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ICAM-1-Mediated, Src- and Pyk2-Dependent Vascular Endothelial Cadherin Tyrosine Phosphorylation Is Required for Leukocyte Transendothelial Migration

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Leukocyte transendothelial migration (TEM) has been modeled as a multistep process beginning with rolling adhesion, followed by firm adhesion, and ending with either transcellular or paracellular passage of the leukocyte across the endothelial monolayer. In the case of paracellular TEM, endothelial cell (EC) junctions are transiently disassembled to allow passage of leukocytes. Numerous lines of evidence demonstrate that tyrosine phosphorylation of adherens junction proteins, such as vascular endothelial cadherin (VE-cadherin) and β-catenin, correlates with the disassembly of junctions. However, the role of tyrosine phosphorylation in the regulation of junctions during leukocyte TEM is not completely understood. Using human leukocytes and EC, we show that ICAM-1 engagement leads to activation of two tyrosine kinases, Src and Pyk2. Using phospho-specific Abs, we show that engagement of ICAM-1 induces phosphorylation of VE-cadherin on tyrosines 658 and 731, which correspond to the p120-catenin and β-catenin binding sites, respectively. These phosphorylation events require the activity of both Src and Pyk2. We find that inhibition of endothelial Src with PP2 or SU6656 blocks neutrophil transmigration (71.1 ± 3.8% and 48.6 ± 3.8% reduction, respectively), whereas inhibition of endothelial Pyk2 also results in decreased neutrophil transmigration (25.5 ± 6.0% reduction). Moreover, overexpression of the nonphosphorylatable Y658F or Y731F mutants of VE-cadherin impairs transmigration of neutrophils compared with overexpression of wild-type VE-cadherin (32.7 ± 7.1% and 38.8 ± 6.5% reduction, respectively). Our results demonstrate that engagement of ICAM-1 by leukocytes results in tyrosine phosphorylation of VE-cadherin, which is required for efficient neutrophil TEM. The Journal of Immunology, 2007, 179: 4053–4064.

Inflammation is a component of the normal immune response in which leukocytes leave the bloodstream and pass into tissues to fight infection. However, when dysregulated, inflammation contributes to numerous disease processes. Passage of leukocytes across the endothelium and into tissues, termed transendothelial migration (TEM), is a critical component of the inflammatory response. The multistep paradigm of leukocyte TEM divides this process into three steps: rolling and adhesion; firm adhesion; and diapedesis (1, 2). During each of these steps, signals are initiated that serve to facilitate progression to the next stage of TEM. During rolling and adhesion, selectins on the endothelial cell (EC) establish transient weak adhesive interactions with carbohydrate ligands on the leukocyte (3). At this time leukocytes encounter chemokines displayed on the surface of the activated EC. These chemokines then activate leukocyte β1 and β2 integrins, facilitating firm adhesion (4, 5). During firm adhesion, the endothelial Ig family adhesion molecules VCAM-1 and ICAM-1 are engaged by leukocyte integrins α4β1 and αvβ3, respectively. Engagement of VCAM-1 and ICAM-1 in turn initiates diverse signals within the EC, which are required for diapedesis (6, 7). Leukocytes undergoing diapedesis use one of two pathways: the paracellular, which occurs between adjacent EC, or the transcellular, which occurs through a pore formed in the body of the EC (8). However, the factors that influence the relative use of these two pathways have not yet been fully characterized.

Paracellular leukocyte TEM is regulated in large part by the endothelial adherens junction. The major adhesive component of the adherens junction is vascular endothelial cadherin (VE-cadherin), a calcium-dependent, homophilic cell-cell adhesion molecule. That VE-cadherin is a critical regulator of the leukocyte TEM is demonstrated by studies in which treatment of EC with blocking anti-VE-cadherin Abs caused increased TEM and loss of endothelial barrier integrity (9–11). Interestingly, leukocytes themselves induce transient displacement of VE-cadherin from cell-cell junctions during paracellular passage across the endothelium (11–13). In addition, IL-1β treatment of EC disrupted adherens junctions, resulting in monocytes transmigrating by a predominantly paracellular route (14). Thus, the adherens junction may be a critical regulator of the mode of leukocyte TEM. Specifically, VE-cadherin may be an important target in the signaling pathways that mediate paracellular transmigration of leukocytes.

Numerous lines of evidence have demonstrated that tyrosine phosphorylation of endothelial adherens junction proteins is associated with the dissociation of junctions (15–18). Although much is known about the role of tyrosine phosphorylation in regulating the general maintenance of adherens junctions in endothelia, the role of tyrosine phosphorylation of EC adherens junction proteins as it relates specifically to leukocyte TEM has not been fully explored. Interestingly, it has been shown that engagement of...
ICAM-1 and VCAM-1 generates diverse outside-in signals, which are required for leukocyte TEM (6, 7). Prominent among these signals is the activation of cellular kinases resulting in tyrosine phosphorylation of several proteins (19–22).

