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# Endothelial Cell Cortactin Phosphorylation by Src Contributes to Polymorphonuclear Leukocyte Transmigration In Vitro

Lin Yang, Jennifer R. Kowalski, Xi Zhan, Sheila M. Thomas, Francis W. Luscinskas

**Abstract**—The underlying mechanisms that regulate leukocyte transendothelial migration through the vascular endothelium remain unclear. Cortactin is a substrate of Src tyrosine kinases and a regulator of cytoskeletal dynamics. Previous studies demonstrated a role for Src phosphorylation of cortactin in clustering of E-selectin and intercellular cell adhesion molecule-1 around adherent leukocytes. In the current study, we used an in vitro flow model to investigate the role of Src-induced cortactin phosphorylation in endothelium during polymorphonuclear leukocyte (PMN) transmigration through human umbilical vein endothelium (HUVEC) monolayers preactivated with tumor necrosis factor- $\alpha$ . Inhibition of Src in HUVEC using Src kinase inhibitors PP2 and SU6656 reduced PMN transmigration by  $45 \pm 8\%$  and  $36 \pm 6\%$ , respectively. Live cell imaging of green fluorescent protein–tagged cortactin in HUVEC revealed redistribution of cortactin in the region surrounding transmigrating PMN. Knockdown of cortactin in HUVEC by small interfering RNA also impaired transmigration to a similar degree, and this phenotype was rescued by reexpression of wild-type cortactin. Analysis of the location of initial arrest and locomotion of PMN adherent to HUVEC demonstrated that inhibition of Src tyrosine kinases or pretreatment with cortactin small interfering RNA reduced PMN transmigration at endothelial cell-to-cell junctions and not adhesion. Tyrosine phosphorylation of cortactin was important for transmigration, because expression of a mutant, in which the tyrosine phosphorylation sites were mutated to phenylalanine (cortactin3F), failed to rescue PMN transmigration. Moreover, expression of cortactin3F alone partially blocked PMN transmigration. These data suggest a model whereby tyrosine phosphorylation of cortactin by Src family kinases regulates PMN transmigration (*Circ Res.* 2006;98:394-402.)

**Key Words:** leukocytes ■ endothelium ■ transendothelial migration

A key component of the inflammatory response is leukocyte recruitment to sites of injury, immune response, or infection. Leukocytes interact with the vascular endothelium through sequential, overlapping steps including leukocyte arrest, rolling, firm adhesion, and ultimately transendothelial migration (TEM) through the vascular endothelium to underlying tissues.<sup>1</sup>

Leukocyte adhesion or crosslinking of the endothelial adhesion molecules E-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, or P-selectin triggers association of these molecules with the endothelial cytoskeleton and transmits outside-in signals that include increases in both intracellular  $\text{Ca}^{2+}$  concentration and myosin contractility and activation of p38 kinase, pp<sup>60src</sup> (Src), and Rho GTPase family.<sup>2,3</sup> These signals lead to rapid cytoskeletal reorganization.<sup>1–3</sup> Previous studies have shown that treatment of endothelium with cytoskeletal disrupting agents or inhibitors of myosin light-chain kinase (MLCK)

blocks leukocyte transmigration,<sup>4</sup> suggesting that cytoskeletal reorganization is necessary for leukocyte TEM.

Cortactin is an F-actin binding protein and an important substrate of Src tyrosine kinases.<sup>5</sup> Cortactin contains an N-terminal acidic domain that binds the Arp2/3 complex and a central repeat domain consisting of 6.5 tandem repeats of 37 amino acids that binds F-actin. The C-terminal region contains a Src homology 3 domain (SH3) that interacts with a variety of cellular proteins. A proline-rich region (PRR) between the tandem repeats and SH3 domain contains tyrosine, serine, and threonine residues that are targets of phosphorylation.<sup>6</sup> Tyrosine residues 421, 466, and 482 (within PRR) of murine cortactin are important targets of Src tyrosine kinases. Expression of a cortactin mutant with these tyrosine residues mutated to phenylalanine impaired endothelial cell migration.<sup>7</sup> Thus, tyrosine phosphorylation of cortactin is important for its role in cytoskeletal regulation.

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Recent studies reported that Src and cortactin associate with ICAM-1 and E-selectin, providing connections between these adhesion molecules and the cytoskeleton.<sup>8</sup> Crosslinking of E-selectin or ICAM-1 on endothelial cells or adhesion of leukocytes (THP-1) to endothelial cells triggered Src-dependent tyrosine phosphorylation of cortactin. Inhibition of Src by PP2 did not prevent association of E-selectin and ICAM-1 with cortactin but did reduce THP-1 adhesion.<sup>9</sup> However, the effect of PP2 on leukocyte TEM was not addressed. Hence, the role of cortactin in leukocyte recruitment is of interest because it associates with ICAM-1 and E-selectin, and its tyrosine phosphorylation by Src correlates with adhesion of THP-1 cells. Recently we have found that leukocyte function-associated antigen-1 on PMN rapidly redistributes to form ring-like structures during PMN transmigration and colocalizes with its endothelial ligand ICAM-1.<sup>10</sup> Further studies by our laboratory showed that the ICAM-1 cytoplasmic tail is required for TEM of PMN,<sup>11</sup> in line with previous reports that T cells have a similar requirement.<sup>12</sup> Based on these observations, we hypothesized that leukocyte adhesion triggers Src phosphorylation of cortactin, which subsequently enables cortactin to integrate ICAM-1 engagement and cytoskeletal remodeling. Such a mechanism might stabilize ICAM-1 clustering around adherent leukocytes and/or facilitate loosening of endothelial cell-cell junctions during leukocyte transmigration. Herein, our results indicate that Src-mediated tyrosine phosphorylation of cortactin in endothelial cells plays a significant role in PMN transmigration.

## Materials and Methods

### Materials

Human recombinant tumor necrosis factor (TNF)- $\alpha$  was purchased from Genzyme (Cambridge, Mass). PP2 and SU6656 were from Calbiochem (San Diego, Calif). Hec-1 (a kind gift from Dr William Muller, Weill Medical College, Cornell University, NY) is a non-blocking monoclonal antibody (mAb) to human VE-cadherin. Hec-1 was conjugated to Alexa 568 (Molecular Probes, Eugene, Ore) for identification of endothelial cell-cell junctions.<sup>11</sup> mAb to ICAM-1 (HU5/3), E-selectin (H18/7), VCAM-1 (E1/6), and nonbinding control mAb K16/16 were used in fluorescence-activated cell sorter (FACS) studies.<sup>13</sup> For Western blotting, mAb to cortactin (4F11) was purchased from Upstate Biotechnologies (Lake Placid, NY).

