation in the VE-cadherin barrier, a second rate-limiting event is involved during lymphocyte interpenetration of the adjacent EC as the EC accommodates leukocyte transmigration. Therefore, our experiments have identified a role for endothelial PI 3-kinase activity in lymphocyte diapedesis at a late step in the transmigration pathway.

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