We tested the hypothesis that signals initiated by leukocyte adhesion could induce tyrosine phosphorylation of adherens junction proteins within the EC. We show that coincubation of leukocytes with TNF-α-treated EC enhances tyrosine phosphorylation of VE-cadherin, specifically on residues Y658 and Y731. Furthermore, we observe that although ICAM-1 is widely distributed across the apical surface of the EC, it is significantly enriched near cell-cell junctions. We therefore tested the role of ICAM-1-mediated signaling in leukocyte-mediated VE-cadherin phosphorylation and found that engagement of ICAM-1 is sufficient to induce tyrosine phosphorylation of VE-cadherin on Y658 and Y731. In agreement with previous studies, we find that ICAM-1 engagement enhances the activity of Src kinase (19, 21). Additionally, we have demonstrated for the first time that ICAM-1 engagement results in activation of endothelial proline-rich tyrosine kinase 2 (Pyk2). Moreover, we observed that the active forms of both kinases are recruited to sites of ICAM-1 engagement. Accordingly, inhibition of Src or Pyk2 decreases ICAM-1-mediated VE-cadherin phosphorylation. Finally, we show that activation of endothelial Src and Pyk2, as well as VE-cadherin phosphorylation on Y658 and Y731 are all required for efficient TEM of neutrophils. These data represent the first direct evidence that endothelial Pyk2 and VE-cadherin phosphorylation play a role in mediating leukocyte TEM.

Materials and Methods

Reagents and Abs
Phospho-specific polyclonal Abs against Src (pY418), Pyk2 (pY402), VE-cadherin (pY658), and VE-cadherin (pY731) were obtained from Bio-source International. Monoclonal PY-20 anti-phosphotyrosine Abs were obtained from BD Transduction Laboratories. Monoclonal Abs against VE-cadherin were obtained from Santa Cruz Biotechnology. Recombinant TNF-α, anti-ICAM-1, anti-VCAM-1, and IgG mAbs were from R&D Systems. Src kinase inhibitors PP2, SU6656 as well as the control compound PP3 were purchased from Calbiochem. The wild-type VE-cadherin GFP adenovirus was a gift of Drs. F. Nwariaku and D. Nahari (University of Texas Southwestern Medical Center, Dallas, TX). CRNK (CADTK/CARK-related non-kinase) adenovirus was a gift of Dr. L. Graves (University of North Carolina, Chapel Hill, NC). All other adenosinergic constructs were generated using the Virapower Adenoviral Expression System (Invitrogen Life Technologies) according to manufacturer’s instructions.

Cell cultures, treatments, and transfections
HUCVE were obtained from Cambrex and cultured as previously described (23). To mimic inflammation and to enhance ICAM-1 expression, EC were activated with 10 ng/ml TNF-α overnight as indicated. To block ICAM-1 function, anti-ICAM-1 Abs were used at 10 μg/ml for 1 h. All cell lines were cultured or incubated at 37°C at 10% CO2. THP-1 cells were obtained from the University of North Carolina Lineberger Comprehensive Cancer Center (UNC-LCCC) Tissue Culture Facility and grown in RPMI 1640 plus 10% FBS. HL-60 cells were obtained from the American Type Culture Collection (ATCC). HL-60 and THP-1 cells were cultured. After 10 min, unbound beads were washed away, and coverslips were subsequently processed for immunofluorescence. For biochemistry, 105 neutrophils were then diluted with 20 volumes of 1% Triton X-100, 1% DOC in TBS. A total of 2 μg of PY-20 monoclonal anti-phosphotyrosine Ab and protein G-Sepharose were added and samples were incubated 4 h at 4°C. Samples were then washed five times in 1% Triton X-100 and 1% DOC in TBS, and analyzed by Western blot using anti-VE-cadherin Ab.

Western blotting
For biochemistry, 10 μg/ml Ab-coated beads were incubated on cells that were then washed twice gently with ice-cold Ca2+ - and Mg2+-containing PBS and lysed in 35 μl of boiling sample buffer. Cells were scraped from the dish and samples were boiled immediately for 10 min. Samples were then analyzed by Western blotting using an ECL detection system from Amersham Biosciences. The intensity of bands was quantified using the NIH Image J1.36, developed at the National Institutes of Health (freely available at http://rsb.info.nih.gov/nih-image/). Results were then subjected to statistical analysis using Student’s t test. Values of p < 0.05 were considered significant.