### Endothelial Cell Culture and Leukocyte Isolation

Human umbilical vein endothelial cells (HUVEC) were isolated, pooled, and cultured in M199 (BioWhittaker Bioproduct, Walkersville, Md) containing 10% FBS as previously described.<sup>14</sup> Human PMNs (>95% pure) were isolated from whole blood drawn from healthy volunteers, anticoagulated with sodium citrate.<sup>11</sup> The isolated PMNs were kept at 8°C and used in studies immediately and for no more than 3 hours after isolation. Blood was drawn and handled according to protocols for protection of human subjects approved by the Brigham and Women's Hospital Institutional Review Board, and all volunteer subjects gave informed consent.

### Cortactin Small Interfering RNA and Transfection

A predesigned small interfering RNA (siRNA) oligonucleotide targeting human cortactin mRNA and a scrambled version of this sequence were purchased from Ambion Inc (Austin, Tex). BLAST searches were performed on both oligonucleotides to ensure the absence of nonspecific targets. The sequences of the oligonucleotides are: cortactin siRNA oligonucleotide (sense), GGGAGAAUGUCUUUCAAGATT; scram-

bled control, UAAGCUGAUAGUACUGGAGTT. A second cortactin siRNA oligonucleotide (GGAGAGGAACUUGAAACATT; Ambion) was also used. HUVEC were transfected with 50 nmol/L of siRNA using Oligofectamine (Invitrogen, Carlsbad, Calif). After 3 days, PMN transmigration studies were performed.

### Retroviral Cortactin Production and Infection

Retrovirus production and infection were performed as previously described.<sup>15</sup> HUVEC were exposed to 2 rounds of retroviral infection, 6 and 3 days before being assayed in transmigration experiments. The expression level of green fluorescent protein (GFP)-tagged cortactin relative to endogenous cortactin was determined by Western blot. The level of cortactin was normalized to FAK to correct for sample loading. The infection efficiency of the retroviral constructs in HUVEC was variable (20% to 50%) as assessed by fluorescence microscopy. For TEM studies, we addressed this variability in infection efficiency by selecting microscope fields with relatively high percentages of GFP-positive cells (GFP: 63±10%; GFP-cortactin: 60±7%; cortactin3F-GFP: 59±6%; n=3 independent experiments, 30 fields for each cell type) that had similar levels of GFP fluorescence per field as shown in Figure 6B.

The following constructs were used for expression of murine cortactin in HUVEC cells: pBabe-GFP,<sup>15</sup> pBabe-GFP-cortactin,<sup>15</sup> MGIN-cortactin-GFP,<sup>7</sup> and MGIN-cortactin3F-GFP (tyrosine 421, 466, and 482 residues mutated to phenylalanine<sup>7</sup>).

### PMN Adhesion and Transmigration Assay

The live cell fluorescence microscopy flow model has been described.<sup>10,11</sup> Confluent HUVEC monolayers were activated with TNF- $\alpha$  (25 ng/mL, 4 hour) and inserted into the flow chamber. Where indicated, HUVEC were preincubated with Alexa-568 tagged anti-VE-cadherin mAb (10 minutes, 0.7  $\mu$ g/mL) to identify cell-cell junctions.<sup>11</sup> PMN ( $1 \times 10^6$ /mL) suspended in flow buffer (DPBS containing 0.1% human serum albumin, 0.75 mmol/L Ca<sup>2+</sup> and Mg<sup>2+</sup>) were drawn across HUVEC at 1.0 dyne/cm<sup>2</sup> for 3 minutes, followed by flow buffer alone for 10 minutes.

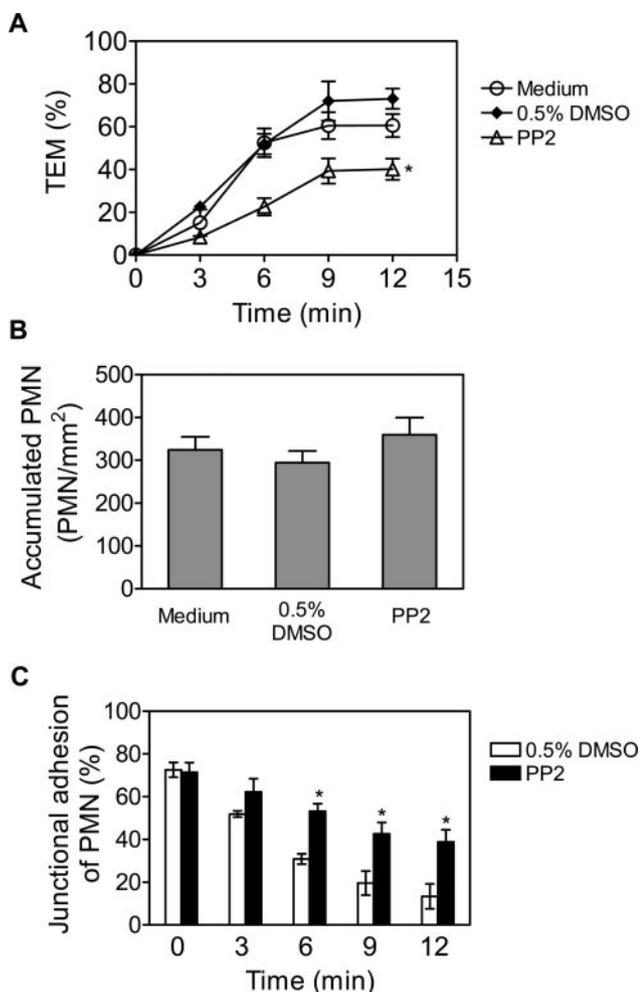
### Image Acquisition and Analysis

Live cell imaging of leukocyte TEM was performed using a digital imaging system coupled to a Nikon TE2000 inverted microscope equipped for differential interference contrast (DIC) microscopy as detailed previously.<sup>11</sup> For TEM experiments, sequential images of VE-cadherin (red) or GFP-cortactin (green) and DIC were taken every 15 sec for 10 minutes (unless noted otherwise) in a representative field, and at the end of flow, 4 additional fields were recorded (each field/30 seconds). The number of accumulated leukocytes was determined by counting the total number of adhered and transmigrated cells per field. The %TEM = total transmigrated leukocytes/[total adhered + transmigrated leukocytes] × 100. The location of adherent PMN was calculated as the distance from the center of the PMN to the nearest junction as identified by VE-cadherin staining. Distances less than 5  $\mu$ m from the PMN to the junction were considered as "junctional adhesion."

