Eosinophil adhesion under flow conditions activates mechanosensitive signaling pathways in human endothelial cells

Susan L. Cuvelier,[1] Smitha Paul,[1] Neda Shariat,[1] Pina Colarusso,[1] and Kamala D. Patel[1,2]

[1]Department of Physiology and Biophysics and [2]Department of Biochemistry and Molecular Biology, Immunology Research Group, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Leukocyte transmigration can be affected by shear stress; however, the mechanisms by which shear stress modulates transmigration are unknown. We found that adhesion of eosinophils or an eosinophilic cell line to intereukin 4–stimulated endothelial cells led to a shear-dependent increase in endothelial cell intracellular calcium and increased phosphorylation of extracellular signal-regulated kinase (ERK) 2, but not c-Jun NH_2-terminal kinase or p38 mitogen-activated protein kinase. Latex beads coated with antibodies were used to characterize the role of specific endothelial cell surface molecules in initiating signaling under shear conditions. We found that ligation of either vascular cell adhesion molecule–1 or E-selectin, but not major histocompatibility complex class I, induced a shear-dependent increase in ERK2 phosphorylation in cytokine-stimulated endothelial cells. Disassembly of the actin cytoskeleton with latrunculin A prevented ERK2 phosphorylation after adhesion under flow conditions, supporting a role for the cytoskeleton in mechanosensing. Rapid phosphorylation of focal adhesion kinase and paxillin occurred under identical conditions, suggesting that focal adhesions were also involved in mechanotransduction. Finally, we found that Rho-associated protein kinase and calpain were both critical in the subsequent transendothelial migration of eosinophils under flow conditions. These data suggest that ligation of leukocyte adhesion molecules under flow conditions leads to mechanotransduction in endothelial cells, which can regulate subsequent leukocyte trafficking.

CORRESPONDENCE
Kamala D. Patel:
kpatel@ucalgary.ca

Abbreviations used: ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HSA, human serum albumin; HUVEC, human umbilical vein endothelial cell; JNK, c-jun NH_2-terminal kinase; Lat A, latrunculin A; MAPK, mitogen-activated protein kinase; ROCK, Rho-associated protein kinase; VCAM-1, vascular cell adhesion molecule–1.

The binding of leukocytes to the vessel wall and subsequent migration into the tissue occurs under flow conditions and is required for normal host defense (1). This stepwise process is initiated by the tethering and rolling of leukocytes on activated endothelium, which is followed by leukocyte activation, firm adhesion, and transendothelial migration. Leukocyte recruitment studies frequently use parallel plate flow chambers to mimic the flow conditions that are found in vivo; however, because cells are firmly adherent before transmigration, most in vitro studies examining transmigration have been performed under static conditions. It is becoming increasingly evident that shear stress is an important regulator of leukocyte transmigration. For example, lymphocyte transmigration is dependent on shear stress (2), whereas the rate of neutrophil transmigration is increased by shear stress (3). We previously demonstrated that a few eosinophils could transmigrate across IL-4–stimulated endothelial cells under static conditions; however, shear stress must be present for maximum transmigration to occur (4). Although these studies have established that shear stress modulates leukocyte transmigration, the precise mechanisms by which this occurs are unknown. This study addresses the molecular mechanisms by which shear stress regulates eosinophil transmigration.

We hypothesized that endothelial cell signaling events contribute to the shear dependence of leukocyte transmigration. Signaling within endothelial cells has previously been shown to regulate leukocyte transmigration. Several groups have demonstrated a role for increased endothelial intracellular calcium in neutrophil (5–7) and monocyte (8) transmigration. Several protein kinases have also been implicated in transmigration, including extracellular signal-regulated kinase

S.L. Cuvelier and S. Paul contributed equally to this work.

(ERK) 1/2 in eosinophil transmigration (4), myosin light chain kinase in neutrophil transmigration (6), and Rho-associated protein kinases (ROCKs) in both lymphocyte and monocyte transmigration (9, 10). Exposure of endothelial cells to high shear stress is sufficient to initiate signaling events within endothelial cells (11–18); however, several groups have shown that preexposing endothelial cells to the low shear stresses typical of postcapillary venules does not enhance subsequent leukocyte transmigration (2–4). These results suggest that shear stress alone is not responsible for initiating the signaling events that are associated with leukocyte transmigration. Instead, shear stress present during the tethering, rolling, and firm adherent phases of leukocyte recruitment affects the subsequent transmigration (2–4).

In this study, we examined the effect of adhesion under flow conditions on endothelial cell signaling. We found that binding of an eosinophilic cell line to endothelial cells induced shear-dependent increases in intracellular calcium and ERK2 phosphorylation, which are two pathways critical for transmigration. ERK2 activation was preceded by phosphorylation of focal adhesion kinase (FAK) and paxillin. Furthermore, disassembly of the actin cytoskeleton prevented ERK2 phosphorylation, suggesting that mechanosensing involved the cytoskeleton and focal adhesions. Calpain is activated downstream of both calcium and ERK (19, 20), and our data showed that blocking calpain dramatically reduced eosinophil transmigration. Together, these data suggest that vascular adhesion molecules can act as mechanosensors, converting the mechanical force of leukocyte adhesion into biochemical signals within the endothelium that can regulate subsequent steps in the recruitment cascade.

RESULTS

Endothelial intracellular calcium and tyrosine kinases are involved in eosinophil transendothelial migration

Eosinophil transmigration is shear dependent, with robust transmigration occurring at shear stresses ranging from 0.5 to 2 dyn/cm^2, but little transmigration occurring under static conditions (Fig. 1 A) (4). To further elucidate the role of endothelial cell signaling in eosinophil transmigration, we performed experiments in which we used either fixation or treatment with azide and deoxyglucose to inhibit the metabolic activity of IL-4–stimulated human umbilical vein endothelial cells (HUVECs). After treatment, the flow chamber was assembled, and freshly isolated human eosinophils were perfused through the chamber at a wall shear stress of 2 dyn/cm^2. As expected, fixation completely blocked transmigration (Fig. 1 B). Blocking metabolic activity in the endothelial cell monolayer reduced eosinophil transmigration by ~50% (Fig. 1 B) without having an effect on eosinophil accumulation (not depicted). These data