Immunocytochemistry and image analysis
Cells were cultured on glass cover slips, fixed and immunostained with the indicated primary Abs using the protocol previously described (23). Subsequent visualization was performed with Alexa-conjugated secondary Abs obtained from Molecular Probes/Invitrogen Life Technologies. Images were recorded with a Zeiss LSM510 confocal microscope with appropriate filter settings. Bleed through between channels was avoided by use of sequential scanning. To assess the role of VE-cadherin phosphorylation in paracellular TEM, EC on coverslips was transduced with wild-type or mutant GFP-tagged VE-cadherin and then TNF-α-treated. EC were subsequently incubated with 5 ng/ml IL-8 as chemoattractant, then washed thoroughly and incubated with primary human neutrophils in assay medium (DMEM plus 0.25% BSA) at 37°C, 10% CO2 for 30 min. Cells were then fixed and stained for ICAM-1. Initiation of TEM was marked by the presence of ICAM-1-positive gap structures surrounding neutrophils and was considered paracellular if the leukocyte was within 1 micron of cell junctions and if VE-cadherin staining was disrupted.

Ab-coated beads
Three-micron polystyrene beads were purchased from Polysciences and were pretreated overnight with 8% glutaraldehyde, washed five times with PBS, and incubated with 300 μg/ml ICAM-1 or VCAM-1 mAb according to the manufacturer’s protocol.

Leukocyte and bead adhesion assays
For immunofluorescence, a 1/100 dilution of bead slurry was incubated in 24 wells containing glass coverslips on which TNF-α-pretreated HUVEC were cultured. After 10 min, unbound beads were washed away, and coverslips were subsequently processed for immunofluorescence. For biochemistry, 900 μl of a 1/30 dilution of bead slurry was incubated on HUVEC cultured in 6-well dishes. For leukocyte adhesion assays, 1 × 105 THP-1 or HL-60 cells were used per well of a 6-well dish. THP-1 cells were washed three times in HUVEC growth media and incubated with EC for various times before lysis in hot sample buffer and subsequent processing.

TEM assay
Migration assays were performed using Transwell filters of 6.5-mm diameter with 8 μm pores as previously described (11). Briefly, EC were grown to confluence on Matrigel-coated Transwell filters and treated with TNF-α overnight. The lower chamber was filled with assay medium (DMEM plus 0.25% BSA) plus 5 ng/ml IL-8 as chemoattractant. EC were washed twice with assay medium and subsequently incubated with 1 × 105 neutrophils at 37°C, 10% CO2 for 1 h. Transmigration was quantified by counting transmigrated cells in the lower chamber using a hemocytometer. To check efficient expression of transduced constructs, cells were simultaneously grown in 24-well dishes and treated with an equivalent amount of adenovirus and analyzed by immunocytochemistry.

Results
Leukocyte adhesion to EC induces tyrosine phosphorylation of VE-cadherin
Previously, it was reported that coinoculation of EC and C5a-activated neutrophils causes increased tyrosine phosphorylation of AJ proteins; however, the specific mechanisms of leukocyte-induced modification of AJ proteins remain unclear (26, 27). To examine
specifically the consequences of leukocyte binding to EC on the phosphorylation of VE-cadherin, we treated EC with TNF-α, which has been shown to increase expression of cell adhesion molecules such as ICAM-1 and VCAM-1 (28). We then incubated EC with monocytic THP-1 cells to avoid inflammatory factors released by activated neutrophils as well as degradation of VE-cadherin by neutrophil proteases during sample preparation as has been previously described (29). After coincubation with THP-1 cells, samples were lysed in gel sample buffer and boiled to inactivate cellular proteases, kinases and phosphatases, and then diluted to allow immunoprecipitation with anti-phosphotyrosine Abs. To determine the amount of phosphorylated VE-cadherin, samples were then analyzed by Western blot using anti-VE-cadherin Abs. Compared with cells incubated with medium alone (left lane), cells incubated with THP-1 cells (right lane) had increased levels of tyrosine phosphorylated VE-cadherin. Data represent quantification of three independent experiments (right), * p < 0.05.

We next wished to determine specific residues on VE-cadherin that may be phosphorylated upon leukocyte adhesion. We therefore took advantage of newly available phospho-specific Abs directed against either Y658 or Y731. After 10 min, increased tyrosine phosphorylation was detected on both residues. Data represent quantification of three independent experiments (right), * p < 0.05.

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Y731. TNF-α-treated EC were again incubated with THP-1 cells for various times and were then lysed directly in sample buffer. Lysates were then analyzed by Western blot using anti-VE-cadherin Abs. Incubation with THP-1 cells for 10 min increased tyrosine phosphorylation of VE-cadherin at both tyrosine residues, demonstrating that adhesive interactions between leukocytes and EC are capable of mediating tyrosine phosphorylation of VE-cadherin (Fig. 1B).

**FIGURE 3.** ICAM-1 engagement induces tyrosine phosphorylation of VE-cadherin. 

**A**. TNF-α-treated EC were incubated with control IgG beads (left lane) or anti-ICAM-1 beads (right lane) after which samples were lysed in hot sample buffer. Samples were then diluted to allow immunoprecipitation with anti-phosphotyrosine Ab. To determine the amount of phosphorylated VE-cadherin, samples were analyzed by Western blot using anti-VE-cadherin Abs. Incubation with anti-ICAM-1 beads induced tyrosine phosphorylation of VE-cadherin. Data represent quantification of three independent experiments (right), *, p < 0.05. 