## Results

### Inhibition of Src Family Kinase Activity in Endothelium Impairs PMN Transmigration

Previous studies<sup>9</sup> have shown that cortactin is tyrosine phosphorylated in response to ICAM-1 crosslinking, which was abrogated by PP2, a Src family kinase-specific inhibitor.<sup>16</sup> Similar results were seen in the current HUVEC system (Figure I in the online data supplement available at <http://circres.ahajournals.org>). Because ICAM-1 and its downstream signaling capabilities are important for leukocyte TEM, we examined whether Src family kinases are required for leukocyte TEM. Here, we used PP2 to inhibit Src family kinases in HUVEC and determined whether PMN transmi-



**Figure 1.** Inhibition of Src tyrosine kinase activity in HUVEC by PP2 impairs PMN transmigration. HUVEC monolayers stimulated with 4 hours TNF- $\alpha$  were incubated with 10  $\mu$ M PP2, 0.5% DMSO carrier, or medium alone for 30 minutes. Endothelial junctions were stained by Alexa 568-conjugated VE-cadherin mAb (Hec-1) as previously described<sup>11</sup> to identify the location of PMN arrest. PMN were drawn across HUVEC at 0.5 mL/min (estimated shear stress, 1 dyne/cm<sup>2</sup>). The percentage of PMN transmigration (A) and PMN accumulation (at 12 minutes) (B) were determined by live cell imaging. C, The junctional and non-junctional locations of adherent PMN on the endothelial monolayers at various time points were measured by overlapping images of DIC and corresponding fluorescent images of VE-cadherin staining as detailed in Materials and Methods. Data shown represent the ratio of adherent PMN located at junctions to the total accumulated PMN. \* $P < 0.05$  vs 0.5% DMSO at the same time point. Data are mean  $\pm$  SEM;  $n = 3$  independent experiments.

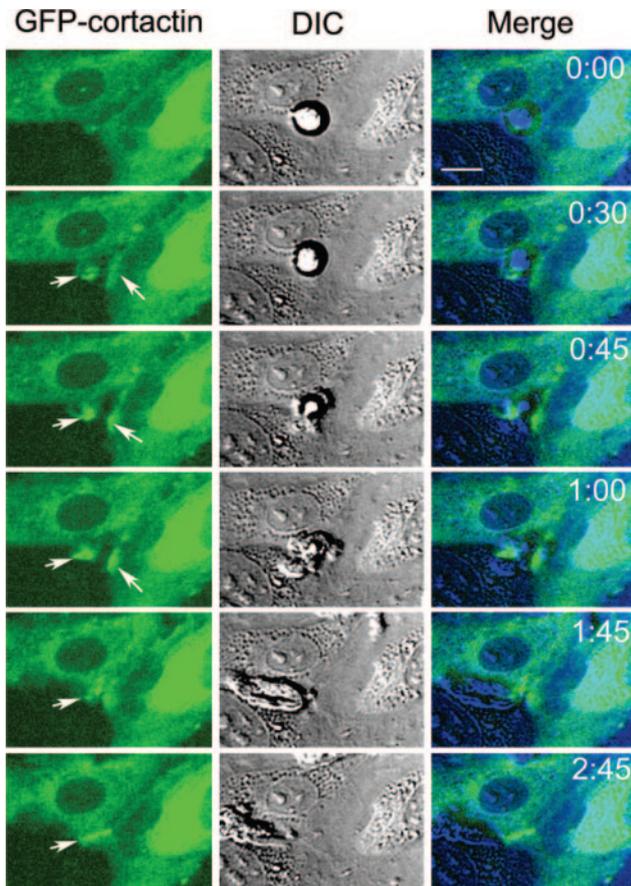
gration was affected. Robust PMN transmigration through HUVEC monolayers treated with media or 0.5% dimethyl sulfoxide (DMSO) (carrier) was observed, and the percentage of PMN that transmigrated reached a plateau by 9 minutes (Figure 1A). These data are consistent with our previous observations.<sup>11</sup> Pretreatment of HUVEC with PP2 reduced PMN transmigration by  $45 \pm 8\%$  (mean  $\pm$  SD,  $P < 0.05$ ) as compared with control carrier (Figure 1A). One possible explanation for the reduction in TEM is that PP2 treatment reduced PMN adhesion. Although a previous report observed that PP2 reduced adhesion of THP-1 cells under static

conditions,<sup>9</sup> no such effect was seen on PMN adhesion under the defined laminar shear stress in these studies (Figure 1B). The lack of effect on adhesion here could be explained by the differences in leukocyte types studied and the adhesion assays used. As a control experiment, treatment with PP2 neither reduced surface expression of adhesion molecules (ICAM-1, E-selectin, or VCAM-1) nor disrupted VE-cadherin staining at HUVEC cell-cell junctions (data not shown). It is also unlikely that PP2 carryover affected PMN functions, because HUVEC were washed extensively before introducing PMN into the assay. Additionally, a study of PP2 effect on TEM of PMN was performed using flow buffer containing 10% autologous plasma to mimic the in vivo environment. Essentially the same level of inhibition on TEM by PP2 was observed ( $52 \pm 9\%$ ).

We next used live cell imaging microscopy and the in vitro flow model to evaluate whether PP2 altered the migratory behavior of PMN adherent to HUVEC. We<sup>10,11</sup> and others<sup>17</sup> have shown that 70% to 80% of PMN initially attach close to endothelial junctions and subsequently transmigrate at cell junctions. To identify HUVEC cell-cell junctions, HUVEC were stained with a nonblocking fluorescent-tagged VE-cadherin mAb as described previously.<sup>11</sup> This mAb neither alter HUVEC permeability, nor the rate or extent of leukocyte TEM.<sup>18</sup> In HUVEC pretreated with carrier, most PMN initially attached at endothelial junctions and subsequently transmigrated through junctions and beneath the endothelial monolayer. This process led to a reduction in PMN adhesion at cell junctions over time (Figure 1C). In contrast, PP2 treatment of HUVEC reduced transmigration and significantly more PMN remained adherent at junctions, indicating an effect on the transmigration step, rather than on adhesion. Similar results for adhesion and TEM were obtained using a second Src family kinase inhibitor, SU6656<sup>19</sup> (supplemental Figure II). Taken together, these data argue that Src family kinase activity is required for PMN transmigration.