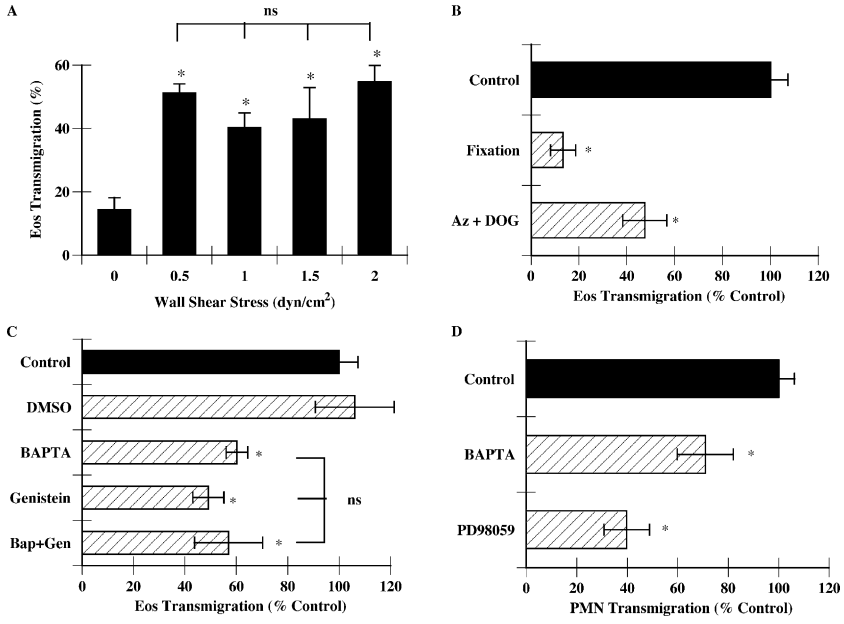

Figure 1. Eosinophil and neutrophil transmigration under shear stress require endothelial cell signaling. HUVECs were stimulated with IL-4 (A–C) for 24 h or TNF-α (D) for 4 h as described in Materials and methods. (A) 5 × 10^5 eosinophils/ml were perfused over IL-4–stimulated HUVECs at the specified wall shear stresses, and transmigration was determined as described in Materials and methods. Transmigration is expressed as a percentage of the total cells interacting with the monolayer. (B) IL-4–stimulated HUVECs were pretreated with a DMSO control, 1% formalin (Fixation), or 0.06% sodium azide and 50 mM deoxyglucose in glucose-free HBSS (Az + DOG) for 30 min. (C) IL-4–stimulated HUVECs were pretreated with a DMSO control or 50 μM BAPTA-AM for 15 min and/or 100 μM genistein for 30 min. (D) TNF-α–stimulated HUVECs were pretreated with a DMSO control or 50 μM BAPTA-AM for 15 min or 20 μM of the MEK (MAPK kinase) inhibitor PD98059 for 15 min. The flow chamber was assembled and 5 × 10^5 eosinophils/ml (B and C) or 10^6 neutrophils/ml (D) were perfused over the monolayers for 4 min at 2 dyn/cm^2. Transmigration was measured as described in Materials and methods and is expressed as the percentage of control. Data represent the mean ± SEM of at least three experiments. *, $P < 0.05$.

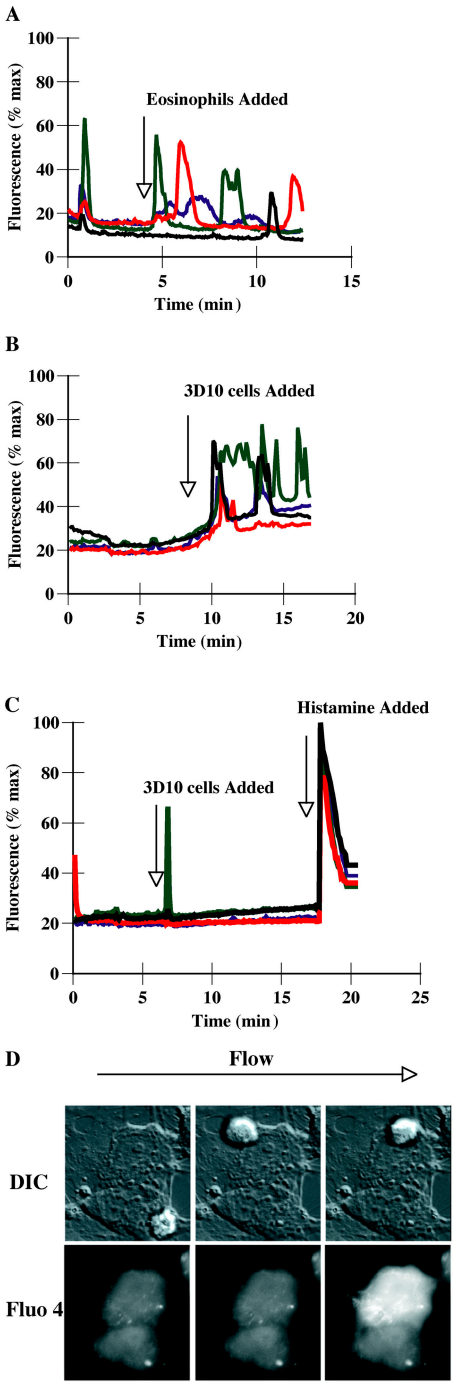

Figure 2. Eosinophil or 3D10 binding under flow conditions induces calcium signaling in IL-4–stimulated HUVECs. HUVECs were stimulated with IL-4 for 24 h and loaded with the calcium-sensitive dye Fluo-4 AM as described in Materials and methods. (A, B, and D) The flow chamber was assembled, and buffer was perfused over the monolayer at 0.25 dyn/cm^2. After 4 min of buffer, 5×10^5 eosinophils/ml (A) or 5×10^5 3D10 cells/ml (B and D) were introduced into the chamber, and the flow was maintained at 0.25 dyn/cm^2. (C) Alternatively, 3D10 cells were added to Fluo-4–loaded IL-4–stimulated endothelial cells under static conditions, and 10^{-5} M histamine was added as a positive control at the end of the experiment. Data show the Fluo-4 emission intensities of cells at 520 nm and are expressed as a percentage of the maximal signal. Traces from four representative cells are shown. (D) The Fluo-4 emission intensities at 520 nm (bottom row) and differential interference contrast (DIC) images (top row) of two representative endothelial cells are shown. A 3D10 cell becomes bound to the top endothelial cell in panel 2 (middle), inducing an increase in endothelial cell intracellular calcium in panel 3 (right). The bottom endothelial cell is not bound by a 3D10 cell and does not undergo an increase in intracellular calcium. The arrow indicates the direction of flow. 4 s elapsed between panels 1 (left) and 2 and between panels 2 and 3. Data are representative of at least three independent experiments.

suggest that ATP production by endothelial cells is required for eosinophil transmigration, but not for the initial accumulation step, and are consistent with a role for endothelial cell signaling in transmigration.

Both neutrophil and monocyte transmigration are regulated by changes in intracellular calcium within endothelial cells (5–8). The role of calcium in eosinophil transmigration has not been determined. To assess the role of intracellular calcium in eosinophil transmigration, we pretreated IL-4–stimulated HUVECs with the intracellular calcium chelator BAPTA-AM before measuring transmigration. BAPTA-AM attenuated transmigration (Fig. 1 C), indicating that eosinophil transmigration is partially dependent on changes in endothelial intracellular calcium. We next examined the role of tyrosine kinases in eosinophil transmigration by using genistein to block the activity of protein tyrosine kinases. Pretreatment of IL-4–stimulated HUVECs with genistein also reduced transmigration (Fig. 1 C), demonstrating that endothelial protein tyrosine kinases are partially required for eosinophil transmigration. Interestingly, when BAPTA and genistein were used in combination, transmigration was blocked to the same extent as when either inhibitor was used alone (Fig. 1 C). Control experiments demonstrated that the concentrations of BAPTA and genistein that were used in these experiments blocked calcium mobilization and tyrosine phosphorylation, respectively, in histamine-stimulated endothelial cells (unpublished data). These data suggest that intracellular calcium and protein tyrosine kinases act within a single pathway in the endothelium to facilitate eosinophil transmigration.

Neutrophil transmigration is accelerated under flow conditions. We used BAPTA and PD98059 to determine if calcium and the ERK1/2 pathway regulate neutrophil transmigration under flow conditions. Both of these inhibitors attenuated neutrophil transmigration under flow conditions (Fig. 1 D), showing that these pathways are critical for neutrophil transmigration under shear conditions. Furthermore, these data suggest that similar signaling pathways regulate the transmigration of at least two major leukocyte subclasses.

Binding of eosinophils or 3D10 cells to IL-4–stimulated HUVECs under flow conditions induces calcium fluxes within endothelial cells

Pharmacological agents directed against intracellular calcium (Fig. 1 C) and the ERK1/2 pathway (4) in endothelial cells block eosinophil transmigration, suggesting that these pathways are activated during eosinophil recruitment. To address this,

we directly examined activation of these signaling pathways in IL-4–stimulated HUVECs. We have previously shown that eosinophils interacting with IL-4–stimulated endothelial cells rapidly transition from rolling to firm adhesion (21); thus, we did not differentiate between rolling and firm adhesion in these experiments. Endothelial cells were stimulated with IL-4 and then loaded with the calcium-sensitive dye Fluo-4 AM. In some experiments, the initiation of shear stress resulted in a spike of increased calcium that rapidly returned to baseline; continued perfusion of buffer had no effect on endothelial cell calcium levels (Fig. 2 A). Intracellular calcium levels rose dramatically when eosinophils were introduced into the chamber under shear conditions (Fig. 2 A). In some endothelial cells, eosinophil binding induced oscillations in the intracellular calcium level, whereas in other endothelial cells a single peak in intracellular calcium levels was observed (Fig. 2 A).