**B**. TNF-α-treated EC were left untreated (left and middle lanes) or treated with ICAM-1 blocking Ab (right lane) and then incubated with granulocytic HL-60 cells (middle and right lanes) after which samples were lysed in hot sample buffer. Samples were then diluted to allow immunoprecipitation with anti-phosphotyrosine Ab. To determine the amount of phosphorylated VE-cadherin, samples were then analyzed by Western blot using anti-VE-cadherin Abs. Incubation with HL-60 cells induced tyrosine phosphorylation of VE-cadherin, which was inhibited by blocking ICAM-1. Blot shown is representative of three independent experiments. 

**C**. TNF-α-treated EC were incubated with control IgG beads (left lane) or anti-ICAM-1 beads (middle and right lanes) for various times. Cells were then lysed directly in hot sample buffer and lysates were analyzed by Western blot using phospho-specific Abs directed against pY658 (top) or pY731 (bottom) on VE-cadherin. After 10 min, tyrosine phosphorylation of both Y658 and Y731 was observed. Data represent quantification of three independent experiments (right), *, p < 0.05.

ICAM-1 localizes to cell-cell junctions in TNF-α-treated EC where ICAM-1 engagement increases local tyrosine phosphorylation

We hypothesized that molecules involved in the firm adhesion of leukocytes, such as ICAM-1, could signal to EC cell-cell junctions during leukocyte TEM. To examine the localization of ICAM-1 relative to cell junctions, we stained ICAM-1 and VE-cadherin in
TNF-α-treated EC. In agreement with previous reports, we found that ICAM-1 is enriched at sites of cell-cell contact (Fig. 2A) (30, 31). Previously, ICAM-1 engagement has been shown to induce tyrosine phosphorylation of several molecules (19–22). To specifically examine signals within EC, we used anti-ICAM-1 Ab-coated polystyrene beads to cross-link ICAM-1 on TNF-α-treated EC as described in Materials and Methods. Carman et al. (32) demonstrated that incubating anti-ICAM-1 beads with EC drives the formation of ICAM-1-enriched, cup-like structures that protrude above the apical plane of the EC and which partially surround the bead. To avoid imaging other sources of phosphotyrosine present in the cell body, we used confocal microscopy to visualize phosphotyrosine in ICAM-1-dependent cup structures, which only contain signaling proteins generated or recruited by ICAM-1 engagement. By looking at a single confocal slice from ~1 micron above the apical surface of the endothelium, we observed that phosphotyrosine staining was increased specifically at sites where anti-ICAM-1 beads adhered (Fig. 2B). Thus, ICAM-1 engagement induces a local increase in phosphotyrosine.

The importance of ICAM-1 in TEM and the observation that ICAM-1 engagement leads to local increases in tyrosine phosphorylation led us to test whether increased tyrosine phosphorylation of AJ proteins would increase leukocyte TEM. Pervanadate treatment of EC has previously been shown to increase tyrosine phosphorylation of AJ proteins as well as increasing neutrophil TEM (16, 18). We confirmed these findings in our system as well (data not shown). This response suggests that tyrosine phosphorylation of AJ proteins may indeed be important in regulating leukocyte TEM.

ICAM-1 engagement results in tyrosine phosphorylation of VE-cadherin

The observation that ICAM-1 localizes to cell-cell junctions and that ICAM-1 engagement increases localized phosphotyrosine levels led us to examine whether ICAM-1 signaling plays a role in leukocyte-mediated tyrosine phosphorylation of VE-cadherin. To specifically activate ICAM-1-mediated signals, beads coated with anti-ICAM-1 Ab or with control IgG were added to TNF-α-treated EC, and anti-phosphotyrosine immunoprecipitation was performed followed by Western blot analysis with anti-VE-cadherin Abs as before. Incubation of cells with anti-ICAM-1 beads, but not control IgG beads, caused an increase in VE-cadherin phosphorylation (Fig. 3A).
The observation that ICAM-1 engagement leads to tyrosine phosphorylation of VE-cadherin led us to examine whether leukocyte binding to ICAM-1 is the only signal that regulates tyrosine phosphorylation of VE-cadherin. To test this observation, we pretreated EC with blocking anti-ICAM-1 Abs, and coincubated these EC with THP-1 cells and assayed for VE-cadherin phosphorylation as before. To our surprise, blocking ICAM-1 did not significantly inhibit VE-cadherin phosphorylation (data not shown). This led us to test whether other signals might contribute to the tyrosine phosphorylation of VE-cadherin during THP-1 adhesion. Because monocytic cells such as THP-1 also bind VCAM-1, which is known to generate signals in EC, we asked whether VCAM-1 also mediates tyrosine phosphorylation of VE-cadherin. To test this effect, we cross-linked VCAM-1 using anti-VCAM-1 beads and assayed for VE-cadherin phosphorylation as before. Interestingly, we found that VCAM-1 is also capable of inducing tyrosine phosphorylation of VE-cadherin (data not shown). To avoid effects due to VCAM-1 signaling, we coincubated EC with granulocytic HL-60 cells, which in contrast to THP-1 cells, do not express the VCAM-1-binding integrin VLA-4 (33). When we assayed for VE-cadherin phosphorylation, we found that adhesion of HL-60 cells induced robust phosphorylation of VE-cadherin. This effect was completely blocked by pretreatment of EC with ICAM-1 blocking Abs (Fig. 3B).