### Endothelial Cortactin Redistributes to the Site of PMN Transmigration

As indicated above, cortactin is a substrate of Src family kinases, and tyrosine phosphorylation of cortactin is detected on engagement of adhesion molecules involved in TEM of PMN.<sup>9</sup> We hypothesized, therefore, that cortactin participates in the regulation of PMN transmigration. To test this hypothesis, we first examined the distribution of cortactin in HUVEC during PMN transmigration using live cell imaging of GFP-tagged murine cortactin. Murine and human cortactin are highly homologous, and murine GFP-cortactin has been demonstrated to be functional in human cells.<sup>7</sup> HUVEC monolayers activated with TNF- $\alpha$  supported robust PMN adhesion and transmigration at junctions in the in vitro flow system, consistent with our previous observations.<sup>11</sup> Analysis of GFP-cortactin distribution in images obtained from 3 independent experiments showed that a small cluster of GFP-cortactin appeared at the contact region between the adherent PMN and endothelium. The GFP-cortactin became more localized to PMN actively migrating at endothelial cell-to-cell junctions and was often retained at endothelial cell borders until well after PMN had completed transmigration.



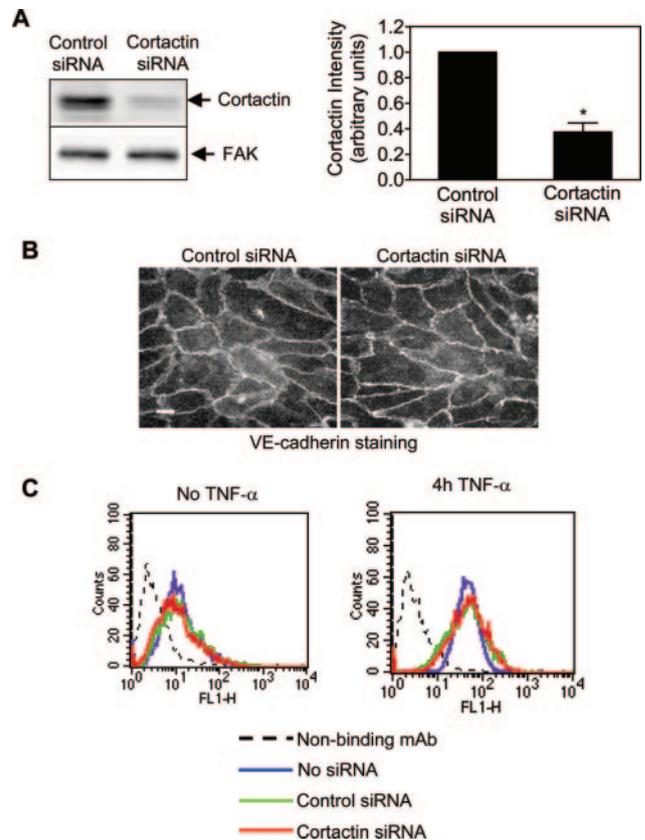
**Figure 2.** Endothelial cortactin redistributes to the site of PMN transmigration. Real-time and live cell microscopy of GFP-cortactin during PMN transmigration through 4-hour TNF- $\alpha$ -activated HUVEC monolayers expressing murine GFP-cortactin under flow conditions, as described in Materials and Methods. GFP-cortactin distribution (first column, green), corresponding DIC images (second column, gray) and overlay of fluorescence, and DIC (third column; DIC channel has been pseudocolored in blue and overlaid to ensure clear separation from green) are shown from PMN adhesion (time=0) to completion of transmigration (2:45 minutes). Arrows indicate clusters of GFP-cortactin. Time is shown in minute:second. Bar, 10  $\mu$ m. The figure represents a typical sequence of events during PMN transmigration from 3 independent experiments using HUVEC and PMNs from multiple donors.

tion (Figure 2 and supplemental Video 1). Supplemental Video 2 shows the distribution of GFP during PMN adhesion and transmigration, as control. These data show that cortactin is actively recruited to adherent PMN and accumulates at HUVEC cell junctions during PMN transmigration.

### Characterization of HUVEC Transfected With Cortactin siRNA

To confirm the role of cortactin in the process of PMN transmigration, we used cortactin siRNA to interfere with cortactin expression in HUVEC. Transfection with cortactin siRNA caused a 60% reduction in cortactin protein levels in rest HUVEC (Figure 3A) or HUVEC activated with TNF- $\alpha$  for 4 hours (Figure 5A).

We next determined whether cortactin siRNA treatment affected HUVEC junctions or expression of adhesion molecules induced by TNF- $\alpha$ . Live cell epifluorescence micros-

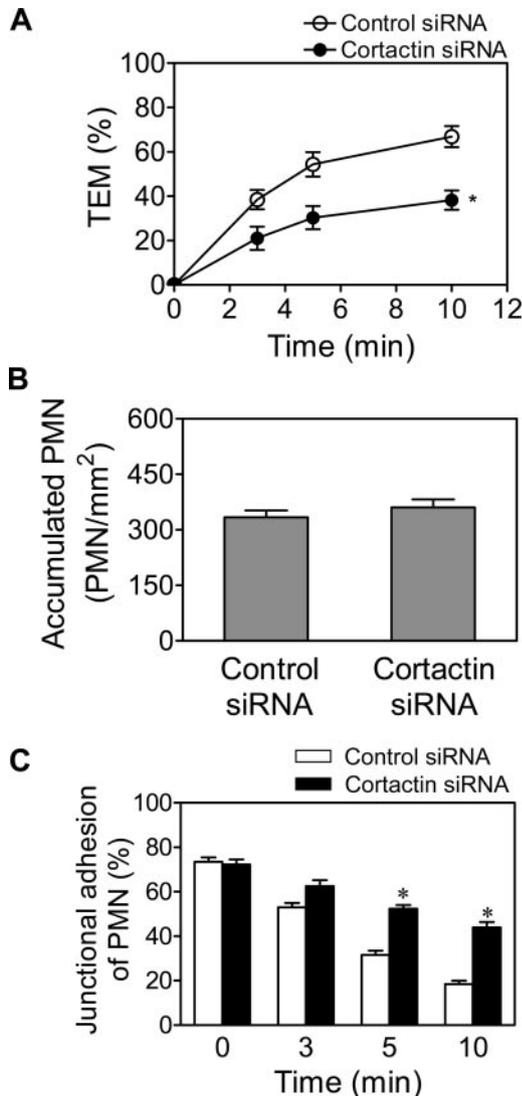


**Figure 3.** Characterization of HUVEC transfected with cortactin siRNA. A, left, Western blot of cortactin in HUVEC transfected with control or cortactin siRNA. FAK was used to control for sample loading. Right, Cortactin protein levels on Western blots were normalized to FAK protein. Data represent mean  $\pm$  SD; n=3 independent experiments. \* $P$ <0.05 vs control siRNA treatment. B, Fluorescent micrographs of VE-cadherin staining of live, confluent HUVEC transfected with control or cortactin siRNA. Bar, 20  $\mu$ m. C, Surface expression of ICAM-1 on HUVEC without or with 4 hours of TNF- $\alpha$  stimulation as assessed by indirect immunofluorescence staining and FACS. HUVEC were treated with media, control siRNA, or cortactin siRNA.

copy using fluorescent-tagged VE-cadherin mAb showed no obvious differences in the density or distribution pattern of VE-cadherin between control (scrambled) and cortactin siRNA-treated HUVEC (Figure 3B). FACS analysis demonstrated that transfection of cortactin siRNA did not alter TNF- $\alpha$  induced surface expression of ICAM-1 (Figure 3C) or E-selectin (data not shown).