Eosinophils tether and roll on IL-4–stimulated HUVECs through interactions with P-selectin and vascular cell adhesion molecule–1 (VCAM-1) (21). Eosinophils subsequently become activated and transmigrate as a result of interactions between eotaxin-3 expressed by the endothelial cells and CCR3 expressed on the eosinophils (4). We used an eosinophilic cell line that lacks CCR3 to differentiate between the effects of binding under shear stress and the effects of transmigration. Eosinophilic 3D10 cells roll and adhere to IL-4–stimulated endothelial cells under flow conditions through interactions with P-selectin and VCAM-1; however, they do not transmigrate because of a lack of CCR3 expression (unpublished data) (22). Rolling and adhesion of 3D10 cells increased endothelial cell intracellular calcium in a manner similar to the rolling and adhesion of eosinophils (Fig. 2, A and B). Increased intracellular calcium was observed in endothelial cells to which 3D10 cells directly adhered, as shown in Fig. 2 D. Because we also observed calcium waves and oscillating increases in intracellular calcium that traveled between endothelial cells (unpublished data), the experiment in Fig. 2 D was performed on a subconfluent monolayer to demonstrate an increase in intracellular calcium in an endothelial cell to which a 3D10 cell was directly bound. When these experiments were performed under static conditions, no change in intracellular calcium was observed (Fig. 2 C), suggesting that 3D10 cell–induced increases in intracellular calcium were shear dependent. Stimulation with histamine at the end of the experiment induced a calcium flux, demonstrating that these cells were capable of mounting a calcium response (Fig. 2 C). Finally, chelating intracellular calcium with BAPTA prevented the increase in intracellular calcium (not depicted). These data suggest that adhesion under flow conditions leads to increased intracellular calcium within endothelial cells.

Binding of 3D10 cells to IL-4–stimulated HUVECs under flow conditions induces shear-dependent ERK2 phosphorylation within endothelial cells

Inhibiting the endothelial ERK1/2 pathway attenuates eosinophil transmigration (4); therefore, we chose to examine ERK1/2 activation after 3D10 cell binding under flow conditions. 3D10 cells were perfused over IL-4–stimulated HUVECs at 1 dyn/cm^2 for either 1 or 5 min, after which the inlet line was switched to ice-cold EDTA to remove adherent 3D10 cells from the monolayer. Endothelial cells were subsequently lysed, and activation of ERK1/2 was measured by quantifying changes in phosphorylation using Western blotting. Adhesion of 3D10 cells to IL-4–stimulated HUVECs caused significant ($P < 0.05$) phosphorylation of ERK2 relative to endothelial cells in which buffer alone was perfused over the monolayer (Fig. 3, A and B), suggesting that leukocyte binding under flow conditions can rapidly activate the ERK1/2 pathway within endothelial cells. In some experiments, ERK1 was also phosphorylated after 3D10 cell adhesion; however, densitometry revealed that only the increase in ERK2 phosphorylation was significant ($P < 0.05$; not depicted). We obtained equivalent results when 3D10 cells were fixed before perfusion over endothelial cells, demonstrating that changes in protein phosphorylation resulted from

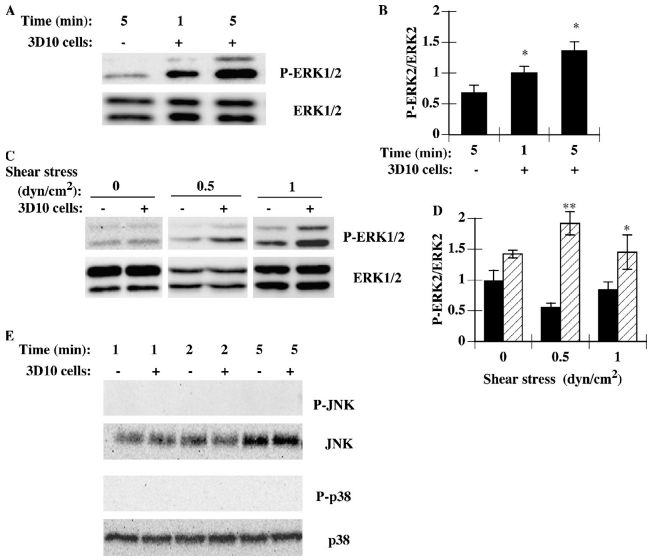

Figure 3. 3D10 cell binding to IL-4–stimulated HUVECs under flow conditions induces phosphorylation of ERK2 but not JNK or p38. HUVECs were stimulated with IL-4 for 24 h, a flow chamber was assembled, and buffer or 5×10^5 3D10 cells/ml were perfused over the monolayer. (A) 3D10 cells were perfused at 1 dyn/cm^2 for 1 or 5 min; (C) fixed 3D10 cells were perfused at 0.5 or 1 dyn/cm^2 for 5 min or the medium was removed, fixed 3D10 cells were added to the monolayer, and the monolayer was incubated at 37°C for 5 min (0 dyn/cm^2); or (E) fixed 3D10 cells were perfused at 0.5 dyn/cm^2 for 1, 2, or 5 min. Adherent 3D10 cells were removed with ice-cold 1× PBS with 5 mM EDTA, after which the HUVECs were lysed, and the lysates were separated by SDS-PAGE. (A, C, and E) Proteins were transferred to polyvinylidene difluoride and Western blotted for phospho-ERK1/2, phospho-JNK, or phospho-p38. Membranes were subsequently stripped and reprobed with an antibody against total ERK1/2, total JNK, or total p38. (B and D) Bands were analyzed by densitometry, and data are expressed as the ratio of phospho-ERK2/total ERK2. In D, the black bars represent buffer alone, and the hatched bars represent 3D10 cells. The blots in A, C, and E are representative of at least three experiments. Data in B and D are mean ± SEM of between four and seven experiments. *, $P < 0.05$; **, $P < 0.01$.

endothelial, not 3D10, cell signaling events (Fig. 3, C and D). Adhesion-induced endothelial cell activation of ERK2 occurred when 3D10 cells were perfused at either 0.5 or 1 dyn/cm^2 (Fig. 3, C and D). When these experiments were performed under static conditions, no change in ERK2 phosphorylation was observed (Fig. 3, C and D), suggesting that ERK2 activation by 3D10 cells was shear dependent. We next examined activation of the other mitogen-activated protein kinases (MAPKs). Binding of 3D10 cells under flow conditions did not increase phosphorylation of either c-jun NH_2-terminal kinase (JNK) or p38 MAPK in IL-4–stimulated HUVECs (Fig. 3 E), suggesting that adhesion of 3D10 cells under flow conditions selectively activates the ERK pathway. Positive controls using anisomysin- or TNF-α–stimulated HUVECs showed that we were able to detect phosphorylated JNK and p38 (not depicted).