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We next examined which VE-cadherin residues became tyrosine phosphorylated downstream from ICAM-1 engagement. TNF-α-treated EC were incubated with anti-ICAM-1 beads for various times, and cells were lysed directly in sample buffer. Samples were then analyzed by Western blot using phospho-specific Abs directed against VE-cadherin phosphorylated on Y658 or Y731. After 10 min, maximal tyrosine phosphorylation was seen at both tyrosine residues (Fig. 3C). By 30 min, levels of tyrosine phosphorylation had declined to near baseline levels (data not shown). These data indicate that ICAM-1-mediated signals can induce VE-cadherin tyrosine phosphorylation and thus may be responsible for leukocyte-induced VE-cadherin phosphorylation.

Activated Src is recruited to sites of ICAM-1 engagement

We next wanted to dissect the signaling pathways by which ICAM-1 clustering leads to VE-cadherin phosphorylation. The observation that ICAM-1 engagement leads to tyrosine phosphorylation of VE-cadherin led us to examine whether leukocyte binding to ICAM-1 is the only signal that regulates tyrosine phosphorylation of VE-cadherin. To test this observation, we pretreated EC with blocking anti-ICAM-1 Abs, and coincubated these EC with THP-1 cells and assayed for VE-cadherin phosphorylation as before. To our surprise, blocking ICAM-1 did not significantly inhibit VE-cadherin phosphorylation (data not shown). This led us to test whether other signals might contribute to the tyrosine phosphorylation of VE-cadherin during THP-1 adhesion. Because monocytic cells such as THP-1 also bind VCAM-1, which is known to generate signals in EC, we asked whether VCAM-1 might also mediate tyrosine phosphorylation of VE-cadherin. To test this effect, we cross-linked VCAM-1 using anti-VCAM-1 beads and assayed for VE-cadherin phosphorylation as before. Interestingly, we found that VCAM-1 is also capable of inducing tyrosine phosphorylation of VE-cadherin (data not shown). To avoid effects due to VCAM-1 signaling, we coincubated EC with granulocytic HL-60 cells, which in contrast to THP-1 cells, do not express the VCAM-1-binding integrin VLA-4 (33). When we assayed for VE-cadherin phosphorylation, we found that adhesion of HL-60 cells induced robust phosphorylation of VE-cadherin. This effect was completely blocked by pretreatment of EC with ICAM-1 blocking Abs (Fig. 3B).

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ICAM-1 engagement has previously been shown to activate Src family kinases, resulting in tyrosine phosphorylation of several proteins (19–21). In support of these findings, incubation of EC with anti-ICAM-1 beads resulted in an increase in Src phospho-
ylated on Y418, a phosphorylation event which has been shown to be critical for activation of the kinase (Fig. 4A). To explore whether activated Src kinase would be recruited to sites of ICAM-1 engagement, EC were incubated with anti-ICAM-1 beads and lysed and analyzed by Western blot as previously described. Anti-ICAM-1 beads, but not control beads, induced tyrosine phosphorylation on Y658 and Y731. However, inclusion of PP2 with anti-ICAM-1 beads inhibited phosphorylation of VE-cadherin at both Y658 and Y731. Data represent quantification of three independent experiments. *, p < 0.05. B, GFP and the dominant negative Pyk2 mutant, CRNK, were expressed in EC using an adenoviral delivery system. EC were then TNF-α-treated and incubated with control IgG or anti-
ICAM-1 beads and lysed and analyzed by Western blot as previously described. Anti-ICAM-1 beads, but not control beads, induced tyrosine phosphorylation of VE-cadherin. However, expression of CRNK inhib-
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tion of VE-cadherin. Data represent quantification of three independent experiments. *, p < 0.05.

Thus, activated Src is specifically recruited to sites of ICAM-1 engagement.