### Cortactin Is Required for PMN Transmigration Through Endothelial Monolayers Activated With TNF- $\alpha$

HUVEC monolayers transfected with control siRNA supported robust PMN transmigration (Figure 4A), similar to that seen in HUVEC in the absence of siRNA (Figure 1A). In contrast, cortactin siRNA pretreatment significantly reduced PMN transmigration (Figure 4A). Impaired TEM was also seen in HUVEC transfected with a second cortactin-specific siRNA (see Materials and Methods; 47  $\pm$  9% inhibition,  $P$ <0.05). Neither control nor cortactin siRNA altered PMN accumulation (Figure 4B). Live cell microscopy revealed that approximately 70% of PMN initially attached (time=0 min-



**Figure 4.** Transfection of HUVEC with cortactin siRNA impairs PMN transmigration. HUVEC monolayers transfected with control or cortactin siRNA were stimulated with TNF- $\alpha$  (25 ng/mL, 4 hours). Endothelial junctions were stained by Alexa 568-conjugated VE-cadherin mAb. The percentage of PMN transmigration over time (A) and PMN accumulation (at 10 minutes) (B) were determined as in the legend Figure 1. C, Locations of adherent PMN at junctional or nonjunctional sites were determined as described in Figure 1C. Data are mean $\pm$ SEM; n=5 independent experiments. \* $P$ <0.05 vs control siRNA at the same time point.

utes) at cell-cell junctions of HUVEC transfected with either control or cortactin siRNA, and significantly more PMN remained adherent at the junctions of cortactin siRNA-treated HUVEC over time as compared with control siRNA (Figure 4C). In addition, we performed experiments with flow buffer containing 10% plasma. A similar level of inhibition of TEM by cortactin siRNA was observed ( $48\pm 8\%$ ).

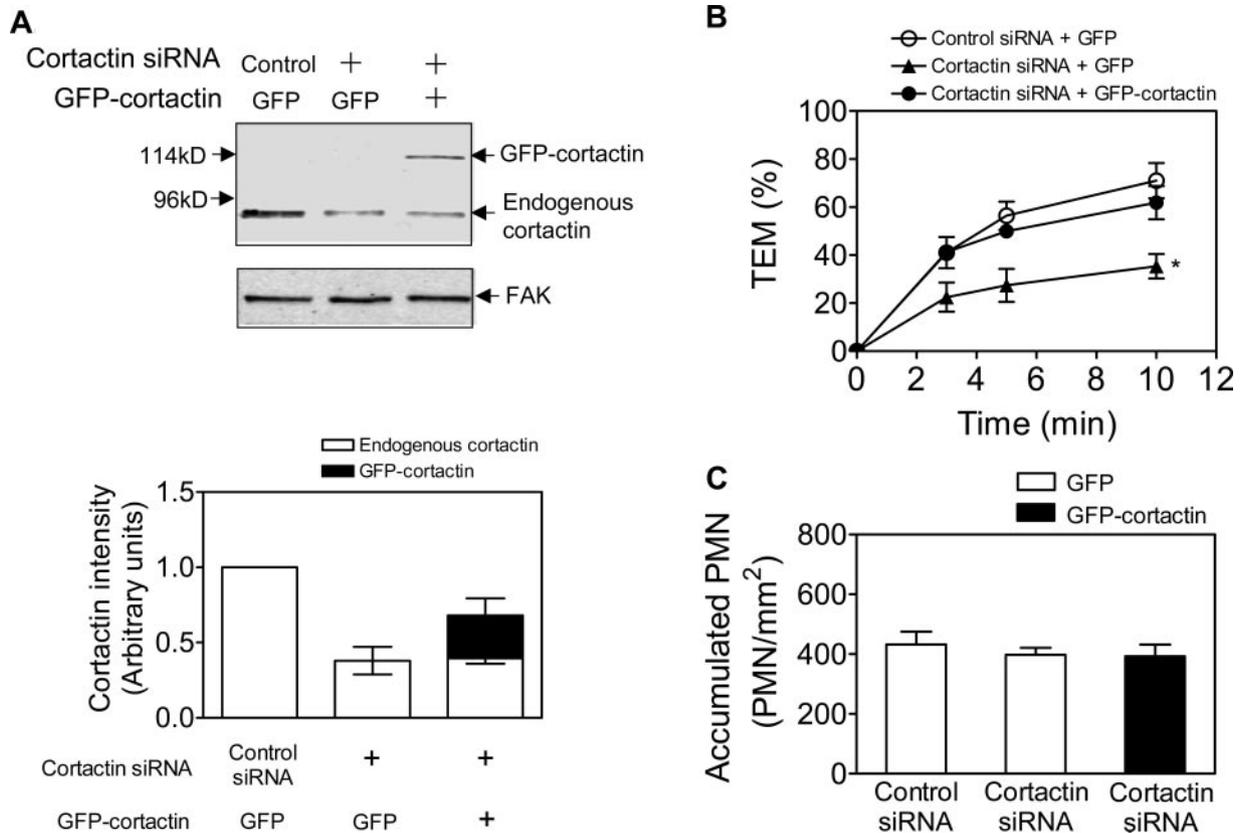
To further corroborate a specific role for cortactin in TEM, studies were designed to reconstitute cortactin levels in cortactin siRNA-transfected HUVEC using infection with retrovirus encoding GFP-tagged murine cortactin.<sup>15</sup> The design of cortactin siRNA was based on the sequence of the human gene, whose sequence has mismatches with murine cortactin. Thus, the cortactin siRNA does not affect murine

GFP-cortactin expression. As shown in Figure 5A, Western blot analysis showed that cortactin siRNA significantly reduced cortactin protein levels by  $\approx 60\%$  ( $n=4$ ,  $P<0.05$ ). Infection with the virus encoding GFP-tagged murine cortactin was able to increase cortactin levels in HUVEC monolayers treated with cortactin siRNA (Figure 5A). For TEM studies, we selected microscope fields with a relatively high percentage of GFP-positive cells ( $\approx 60\%$ ). Whereas expression of GFP did not rescue defects in TEM in cortactin siRNA-treated HUVEC, expression of GFP-cortactin did rescue the TEM defect (Figure 5B). Interestingly, rescue of TEM did not occur in fields with low numbers ( $<20\%$ ) of GFP positive cells (data not shown). Accumulation of PMN was not affected by any of these pretreatments (Figure 5C). Taken together, these results demonstrate the specificity of the cortactin siRNA approach combined with retroviral GFP cortactin and a requirement for cortactin in efficient PMN transmigration.