VCAM-1 ligation leads to shear-dependent ERK2 phosphorylation

To determine whether ligation of VCAM-1 alone can induce signaling in IL-4–stimulated HUVECs under shear conditions, we used 7.2-μm latex microspheres coated with an anti–VCAM-1 mAb to ligate endothelial VCAM-1. The use of antibody-coated microspheres enabled us to ligate VCAM-1 using a particle that is similar in size to a leukocyte, thereby simulating the forces that are exerted on VCAM-1 during leukocyte adhesion under flow conditions. Anti–VCAM-1–coated microspheres were allowed to settle onto and bind to the surface of IL-4–stimulated HUVECs for 5 min, after which buffer was perfused over the surface at 0.2, 1, or 5 dyn/cm^2 for 5 min. Because the initiation of flow alone occasionally led to a spike in intracellular calcium (Fig. 2 A), we were unable to evaluate changes in intracellular calcium using this experimental design. Instead, we focused on the activation of the ERK pathway. Western blotting showed increased ERK2 phosphorylation after VCAM-1 ligation at an applied shear stress of 0.2, 1, or 5 dyn/cm^2 (Fig. 4 A and not depicted). Consistent with published data (23), microspheres that were coated with goat anti–mouse IgG alone had no effect on ERK2 phosphorylation at the lowest shear stress examined but induced increasing activation of ERK2 at the higher shear stresses (unpublished data). As a result, anti–VCAM-1–coated microspheres caused a statistically significant ($P < 0.05$) increase in ERK2 phosphorylation relative to goat anti–mouse–coated microspheres at 0.2 and 1 dyn/cm^2, but not at higher shear stresses (Fig. 4, A and C and not depicted). ERK1 showed increased phosphorylation in some experiments; however, there was no increase in ERK1 phosphorylation when densitometry was performed (not depicted).

We next examined activation of ERK1/2 after VCAM-1 ligation under static and shear conditions. To examine the effect of ligating VCAM-1 under static conditions, suspensions of control microspheres or anti–VCAM-1–coated microspheres were added to monolayers of IL-4–stimulated HUVECs, and the monolayers were incubated at 37°C for 10 min. After this incubation, the microsphere suspensions were removed, and the monolayers were lysed for Western blotting. Western blotting for phospho-ERK1/2 showed that neither ERK1 nor ERK2 was activated after VCAM-1 ligation under static conditions (Fig. 4, B and C). These data indicate that VCAM-1–mediated ERK2 activation is shear dependent.

HUVECs were next stimulated with TNF-α for 4 h to induce the surface expression of VCAM-1 to determine whether VCAM-1–mediated ERK2 activation was stimulus dependent. As was seen for IL-4–stimulated HUVECs, ligation of VCAM-1 on TNF-α–stimulated HUVECs under shear conditions led to increased ERK2 phosphorylation, whereas control microspheres had no effect on ERK2 activation (Fig. 5 A). Activation of ERK2 in TNF-α–stimulated

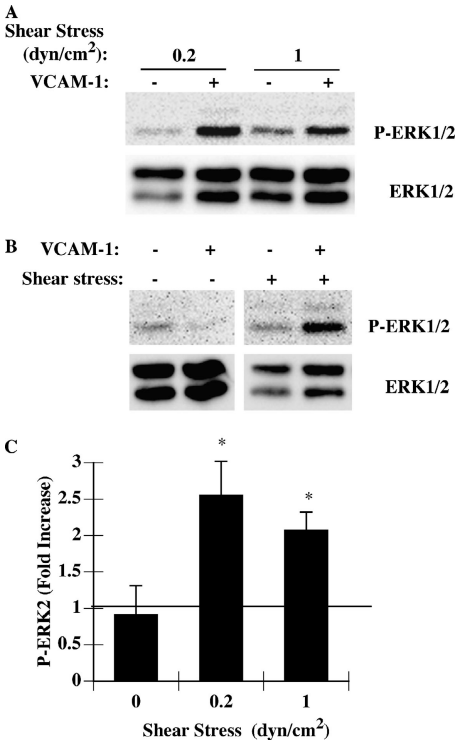

Figure 4. Binding of anti–VCAM-1–coated microspheres to IL-4–stimulated HUVECs induces shear-dependent ERK2 phosphorylation. HUVECs were stimulated with IL-4 for 24 h, the flow chamber was assembled, and goat anti–mouse–coated microspheres or anti–VCAM-1–coated microspheres (10^6/ml) were pulled into the flow chamber and allowed to settle for 5 min. Buffer was then perfused over the monolayer for 5 min at the specified shear stresses, after which the reaction was stopped by perfusing ice-cold 1× PBS with 5 mM EDTA over the monolayer (shear). Alternatively, the media was removed, goat anti–mouse–coated microspheres or anti–VCAM-1–coated microspheres (10^6/ml) were added to the monolayer, and the monolayer was incubated at 37°C for 10 min (0 dyn/cm^2). (A and B) Cells were lysed and Western blotted for phospho-ERK1/2. Membranes were stripped and reprobed for total ERK1/2. (C) Bands were analyzed by densitometry, and data are expressed as the fold increase in normalized phospho-ERK2 induced by anti–VCAM-1–coated microspheres as compared with IgG-coated microspheres. A and B are representative of at least three experiments. Data in C are mean ± SEM of at least three experiments. *, $P < 0.05$.

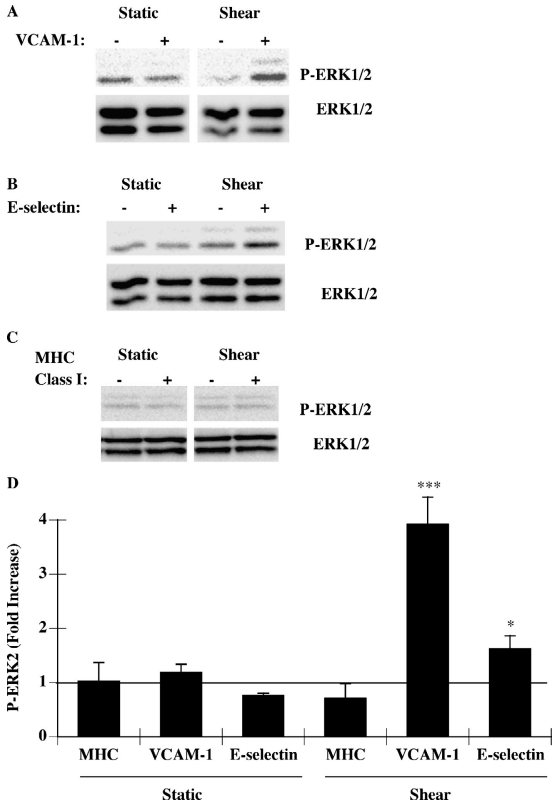

Figure 5. Binding of anti–VCAM-1–coated or anti–E-selectin–coated microspheres to TNF-α–stimulated HUVECs induces shear-dependent ERK2 phosphorylation. HUVECs were stimulated with TNF-α for 4 h, the flow chamber was assembled, and 10^6 of the specified microspheres/ml were used to activate HUVECs as described in Fig. 4. (A–C) Cells were lysed and Western blotted for phospho-ERK1/2. Membranes were stripped and reprobed for total ERK1/2. (D) Bands were analyzed by densitometry, and data are expressed as the fold increase in normalized phospho-ERK2 induced by coated microspheres as compared with IgG-coated microspheres. A, B, and C are representative of at least three experiments. Data in D are mean ± SEM of at least three experiments. *, $P < 0.05$; ***, $P < 0.001$.

HUVECs was also shear dependent (Fig. 5, A and D). Together, these data suggest that VCAM-1 ligation under shear conditions can induce signaling within endothelial cells, regardless of the stimulus used to up-regulate VCAM-1.

E-Selectin ligation leads to shear-dependent ERK2 phosphorylation

We next examined E-selectin, another vascular adhesion molecule, to determine if it can participate in shear-dependent signaling. E-selectin is involved in the recruitment of multiple leukocyte subclasses and has been implicated in signaling within endothelial cells (24, 25). We examined ERK1/2 activation after E-selectin ligation on TNF-stimulated HUVECs using anti–E-selectin–coated microspheres. Ligation of E-selectin caused modest but significant ($P < 0.05$) ERK2 activation under shear conditions but not under static conditions (Fig. 5, B and D). Control microspheres alone had no effect on ERK2 activation (Fig. 5, B and D). These data indicate that E-selectin ligation can also lead to shear-dependent ERK2 activation.