ICAM-1 engagement activates endothelial Pyk2
Previous work has shown that Pyk2 plays a role in junctional dis-
assembly and localizes to cell-cell junctions upon junctional dis-
ruption (34). Thus, Pyk2 is a candidate tyrosine kinase down-
stream from ICAM-1. To test whether ICAM-1 can activate Pyk2, we used a phospho-specific Ab directed against pY402 of Pyk2, which is the auto-phosphorylation site for Pyk2. Again, control or anti-ICAM-1 beads were added to TNF-α-treated EC for 10 min, followed by lysis directly in hot gel sample buffer. Western blot analysis using anti-Pyk2 pY402 revealed that anti-ICAM-1 beads, but not control beads, increased Pyk2 phosphorylation on Y402 (Fig. 5A). Thus, ICAM-1 engagement activates not only Src, but also Pyk2. To test whether the active form of this kinase is recruited to sites of ICAM-1 engagement, we incubated TNF-α-treated EC with...
anti-ICAM-1 beads, then fixed, stained for Pyk2 pY402, and performed confocal analysis as described. We found that the active form of Pyk2 was enriched at sites of ICAM-1 engagement, but that control beads coated with mouse IgG did not recruit active Pyk2 (Fig. 5B). As before, EC were incubated with anti-ICAM-1 beads and stained for Pyk2 pY402 or control proteins, β-catelin or VE-cadherin. ICAM-1 engagement recruited active Pyk2, but not β-catelin or VE-cadherin (Fig. 5C). The enrichment of active Pyk2 at sites of ICAM-1 engagement further supports the role of ICAM-1 in activating Pyk2.

**Src and Pyk2 are required for ICAM-1-mediated tyrosine phosphorylation of VE-cadherin**

Our observation that ICAM-1 engagement can recruit and activate both Src and Pyk2 led us to examine the roles of these kinases in ICAM-1-mediated phosphorylation of VE-cadherin. To test whether Src activity is required, we included PP2 or the inactive control compound, PP3, with anti-ICAM-1 beads and incubated them with EC as previously described. Inclusion of PP2, but not PP3, with anti-ICAM-1 beads completely inhibited ICAM-1-mediated phosphorylation of VE-cadherin on both Y658 and Y731 (Fig. 6A). Similar results were obtained using another Src inhibitor, SU6656 (data not shown). Interestingly, inhibition of Src resulted in a reduction of VE-cadherin phosphorylation even below baseline levels. This result suggests that Src family kinases are required not only for the ICAM-1-mediated increase in VE-cadherin phosphorylation, but also for maintenance of low level phosphorylation, which is perhaps part of normal VE-cadherin function.

Because no pharmacological inhibitor of Pyk2 is available, we used an adenoviral delivery system to express CRNK in EC before treatment with anti-ICAM-1 beads. CRNK is an N-terminal truncated fragment of Pyk2 that acts in a dominant negative manner (35). To rule out nonspecific effects of adenoviral infection, control cells were infected with a GFP-expressing adenovirus. ICAM-1 engagement caused VE-cadherin phosphorylation in GFP-expressing EC. By contrast, expression of CRNK decreased ICAM-1-mediated VE-cadherin phosphorylation at Y658 and Y731 (Fig. 6B). These results demonstrate that both Src and Pyk2 are required for ICAM-1-mediated phosphorylation of VE-cadherin.

**Endothelial Src and Pyk2 activity are required for efficient neutrophil TEM**

Taking into account the role of Src in ICAM-1-mediated phosphorylation of VE-cadherin, we hypothesized that endothelial Src regulates cell-cell junctions during leukocyte TEM. To test this hypothesis, we used Transwell transmigration assays using TNF-α-treated EC, which were pretreated with vehicle control or the Src inhibitor PP2, and then washed thoroughly. For these assays, primary human neutrophils were used. In comparison to vehicle control, PP2 pretreatment dramatically decreased leukocyte transmigration (71.1 ± 3.8% reduction). Similar results were obtained with another Src inhibitor compound, SU6656 (48.6 ± 3.8% reduction) (Fig. 7A). These results suggest that endothelial Src family kinase activity is required for leukocytes to pass between EC.

We next tested whether Pyk2 activity is required for leukocyte TEM. EC were transduced with either GFP or CRNK and then used in transmigration assays. CRNK expression significantly attenuated neutrophil TEM compared with GFP controls (25.5 ± 6.0% reduction) (Fig. 7B), indicating that endothelial Pyk2 also contributes to efficient neutrophil TEM.

**VE-cadherin tyrosine phosphorylation on Y658 and Y731 is required for efficient neutrophil TEM**

To determine the importance of VE-cadherin phosphorylation on Y658 or Y731 in TEM, we generated adenoviruses expressing GFP-tagged mutants of VE-cadherin in which Y658 or Y731 were mutated to phenylalanine. When expressed in EC, mutant and wild-type VE-cadherin localized to cell-cell junctions (Fig. 8A). EC were infected with adenovirus expressing VE-cadherin GFP Y658, VE-cadherin GFP Y731, or wild-type VE-cadherin such that expression was seen in 95–100% of cells. Transmigration assays revealed that expression of either the Y658F or Y731F mutant caused a significant reduction in neutrophil transmigration (32.7 ± 7.1% and 38.8 ± 6.5% reduction, respectively) (Fig. 8B). Next, EC expressing wild-type VE-cadherin, or the Y658F or Y731F mutants, were incubated with primary human neutrophils. Samples were then fixed, processed for immunofluorescence microscopy, and initiation of transmigration was scored as paracellular based on criteria described in Materials and Methods. Neutrophils incubated with endothelial monolayers expressing wild-type VE-cadherin initiated TEM via the paracellular pathway in 76.25 ± 4.7% of cases examined (Fig. 8C). By contrast, neutrophils incubated with monolayers expressing Y658F or Y731F mutants used the paracellular pathway only 37.5 ± 5.4% or 33.3 ± 5.3% of the time, respectively (Fig. 8C). Taken together, these data suggest that ICAM-1-mediated phosphorylation of VE-cadherin is required for efficient neutrophil TEM.
cell-cell junctions. An adenoviral expression system. All VE-cadherin constructs localized to significantly inhibited neutrophil TEM.