### Src-Induced Phosphorylation of Cortactin Is Important for PMN Transmigration

Previous studies have shown that tyrosine residues 421, 466, and 482 in cortactin are important targets of Src kinase and that overexpression of a cortactin mutant deficient in these tyrosine residues in human endothelium led to impaired endothelial cell migration.<sup>7</sup> Given that Src family kinases are required for TEM and that cortactin is regulated by Src family kinases, we examined whether cortactin tyrosine phosphorylation was important for TEM.

Therefore, a GFP-tagged murine cortactin mutant (cortactin3F-GFP<sup>7</sup>) in which tyrosine residues 421, 466, and 482 were substituted by phenylalanine was expressed in HUVEC transfected with cortactin siRNA (Figure 6A). As described in Materials and Methods, we selected microscope fields that had maximal but similar levels of GFP expression (Figure 6B). Consistent with the data in Figure 5, reconstitution with wild-type GFP-cortactin restored TEM to levels seen in control siRNA HUVEC (Figure 6C). Interestingly, expression of cortactin3F-GFP at the similar levels to that of wide-type GFP-cortactin did not rescue PMN transmigration, and, in fact, transmigration was consistently less than in HUVEC transfected with cortactin siRNA alone (Figure 6C). Infection of the mutant cortactin3F-GFP did not change the accumulation of PMN on endothelial monolayers (data not shown). Based on this result, we suspected that the cortactin3F mutant would act as a dominant negative, blocking the function of endogenous cortactin. To test this hypothesis, HUVEC monolayers were infected with retrovirus encoding GFP-tagged wild-type cortactin or cortactin3F, or GFP as control. We selected microscope fields with relatively high percentages of GFP-positive cells that had similar levels of GFP fluorescence between wild-type and mutant cortactin. Expression of wild type GFP-cortactin in HUVEC did not alter TEM; however, similar levels of cortactin3F-GFP expression impaired PMN transmigration ( $29\pm 6\%$  inhibition; Figure 6D) but had no effect on adhesion (data not shown). Taken together, these data suggest that 1 mechanism by which Src family kinases regulate TEM is through modulation of cortactin tyrosine phosphorylation.



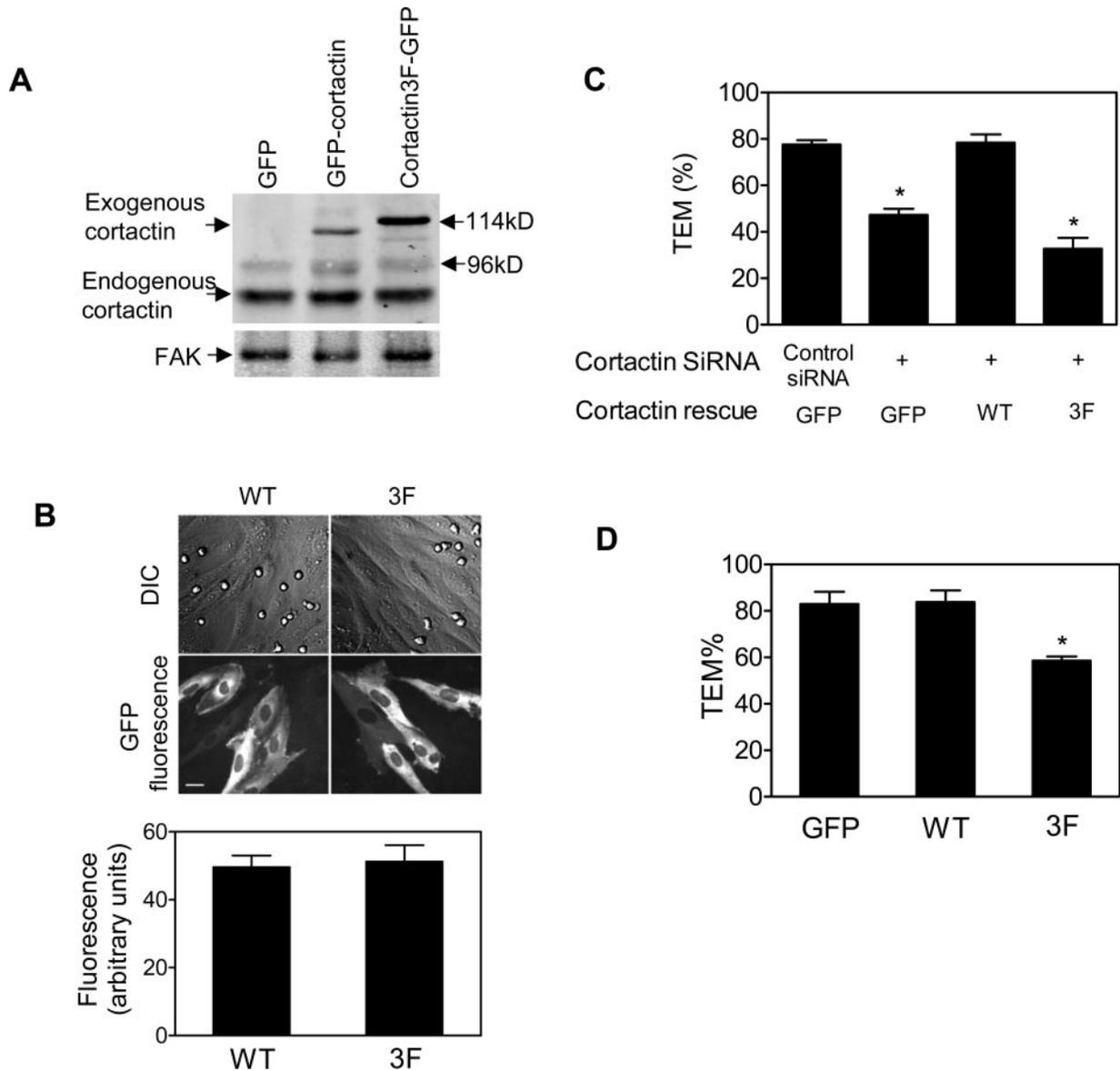
**Figure 5.** Reconstitution of cortactin siRNA-transfected HUVEC with murine GFP-cortactin rescues PMN transmigration. A, Expression of endogenous cortactin and GFP-cortactin in HUVEC treated with siRNA and retrovirus (as indicated) was determined by Western blot as described in Figure 3. Representative blot (top) and quantitation of cortactin levels (normalized to FAK) from 4 experiments (bottom) are shown. Percentage of PMN transmigration through HUVEC monolayers (B) and accumulation of PMN (at 10 minutes) on HUVEC treated with siRNA and retrovirus (as indicated) (C) were determined using live cell imaging. Values are mean  $\pm$  SEM of 3 independent experiments. \* $P < 0.05$  vs control siRNA.