We next examined whether ligation of any cell surface protein under shear conditions is sufficient to activate ERK2 in this system. For these experiments, we used microspheres coated with anti–MHC class I antibody. Ligation of MHC class I under shear conditions had no effect on either ERK1 or ERK2 phosphorylation (Fig. 5, C and D), indicating that distortion of the cell surface alone is not sufficient to induce ERK2 activation.

Adhesion-induced signaling under flow conditions involves the actin cytoskeleton and focal adhesion proteins

Because our data indicated that endothelial cells responded to mechanical force associated with leukocyte binding, we investigated whether the actin cytoskeleton and endothelial cell focal adhesions participate in adhesion-induced mechanosignaling. We first examined the role of the actin cytoskeleton in ERK activation by disassembling the endothelial actin cytoskeleton with latrunculin A (Lat A) before perfusion of 3D10 cells over IL-4–stimulated HUVECs. Immunofluorescence was used to determine the concentration of Lat A that would lead to the loss of a majority of stress fibers while maintaining the integrity of the endothelial monolayer (Fig. 6 A). We then determined the effect of stress fiber loss on adhesion-induced ERK2 activation and found that Lat A pretreatment of endothelial cells led to a significant ($P < 0.05$) decrease in ERK2 phosphorylation (Fig. 6, B and C). Based on these results, we anticipated that Lat A pretreatment would block transmigration to the same extent as ERK2 inhibition; however, we found that Lat A did not reduce eosinophil transmigration (unpublished data). Because Lat A acts generically on stress fibers, this finding is likely caused by stress fibers acting on targets in addition to ERK2 phosphorylation that influence subsequent eosinophil transmigration.

An early event in mechanosignaling in many cell types is the phosphorylation of focal adhesion proteins such as FAK and paxillin (26–28). These signaling events have been shown to be upstream of ERK phosphorylation (16, 29, 30). We therefore determined whether there was a change in either FAK or paxillin phosphorylation after adhesion under flow conditions. Binding of 3D10 cells under flow conditions induced rapid phosphorylation of both of these proteins (Fig. 6 D). Phosphorylation peaked between 1 and 2 min and returned to baseline by 5 min (Fig. 6 D). Src family kinases have also been implicated in mechanical signaling through focal adhesion; however, we found no evidence for increased src family kinase phosphorylation (unpublished data). These data suggest that the actin cytoskeleton may participate in endothelial mechanosignaling downstream of leukocyte adhesion, potentially through the activation of the focal adhesion proteins FAK and paxillin.

Calcium is not required for ERK2 phosphorylation after adhesion under flow conditions

In this study, calcium mobilization occurred within seconds (Fig. 2) and ERK2 phosphorylation occurred between 1 and

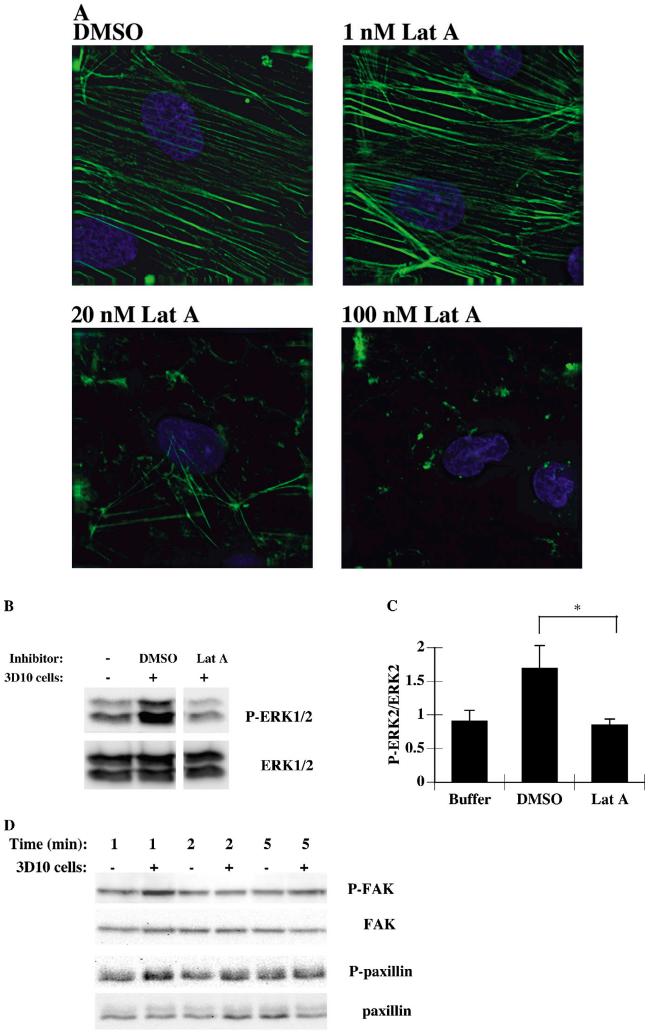

Figure 6. Adhesion-induced signaling under flow conditions involves stress fibers and focal adhesion proteins. (A) HUVECs were pretreated with HBSS containing a 1:1,000 dilution of DMSO or the specified concentrations of Lat A. The cells were then fixed, permeablized, and stained with Alexa 488–phalloidin for stress fibers and with DAPI to stain the nucleus. The cells were optically sectioned as described in Materials and methods. Buffer- and DMSO-treated cells were identical (not depicted). (B–D) IL-4–stimulated HUVECs were pretreated with DMSO alone or with 20 nM Lat A for 15 min, the flow chamber was assembled, and buffer or 10^6 fixed 3D10 cells/ml were perfused over the monolayer at 1.0 dyn/cm^2 for either 5 min or the times indicated. Phosphorylation of ERK1/2, FAK, or paxillin was measured by Western blotting as described in Fig. 3. The bands shown in B were from a single membrane. (C) Bands were analyzed by densitometry, and data are expressed as mean ± SEM of the ratio of phospho-ERK2/total ERK2. The blots shown are representative of four experiments. *, $P < 0.05$.

5 min (Fig. 3); our previous data show that maximal eosinophil transmigration occurs by 15 to 20 min (4). Our transmigration data in Fig. 1 suggested that calcium and ERK1/2 were acting on a single pathway. One could envision at least two mechanisms for this: one in which calcium is required for ERK2 phosphorylation and one in which both ERK and calcium act on a single target. To address the first mechanism, we exposed IL-4–stimulated endothelial cells to BAPTA before binding 3D10 cells under flow conditions and then determined the effect of ERK2 phosphorylation. We found that BAPTA had no effect on adhesion-induced ERK2 phosphorylation (1.99 ± 0.33-fold increase with adhesion alone and 1.62 ± 0.52-fold increase in HUVECs treated with 50 μM BAPTA; $n = 5$). Thus, ERK2 phosphorylation was not dependent on increased calcium after adhesion under flow conditions, suggesting that calcium and ERK2 may be acting on a single target.