\[ Y_{658}, Y_{731} \] were expressed using an adenoviral expression system. EC were then incubated with freshly isolated human neutrophils. Both nonphosphorylatable mutants significantly inhibited neutrophil TEM. Data are representative of at least three independent experiments with each condition performed in quadruplicate. EC were grown on matrigel-coated glass coverslips and GFP-tagged VE-cadherin wild type (a), Y658F (b), or Y731F (c) were expressed using an adenoviral expression system. EC were then incubated with freshly isolated human neutrophils. Both nonphosphorylatable mutants significantly reduced the proportion of neutrophils using the paracellular pathway. Data are representative of ~100 transmigration events per condition analyzed from three independent experiments.

on Y658 and Y731 plays an important role in paracellular neutrophil TEM.

**Discussion**

In recent years, it has become clear that the process of leukocyte TEM involves a complex set of signals in both leukocytes and the endothelia. Cues from adhesive interactions between the two cell types are responsible for coordination of these signals leading to efficient leukocyte TEM. ICAM-1 has emerged as a critical regulator of this process due to its role in leukocyte adhesion as well as its role in outside-in signaling pathways.

Previous studies have shown that ICAM-1 engagement has multiple effects on the actin cytoskeleton. For example, ICAM-1 cross-linking activates RhoA and induces stress fiber formation (36). Resultant tension generated in the EC may stimulate the opening of cell-cell junctions (37–39). ICAM-1 interacts with several actin-binding proteins including α-actinin 4, and ERM proteins (40, 41). Celli et al. (40) demonstrate a functional requirement for the interaction between ICAM-1 and α-actinin 4 during leukocyte TEM. Similarly, sequestration of endothelial ezrin or moesin away from sites of leukocyte adhesion blocks formation of endothelial docking structures as well as leukocyte TEM (41, 42).

ICAM-1 has also been shown to regulate the phosphorylation of the cytoskeletal regulatory protein, cortactin. Yang et al. (22, 43) have demonstrated a requirement for Src-mediated phosphorylation of cortactin specifically in paracellular leukocyte TEM. It was also shown that cortactin phosphorylation is required for leukocyte-mediated cytoskeletal remodeling as well as clustering of ICAM-1 (43). During, TEM, ICAM-1 clustering is required for numerous signaling processes in EC, thus Src-cortactin signaling may be the initiating event upstream of other ICAM-1-dependent signals (43). In line with this, expression of VE-cadherin Y658F or Y731F mutants had no effect on the distribution or recruitment of ICAM-1 to sites of leukocyte adhesion (data not shown) strengthening the idea that the ICAM-Src-cortactin pathway functions upstream of VE-cadherin phosphorylation. It is likely that Src activated downstream of ICAM-1 engagement has numerous targets during leukocyte TEM. The fact that Src has at least two targets that are localized to cell-cell junctions, cortactin and now VE-cadherin, suggests that Src has important functions in paracellular leukocyte TEM. In our system, we find that inhibition of endothelial Src strongly attenuates TEM of leukocytes. This result is in accordance with Yang et al. (22, 43), who have shown that inhibition of endothelial Src reduces TEM of neutrophils by roughly 40%. The multifaceted role of Src in paracellular TEM provides a likely explanation for why inhibition of Src blocks leukocyte TEM to a greater extent than Pyk2 inhibition, or specific blockade of VE-cadherin phosphorylation (Figs. 7 and 8).