## Discussion

In this study, we have identified a role for Src family kinases and the cytoskeletal scaffold, cortactin, in regulation of TEM. Our data argue that Src-dependent phosphorylation of cortactin is 1 mechanism by which Src family kinases regulate leukocyte TEM. Using HUVEC activated with TNF- $\alpha$ , which have been previously reported to support robust PMN transmigration at cell–cell junctions under flow,<sup>11</sup> we show here that endothelial cortactin dynamically redistributes to cell–cell junctions where PMN transmigrate. Pharmacological inhibition of Src family kinases and siRNA knockdown of endothelial cell cortactin reduced PMN transmigration at cell–cell borders (paracellular TEM) without affecting PMN initial attachment or localization to cell–cell junctions. We further show that Src-induced cortactin phosphorylation at tyrosine 421, 466, and 482 is critical for cortactin-mediated PMN transmigration.

The live cell imaging of PMN transmigration revealed that GFP-cortactin formed small, punctate clusters around PMN before and during TEM and subsequently redistributed to the leading edge of the endothelial membranes that resealed the junctions once the PMN completed TEM (Figure 2 and supplemental Videos 1 and 2). Recently, cortactin and Arp2/3 were shown to mediate endothelial cell cytoskeletal remodeling triggered by Sphingosine-1-phosphate through promot-

ing actin assembly at the cell leading edge.<sup>20</sup> Hence, the behavior of GFP-cortactin during TEM suggests that, in addition to a role in localizing and stabilizing the association of actin with adhesion molecules, cortactin, in association with the Arp2/3 complex and cofactors, such as neural Wiskott–Aldrich syndrome protein, promotes new actin polymerization and assembly into cortical actin at cell–cell junctions. The newly formed actin filaments may provide a source of rapid F-actin assembly at the cell junctions to reseat the gap in VE-cadherin made by transmigrating PMN,<sup>18,21</sup> as well as give rise to actin rings, termed transmigration tunnels, that surround leukocytes as they transmigrated in another TEM assay system.<sup>22</sup> The current finding that cortactin clusters around adherent leukocytes is consistent with the work of several groups showing that actin–cytoskeletal linker proteins including cortactin, moesin, vinculin,  $\alpha$ -actinin, and vasodilator-stimulated phosphoprotein associate with endothelial cell adhesion molecules and cocluster with adherent leukocytes.<sup>9,23–26</sup> These associations regulate and stabilize the location of adhesion molecules, and one could envision that such regulation would be critical for the tracking of adherent leukocytes as they position themselves to transmigrate. Future studies are necessary to evaluate the dynamics of cortactin and actin in combination with junctional molecules during transmigration and to delineate the mechanisms con-



**Figure 6.** Tyrosine phosphorylation of cortactin is important for PMN transmigration. **A**, Expression of endogenous cortactin and murine GFP-tagged wild-type or cortactin3F in the confluent HUVEC monolayer was determined by Western blot. **B**, Paired DIC and GFP fluorescence images of representative microscope fields of confluent HUVEC monolayers captured after 1 minute of PMN perfusion. Micrographs of GFP fluorescence demonstrate that wild-type GFP-cortactin (WT) and cortactin3F-GFP mutant (3F) have a similar percentage of GFP-positive cells. The DIC images show attachment of PMN at the beginning of the flow. The average fluorescence intensity of GFP-cortactin (WT) or cortactin3F-GFP mutant (3F) in the microscope fields used for TEM studies were quantitated using MetaMorph software. Data represent mean  $\pm$ SD of 3 experiments (total 30 fields for each cell type). Bar, 20  $\mu$ m. **C**, HUVEC were transfected with control or cortactin siRNA and then infected with murine GFP-tagged wild-type (WT) or cortactin3F-GFP mutant (3F) retroviruses, as indicated. PMN transmigration was determined at 1 dyne/cm<sup>2</sup> using time-lapse live cell imaging. \**P*<0.05 vs control siRNA. **D**, PMN transmigration was determined in HUVEC expressing wild-type GFP-cortactin (WT) or cortactin3F-GFP mutant (3F) under flow. Data represent mean  $\pm$ SEM of 3 independent experiments. \**P*<0.05 vs GFP control.

trolling the initiation and release of adhesion molecules from actin binding proteins.

The PMN transmigration in this system is dependent on ICAM-1.<sup>11</sup> The association of cortactin with ICAM-1 and subsequent tyrosine phosphorylation of cortactin by Src tyrosine kinase in HUVEC is triggered by leukocyte adhesion or crosslinking of ICAM-1.<sup>9</sup> How cortactin associates with ICAM-1 is unclear. The short cytoplasmic tail of ICAM-1 has no obvious binding sites for cortactin. Thus the interaction between cortactin and ICAM-1 could be indirectly through other molecules like Src<sup>27</sup> or SHP-2 (Src homology 2-con-

taining protein-tyrosine phosphatase-2).<sup>28</sup> We speculate that such associations bring Src and cortactin into close proximity, at which point Src phosphorylates cortactin at tyrosine residues. Future studies elucidating the precise nature of these interactions may shed light on the signal relay between these molecules during TEM.

Regardless of the nature of this association, our studies clearly implicate a role for cortactin in regulation of TEM via its tyrosine phosphorylation sites. Although cortactin tyrosine phosphorylation has been shown to have both positive and negative effects on cortactin function in various systems,<sup>29</sup>

transmigration was clearly impaired by preventing tyrosine phosphorylation of cortactin (Figure 1A) or by overexpression of cortactin3F in endothelial cells (Figure 6C). At least 2 potential models can be envisioned to explain how tyrosine phosphorylation contributes to cortactin function in this system. First, these sites may be important for recruiting phosphotyrosine binding domain proteins. For example, these sites have been shown to bind Src and the adaptor proteins Crk.<sup>27,30</sup> In the case of Src, it is possible that such an interaction would potentiate activation of Src family kinases, thus providing a positive feedback loop. Alternatively, phosphorylation of these residues may regulate other domains. For example, Src-induced tyrosine phosphorylation of cortactin decreases its F-actin cross-linking ability. Tyrosine phosphorylation of cortactin can also modulate its interaction with MLCK,<sup>31,32</sup> which binds to the cortactin SH3 domain. Interestingly, MLCK activity in HUVEC is required for TEM.<sup>4</sup> Structure/function studies examining the role of other domains of cortactin in TEM may help to define how tyrosine phosphorylation modulates TEM and further elucidate the role of cortactin in TEM.