Calpain and ROCK regulate eosinophil transmigration

Once proteins within focal adhesions are activated, reorganization and remodeling occur via changes in the cytoskeleton, as well as by the activation of proteases that can degrade focal adhesion proteins. Calpains are calcium-dependent proteases that can be activated downstream of focal adhesion signaling and can also serve as targets for ERK1/2 (19, 20). We used two structurally different inhibitors of calpain, calpeptin and PD 150606, to determine whether blocking calpain activity can regulate eosinophil transmigration. Calpeptin has previously been shown to block calpain-dependent degradation of the focal adhesion protein, talin, in HUVECs at concentrations between 10 and 20 μM (31). We replicated these experiments and found that calpeptin had the same effective concentrations in our system (unpublished data). Both

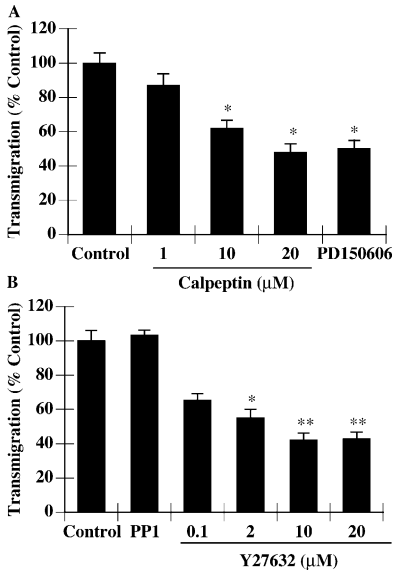

Figure 7. Calpain and ROCK regulate eosinophil transmigration across IL-4–stimulated HUVECs. IL-4–stimulated HUVECs were pretreated with (A) DMSO alone, 5 μM PD150606 , or the specified concentrations of calpeptin for 15 min or (B) DMSO alone, 10 μM of the src family kinase inhibitor PP1, or the specified concentrations of the ROCK inhibitor Y27632 for 15 min. The flow chamber was assembled, and 5×10^5 eosinophils/ml were perfused over the monolayers for 4 min at 2 dyn/cm^2. Transmigration was measured as described in Materials and methods and is expressed as the percentage of control. Data represent the mean ± SEM of between three and six experiments. *, $P < 0.05$; **, $P < 0.01$.

calpeptin and PD 150606 significantly ($P < 0.05$) reduced eosinophil transmigration (Fig. 7 A).

Focal adhesions serve as a nexus for many signaling pathways, including activation of src family kinases and reorganization of the cytoskeleton through activation of the small GTPases RhoA, Rac, and Cdc42 (32). We specifically examined the effects of blocking src family kinases and ROCK on eosinophil transmigration under flow conditions. We found that blocking endothelial cell src family kinases with the inhibitor PP1 had no effect on eosinophil transmigration (Fig. 7 B). This was consistent with a lack of src family kinase activation after adhesion under flow conditions (unpublished data). We have previously demonstrated increased src family kinase phosphorylation and activity downstream of neutrophil integrins (unpublished data). Y27632 has been used by other groups to block the activity of ROCK in human endothelial cells (33–35), and we also found that Y27632 effectively blocked ROCK activity in our system (unpublished data). We found that Y27632 led to a dose-dependent decrease in eosinophil transmigration (Fig. 7 B), suggesting a role for ROCK in eosinophil transmigration. Together, these data show that shear-dependent eosinophil transmigration can be regulated by three pathways that are downstream of focal adhesion signaling: the ERK1/2, calpain, and ROCK pathways.

DISCUSSION

For an organism to adapt to its physical environment, it must be capable of responding to changes in mechanical forces. Multiple cell types, ranging from specialized hair cells within the inner ear to endothelial cells within the bloodstream, are capable of sensing and responding to mechanical force. For example, bone remodeling requires the response of osteocytes, osteoblasts, and osteoclasts to changes in the compressive load, whereas maintenance of blood pressure homeostasis requires vascular smooth muscle cells to respond to changes in the stretch experienced by the vessel (26). The response to mechanical force varies between cell types but can include alterations in cellular functions such as gene expression, growth, differentiation, migration, and apoptosis. Given the importance of mechanical force to multiple physiologic processes, uncovering the mechanisms by which mechanical forces are sensed and responded to at the cellular level is likely to have widespread applications.

Within the vasculature, endothelial cells are constitutively exposed to mechanical force in the form of fluid shear stress. Endothelial cells respond to long-term exposure to shear stress with changes in cell morphology (36, 37) and gene expression (38). Multiple signaling pathways are activated within endothelial cells in response to shear stress and may be involved in mediating the functional responses to shear stress. Exposure of endothelial cells to shear stress induces the generation of second messengers, including intracellular calcium (11, 12), inositol 1,4,5-triphosphate (IP_3) (39), diacyl glycerol (40), and cyclic GMP (cGMP) (41), as well as the activation of protein kinases, including ERK1/2 (13, 15), JNK (14), $p125^{FAK}$ (16, 17), and c-Src (18).

Much effort has been devoted to identifying the specific molecules that sense and respond to shear stress in endothelial cells. Molecules such as ion channels (41–43), G protein–coupled receptors (13, 15, 41, 44), growth factor receptors (45), and integrins (16, 45, 46) have all been proposed to be mechanotransducers of shear stress in endothelial cells. We present data here suggesting that apical adhesion molecules on vascular endothelial cells can function as mechanotransducers. We found that the binding of eosinophils or 3D10 cells to IL-4–stimulated endothelial cells under shear conditions caused intracellular calcium fluxes (Fig. 2) and ERK2 phosphorylation in endothelial cells (Fig. 3), suggesting that endothelial cell adhesion molecules act as signaling molecules during eosinophil recruitment. Inhibiting either intracellular calcium increases or tyrosine phosphorylation blocked eosinophil transmigration (Fig. 1). When antibody-coated latex microspheres were used to ligate specific adhesion molecules under shear stress, we found that the adhesion molecules VCAM-1 (Figs. 4 and 5) and E-selectin (Fig. 5) could mediate activation of ERK2 in cytokine-stimulated HUVECs. Ligation of VCAM-1 or E-selectin under static conditions did not induce ERK2 activation, indicating that this is a shear-dependent process (Figs. 4 and 5). Increased phosphorylation of focal adhesion proteins FAK and paxillin preceded ERK2 activation and did not occur in the absence of 3D10 cell adhesion to endothelial cells (Fig. 6). Our data suggest that leukocyte adhesion to endothelial cells under flow conditions represents a novel mechanism for inducing mechanotransduction that then activates focal adhesion proteins such as FAK and paxillin.

Mechanotransduction through leukocyte adhesion molecules taps into previously described processes by which endothelial cells sense force. The signaling through VCAM-1 and E-selectin that we observed was not the result of initiation of shear stress, but rather the result of adhesion molecule ligation in the presence of laminar shear stress. This was specific for VCAM-1 and E-selectin because ligation of MHC class I had no effect on ERK signaling (Fig. 5 C). It has been proposed that endothelial signaling pathways are not induced by continuous laminar shear stress in vivo because endothelial cells "adapt" to shear stress that is constitutively present. Instead, endothelial signaling pathways become activated when laminar flow is disturbed, as occurs during atherosclerosis. The binding of a leukocyte under shear conditions would also lead to disruption of laminar flow. Recent data from Davies et al. show that leukocyte binding to endothelial cells induces a transient and focal increase in the force exerted on both the leukocyte and the endothelial cell at the point of contact (Fig. 8 A) (47). Thus, endothelial cell mechanotransduction in response to leukocyte binding or adhesion molecule ligation under shear conditions is representative of physiologic conditions that typically occur during leukocyte trafficking. This hypothesis would also support mechanical activation of the attached leukocyte as well, although exploring mechanotransduction in leukocytes was not addressed in our study.