Although many studies of signaling by endothelial adhesion molecules, such as ICAM-1 and VCAM-1, have focused on regulation of the actin cytoskeleton or gene expression (7), the observation that VCAM-1 cross-linking can cause formation of intercellular gaps suggests that signals generated by leukocyte adhesion also target cell-cell junctions (44). In this study, we demonstrate that ICAM-1-mediated signaling also regulates proteins of the adherens junctions during TEM and that these signals are required for TEM of neutrophils. Several lines of evidence lead us to propose a role for ICAM-1-mediated phosphorylation of VE-cadherin in facilitating junctional disruption during the paracellular passage of leukocytes. We and others have observed that, in TNF-α-treated EC, ICAM-1 is enriched at EC cell-cell junctions (30, 31). This localization places ICAM-1 in an appropriate location from which to signal to the AJ protein. Moreover, treatment of coronary artery EC with IL-1β enhanced the proportion of monocytes using the paracellular route and this response was due to IL-1β-mediated weakening of cell-cell contact (14). Thus, junctional integrity is important in regulating the transmigration of leukocytes by the paracellular pathway. Interestingly, ICAM-1 signaling has previously been shown to be required for leukocyte-mediated disruption of cell-cell junctions in vitro. Specifically, adhesion of monocytes to TNF-α-treated EC causes displacement of VE-cadherin and associated catenins from the areas of cell-cell contact (12, 13). This phenomenon was shown to be ICAM-1-dependent, as pretreatment of leukocytes with blocking Abs directed against the ICAM-1 receptor, β1 integrin, inhibited leukocyte-mediated disruption of junctions (12). Another study demonstrated that Ab blockade of ICAM-1-mediated adhesion...
causes monocytes to wander across the endothelium, failing to undergo diapedesis at cell-cell junctions (45). Together, these data suggest that signals downstream from ICAM-1 play an important role in junctional disassembly, which is required for paracellular leukocyte transmigration.

ICAM-1-mediated phosphorylation of VE-cadherin specifically on Y658 and Y731 may cause junctional disassembly by two potential mechanisms. Previous work demonstrated that the phosphomimetic VE-cadherin mutant Y731E exhibits greatly decreased binding to β-catenin (46). Thus, phosphorylation on this residue would lead to uncoupling of VE-cadherin from the actin cytoskeleton, thereby weakening junctions and promoting leukocyte passage through the paracellular pathway. Similar experiments with the Y658E VE-cadherin mutant suggested that phosphorylation of Y658 reduces binding to p120-catenin (46). Because it has been shown that interaction with p120-catenin stabilizes VE-cadherin at the junctions by preventing its clathrin-mediated endocytosis, the loss of p120-catenin binding may remove VE-cadherin from sites of paracellular TEM by an endocytic mechanism (47–49). Based on our data, we propose a model in which ICAM-1 engagement near cell-cell junctions results in localized activation of Src and Pyk2 leading to phosphorylation of VE-cadherin on Y658 and Y731. These signals then mediate junctional disruption at sites of leukocyte paracellular TEM, which allows leukocytes to pass across the endothelium (Fig. 9). In support of this model, we have shown that ICAM-1 is capable of recruiting and activating both Src and Pyk2. Thus, leukocytes transmigrating at cell-cell borders will engage ICAM-1, resulting in local activation of these kinases and accompanying phosphorylation of VE-cadherin.

This work creates several intriguing directions for future studies. For example, it is currently unclear which kinase directly phosphorylates VE-cadherin downstream of ICAM-1 engagement. The means by which Src and Pyk2 may regulate one another downstream from ICAM-1 is also unknown. Pyk2 and Src are known to interact and this interaction has been shown to contribute to the activation of both kinases (50–52). However, differing reports regarding the interdependence of Src and Pyk2 in EC complicate speculation regarding the temporal regulation of these kinases in this pathway (50, 51, 53). Moreover, while we show that Src and Pyk2 are required for ICAM-1-mediated VE-cadherin phosphorylation, other intermediate signals, such as reactive oxygen species, may play a role in this pathway. Finally, it is interesting to consider the role of other adhesion molecules, such as VCAM-1, in the regulation of endothelial AJ proteins during leukocyte TEM. Our preliminary studies suggest...
that VCAM-1 can also mediate phosphorylation of VE-cadherin and that VCAM-1 can substitute for ICAM-1 in this pathway (data not shown). Of note, VCAM-1 engagement has been shown to activate Rac1 resulting in generation of reactive oxygen species, which are capable of activating Pyk2 (44, 54). Therefore, engagement of VCAM-1 may further activate Pyk2 downstream of leukocyte adhesion to EC. The differences between the ICAM-1- and VCAM-1-mediated VE-cadherin phosphorylation pathways deserve further study. It is interesting to note that Pyk2 phosphorylates β-catenin, which also plays a role in regulation of EC junctional stability (34). Thus, additional protein components of cell–cell junctions may be affected by signals triggered by leukocyte adhesion.

Finally, VE-cadherin and its phosphorylation status may contribute to determining the route of leukocyte transmigration. Our data suggest that expression of nonphosphorylatable VE-cadherin reduces paracellular passage of leukocytes (Fig. 8). It is interesting to note that the percent reduction in initiation of paracellular TEM (Fig. 8C) correlates well with the percent reduction in total TEM seen in Fig. 8F. This observation is consistent with a lack of compensation via the transcellular pathway. In the future, we hope to explore this in more detail.

In conclusion, we have identified one step in the pathway by which leukocytes cause junctional disruption during paracellular TEM. Enhanced leukocyte TEM and associated disruption of endothelial junctions has been implicated in pathologies ranging from multiple sclerosis to ischemia-reperfusion injury. Thus, the signaling processes elaborated in this work may serve as potential targets in the treatment of diverse inflammatory diseases.

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