Cortactin can directly engage in actin assembly through interaction with Arp2/3 complex and F-actin by its N-terminal acidic domain (NAD) and tandem repeats, respectively.<sup>6</sup> Given the modular nature of cortactin, however, it is likely that other domains may also be required. For example, recent studies have demonstrated that the C-terminal domain of cortactin (including proline-rich region) and the N-terminal domain can independently enhance cell motility.<sup>15</sup> Thus, cortactin is likely to use multiple mechanisms to regulate transmigration, and structure/function studies should help elucidate the function of these other regions in TEM.

Our data provide strong evidence that Src and cortactin contribute to PMN transmigration. However, the results do not exclude involvement of other signaling pathways or proteins in regulating PMN transmigration. Recently, studies from other groups have shown that other cytoskeleton linker proteins, such as ERM (ezrin and moesin) family members, vinculin and  $\alpha$ -actinin, colocalize with adhesion molecules ICAM-1 and VCAM-1, and form endothelial “docking structures” around adherent or transmigrating leukocytes.<sup>24,26</sup> These structures may be important for strengthening the interaction of adhesion molecules with their leukocyte ligands and facilitating leukocyte TEM. Phosphoinositides and the Rho/p160 ROCK pathway have been implicated in stabilizing endothelial cell adhesion molecules during leukocyte adhesion or TEM.<sup>24</sup> Future studies are required to understand how Src and cortactin integrate into the various signaling networks that regulate leukocyte TEM.

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**Supplemental Data:****Supplemental Fig.1. PP2 treatment abrogates cortactin tyrosine phosphorylation**

**triggered by ICAM-1 crosslinking.** HUVEC were stimulated with TNF- $\alpha$  for 4h and treated with 10 $\mu$ mol/L PP2 or 0.5% DMSO (carrier) for 30 min. ICAM-1 was crosslinked by incubation of HUVEC with 10  $\mu$ g/ml ICAM-1 mAb (HU5/3) for 30 min followed by incubation with goat anti-mouse IgG (10 $\mu$ g/ml, 10min). Cortactin in cell lysates was immunoprecipitated using cortactin mAb. **A.** The level of cortactin and tyrosine phosphorylated cortactin were determined by western blot. **B.** The level of tyrosine-phosphorylated cortactin was normalized to total cortactin levels.

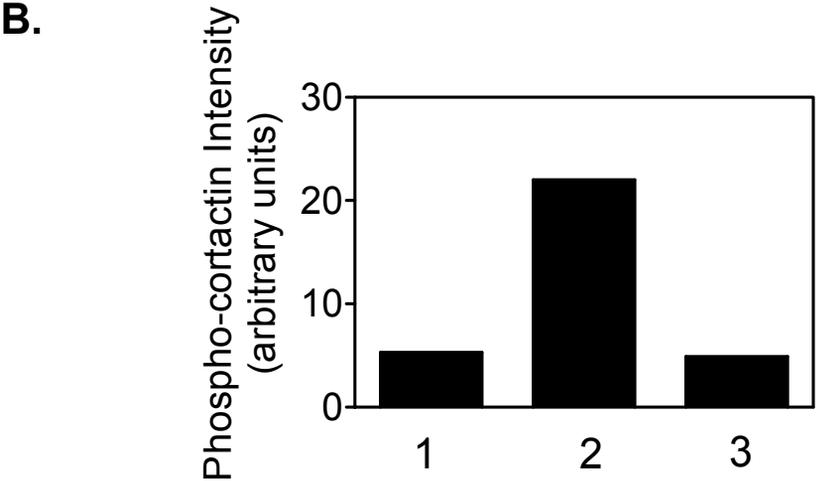
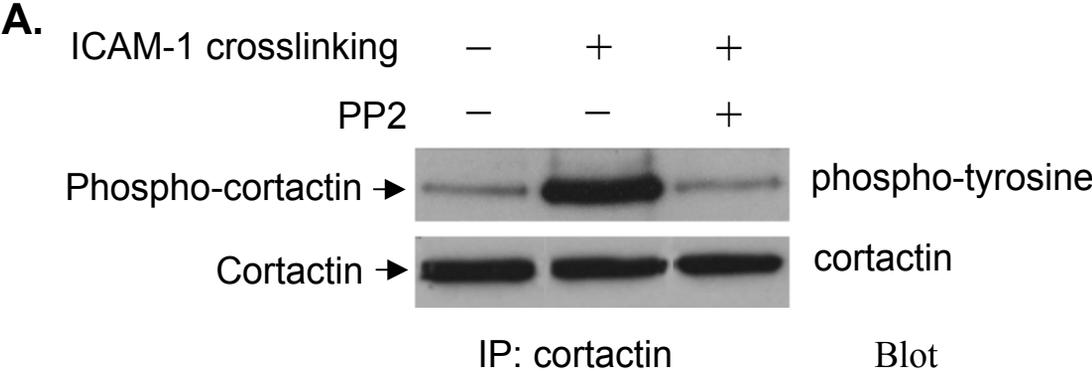
**Supplemental Fig.2. Inhibition of Src tyrosine kinases in HUVEC by SU6656**

**impairs PMN transmigration.** TNF- $\alpha$ -activated endothelial monolayers were incubated with 10 $\mu$ mol/L SU6656 or 0.5% DMSO (carrier), as indicated, for 30 min. Endothelial junctions were stained by non-blocking Alexa 568-conjugated VE-cadherin mAb. **A.** Percentage of PMN transmigration at various time points and **B.** PMN accumulation at 10 min was determined under flow using live cell imaging. **C.** The sites of adherent PMN at junctional and non-junctional locations at various time points were measured by overlapping images of DIC and corresponding fluorescent images of VE-cadherin staining. Data are mean $\pm$ SD of 3 independent experiments. \*  $p < 0.05$ , vs 0.5%DMSO at the same time point.

**Supplemental Video 1 and 2: Endothelial cortactin redistribution during PMN transmigration.** *Supplement to Figure 2.* HUVEC monolayers infected with retrovirus

encoding murine GFP-cortactin (Video 1) or GFP (Video 2, control) were stimulated with TNF- $\alpha$  for 4 hours. PMNs were drawn across the monolayer at 1 dyne/cm<sup>2</sup>. Adhesion and TEM were monitored as described in Materials and methods. GFP-cortactin (green) forms clusters around a transmigrating PMN (Video 1), while no cluster was observed for GFP (Video 2). Bar, 10  $\mu$ m. Time = min:sec (t = 0: initial adhesion of PMN).

# Supplemental Fig.1



## Supplemental Fig.2