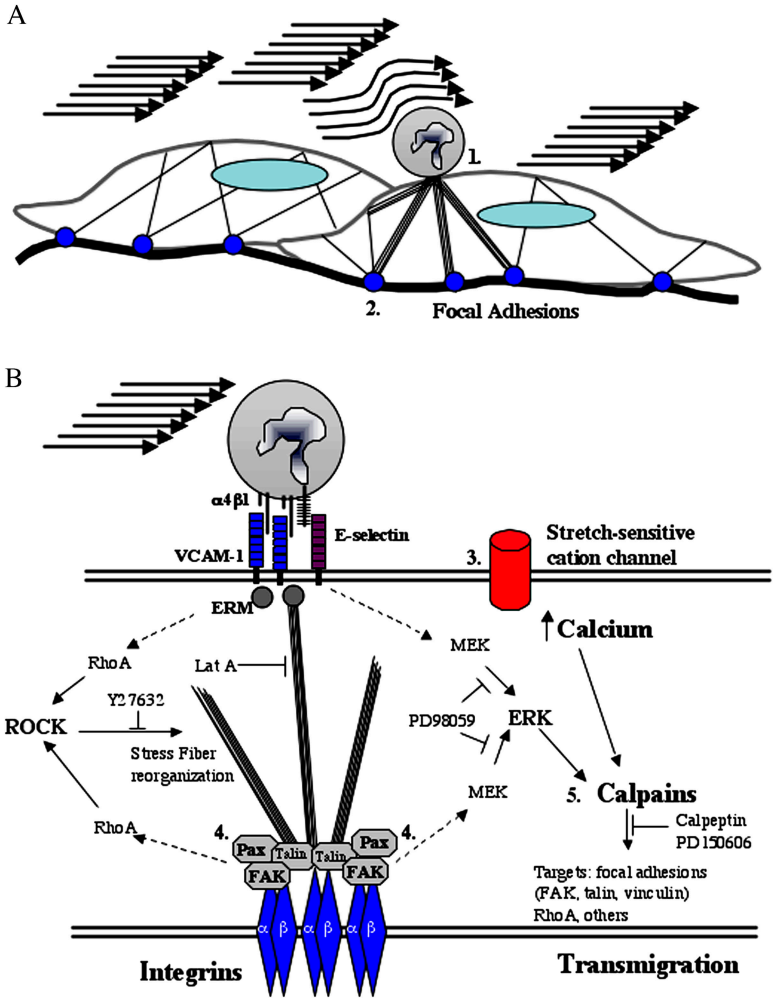

Figure 8. A model for leukocyte-induced mechanotransduction. (A, 1.) Leukocyte binding to adhesion molecules expressed on the luminal side of the endothelial cell under flow conditions changes the local forces experienced by the endothelial cell. (2.) These forces may be transmitted by the cytoskeleton to focal adhesions on the basolateral side of the endothelial cells. (B, 3.) Stretch-sensitive cation channels can lead to increased intracellular calcium. (4.) Integrin-mediated signaling leads to phosphorylation of FAK and paxillin (Pax). These events increase the activity of ERK2 and ROCK. (5.) Calpain activation downstream of calcium influx and ERK activation leads to the degradation of target proteins that influence transmigration. Panel A is adapted from Alenghat and Ingber (reference 26). MEK, MAPK kinase.

We propose that leukocyte tethering, rolling, and adherence to activated endothelial cells under flow conditions pulls on the cytoskeleton through interactions with adhesion molecules like VCAM-1 and E-selectin that associate with the cytoskeleton after leukocyte binding. This would be consistent with the tensegrity model for mechanosensing (26, 48). Force transmitted through the cytoskeleton to integrins on the basolateral side of the endothelial cells could then lead to signaling via focal adhesions as measured by phosphorylation of FAK and paxillin (Fig. 8). In the absence of linkage to the cytoskeleton, signaling via ERK2 does not occur, as demonstrated by our data using Lat A to disrupt stress fibers (Fig. 6) and the inability of ligation of MHC class I under flow conditions to initiate ERK2 activation (Fig. 5). Others have shown that integrins can activate many mechanosensitive signaling pathways in endothelial cells that can then lead to functional changes in the endothelium (26, 49). Our data suggest that ROCK and calpain participate in shear-dependent eosinophil transmigration. Ligation of VCAM-1 or E-selectin alone activates signaling pathways in endothelial cells (24, 25, 50–52) (Fig. 8). Our data suggest that these signaling pathways alone are not sufficient to promote eosinophil transmigration; however, these pathways may cooperate to maximize eosinophil transmigration.

The classical "multistep paradigm" divides the process of leukocyte recruitment into a series sequential steps that includes tethering and rolling, activation, firm adhesion, locomotion, and transmigration. This model describes a very active role for the recruited leukocyte, whereas implying a relatively passive role for the endothelium. It is increasingly appreciated that the endothelium actively participates in leukocyte recruitment (53). Studies have shown that leukocyte

adhesion can activate endothelial cell signaling pathways (5–8, 25, 54, 55), some of which can promote subsequent leukocyte transmigration (5–8, 10, 54). Our data further support an active role for the endothelium in leukocyte transmigration and present the first evidence that mechanotransduction through lumenal adhesion molecules is important for leukocyte transmigration under shear conditions. Based on our data and the data of other groups, we propose a modified multistep paradigm in which mechanotransduction through rolling and adhesion receptors induces signaling within the endothelium, thereby activating the endothelium and promoting transmigration. A central role for adhesion-dependent mechanotransduction in leukocyte transmigration would explain the effects of shear stress on leukocyte transmigration, which have been observed for the transmigration of lymphocytes (2), neutrophils (3), and eosinophils (4).

MATERIALS AND METHODS

Materials. Anti–VCAM-1 mAb (4B9) was a gift from R. Lobb (Biogen, Inc., Cambridge, MA) and anti–E-selectin mAb (ES1) was a gift from R. McEver (University of Oklahoma, Oklahoma City, OK). Anti-CD16 and anti-CD3 paramagnetic beads were obtained from StemCell Technologies Inc. Antibodies directed against phospho-ERK1/2, phospho-p38, p38, phospho-JNK, phospho-FAK, phospho-paxillin, and FAK were purchased from Cell Signaling Technology, Inc. Antibodies directed against ERK1/2 and total paxillin were from Upstate Biotechnology. Anti-JNK antibody was obtained from Santa Cruz Biotechnology, Inc. Mouse anti–human MHC class I antibody (MCA81) was purchased from Serotec. Human recombinant TNF-α and IL-4 were obtained from R&D Systems. Human serum albumin (HSA) was purchased from Bayer, Inc.; M199 and lymphoprep 1077 were purchased from Invitrogen; RPMI 1640 was purchased from BioWhittaker Inc.; and FBS was purchased from Hyclone, USA. Deoxyglucose (2-deoxy-D-glucose grade III), sodium azide, and HBSS containing calcium and magnesium were purchased from Sigma-Aldrich. 7.2-μm goat anti–mouse–coated latex microspheres were obtained from Bangs Laboratories, Inc. BAPTA-AM and Fluo-4 AM were purchased from Invitrogen. ImmunoPure IgG elution buffer was obtained from Pierce Chemical Co. All inhibitors were purchased from Calbiochem-Novabiochem. All other reagents were obtained from VWR International.

Cell isolation and culture. Eosinophils and neutrophils were isolated as previously described (4). In brief, blood from healthy or mildly atopic adults was drawn into heparinized syringes, and granulocytes (neutrophils and eosinophils) were isolated by dextran sedimentation, hypotonic lysis, and density centrifugation on lymphoprep 1077. Eosinophils were further purified by depleting neutrophils and any remaining T lymphocytes using magnetic cell separation with anti-CD16 and anti-CD3 paramagnetic beads. The resulting eosinophils and neutrophils were routinely >95% pure by Kimura staining. Primary or first passage HUVECs were isolated from umbilical cords (Foothills Hospital, Calgary, Canada) as previously described (56) and maintained in M199 with 20% human serum. For all experiments, HUVECs were used 2 d after confluence. Acute myelogenous leukemia 3D10 (3D10) cells were a gift from M. Baumann and C. Paul (Wright State University, Dayton, OH). 3D10 cells were maintained in RPMI 1640 with 10% FBS. The Ethics Committee at the University of Calgary approved all procedures that used human tissue samples.

Latex microsphere labeling. 7.2-μm goat anti–mouse–coated latex microspheres were coated with anti–VCAM-1, anti–E-selectin, or anti–MHC class I mAb according to the manufacturer's instructions. In brief, microspheres were washed with HBSS containing 0.1% Tween 20 to remove the storage buffer. Anti–VCAM-1 or anti–E-selectin mAb was bound to the microspheres by gently rotating a suspension of microspheres and antibody (0.77 μg/10^6 microspheres) at room temperature for 30 min. Microspheres were washed with HBSS containing 0.1% Tween 20 to remove any unbound antibody. Similar numbers of anti–VCAM-1–, anti–E-selectin–, or anti–MHC class I–coated latex beads bound to activated endothelial cells in these studies (unpublished data).

Transmigration. Transmigration under flow conditions was quantified as previously described (4). In brief, 2-d postconfluent HUVEC monolayers were stimulated for 24 h with 10 or 20 ng/ml IL-4. In some experiments cells were stimulated for 4 h with 10 ng/ml TNF-α. After stimulation, a parallel plate flow chamber from Glycotech was assembled onto the monolayer and 5×10^5 freshly isolated eosinophils/ml in HBSS containing 0.5% HSA were perfused across the monolayer at 2 dyn/cm^2. After 4 min of perfusion, the inlet line was transferred to HBSS to prevent the binding of new eosinophils, and shear was maintained at 2 dyn/cm^2. In all experiments, accumulation was measured between 4 and 5 min of perfusion, whereas transmigration was measured between 6 and 7 min of perfusion. Interacting cells were visualized at 400× and recorded via a CCD camera (KP-M1U; Hitachi Denshi, Ltd.) for later analysis. Transmigration is calculated by dividing the number of transmigrated cells by the total number of cells counted. In most experiments, 5–10 fields of view were recorded and 100–150 cells were counted. In some experiments, HUVECs were pretreated with the specified concentrations of inhibitors before assembly of the flow chamber. DMSO alone was used as a solvent control where appropriate. Endothelial cells were exposed to extensive washing before the introduction of eosinophils to prevent carryover of inhibitors. The inhibitors used in this study included BAPTA, genistein, Lat A, calpeptin, PP1, and Y27632. In each case, the ability of the inhibitors to block their intended targets was determined biochemically (unpublished data), and the optimal concentration was then used to determine the effect on endothelial cell signaling and eosinophil transmigration.

Calcium imaging. HUVECs were grown to 2 d after confluence on either 12- or 40-mm gelatin-coated glass coverslips and were stimulated for 24 h with 10 ng/ml IL-4. HUVECs were incubated with cell-permeant esterified Fluo-4 AM in loading buffer (HBSS with 5 mM HEPES and 200 μM probenicid) for 30 min at 37°C and 5% CO_2. After the incubation with Fluo-4 AM, the cells were washed, the media was replaced with loading buffer, and the cells were incubated for an additional 30 min at 37°C and 5% CO_2 to deesterify the Fluo-4. Fluo 4–labeled HUVECs were placed in a parallel plate flow chamber from Bioptechs, and loading buffer was perfused over the monolayer at 0.25 dyn/cm^2. After 5 min, either 5×10^5 3D10 cells/ml or 5×10^5 freshly isolated eosinophils/ml were perfused over the monolayer at 0.25 dyn/cm^2. This shear stress was selected to maximize the number of interactions that occurred in a single field of view. For static experiments, Fluo-4–loaded IL-4-stimulated HUVECs grown on 12-mm coverslips were placed in a Warner chamber and 5×10^5 3D10 cells/ml were added and allowed to settle on the monolayer. Sequential fluorescence and differential interference contrast images were acquired using a 40× 1.35 NA objective on an imaging workstation (DeltaVision; Applied Precision). Changes in intracellular calcium were monitored by exciting Fluo-4 at 490 nm and detecting the emission at 528 nm. The fluorescent images were acquired ~2 s apart and were binned by a factor of two to increase the signal/noise ratio. The relative fluorescent intensities within the cells were measured by selecting standard regions of interest and recording the average pixel intensities. Image analysis was performed using Metamorph version 6.1 (Universal Imaging Corp.). Data were normalized by expressing the values as a percentage of the maximal signal obtained with a positive control.

Stimulation by 3D10 cell binding. HUVECs were grown to 2 d after confluence and were stimulated for 24 h with 10 ng/ml IL-4. The Glycotech flow chamber was assembled onto a monolayer and HBSS alone (buffer), 5×10^6 untreated 3D10 cells/ml, or 5×10^6 fixed 3D10 cells/ml were perfused over the monolayer at 0.5 dyn/cm^2 for 1, 2, or 5 min. Ice-cold 1× PBS with 5 mM EDTA was perfused over the monolayer to remove adherent 3D10

cells, the flow chamber was disassembled, and the monolayer was prepared for Western blotting as described below. Similar results were obtained when 10^6 3D10 cells/ml were used and perfused at 1.0 dyn/cm^2.

Stimulation using antibody-coated microspheres. HUVECs were grown to 2 d after confluence and were stimulated with 10 ng/ml IL-4 or TNF-α for 24 or 4 h, respectively. For shear experiments, the Glycotech flow chamber was assembled onto a monolayer and a suspension (10^6 microspheres/ml) of goat anti–mouse–coated microspheres, anti–VCAM-1–coated microspheres, anti–E-selectin–coated microspheres, or anti–MHC class I–coated microspheres was pulled into the flow chamber. Microspheres were allowed to settle onto the monolayer for 5 min, after which shear was initiated, and HBSS was perfused over the monolayer for 5 min at 0.2, 1, or 5 dyn/cm^2. Ice-cold 1× PBS with 5 mM EDTA was perfused over the monolayer, the flow chamber was disassembled, and the monolayer was prepared for Western blotting as described below. For static experiments, the media was removed and replaced with a suspension (10^6 microspheres/ml) of goat anti–mouse–coated microspheres, anti–VCAM-1–coated microspheres, anti–E-selectin–coated microspheres, or anti–MHC class I–coated microspheres. HUVECs were incubated at 37°C for 10 min, after which the microsphere suspension was removed, and the monolayer was prepared for Western blotting as described below. Extreme care was taken to prevent the introduction of shear during the manipulation of the monolayers.

Western blotting. HUVEC monolayers were lysed in a buffer containing 1% Triton X-100. Soluble lysates were separated by SDS-PAGE, and proteins were transferred to a polyvinyldine difluoride membrane. Membranes were initially probed with the specified phospho-specific antibody, after which they were stripped in IgG elution buffer and reprobed with an antibody directed against the total protein. Bands were visualized using a multiimager (Fluor-S MAX; Bio-Rad Laboratories), and densitometry was performed using Quantity One software (Bio-Rad Laboratories). Densitometry data are presented as the ratio of phospho-ERK2/total ERK2.

Immunofluorescence. Endothelial cells were cultured on #1.5 round glass coverslips that had been pretreated with 0.1% sodium carbonate, pH 9.2, and gelatinized overnight in 0.2% gelatin. Cells were treated for 20 min with DMSO alone or the specified concentrations of Lat A. Cells were fixed in 2% paraformaldehyde, washed with HBSS, and permeabilized in 0.5% Triton X-100. Cells were blocked in HBSS containing 1% HSA with 0.1% Triton X-100. For stress fiber staining, cells were incubated with Alexa 488–conjugated phalloidin for 30 min at 37°C. All coverslips were subsequently stained with the nuclear dye Bisbenzimide H 33258 for 5 min at 37°C and mounted in glycerol. Cells were imaged using a PlanApo 60× (1.40NA) objective, and optical sections were acquired using a Delta Vision digital deconvolution system (Applied Precision) mounted on a microscope (IX70; Olympus). Digital deconvolution was performed using SoftWorx software (Applied Precision).

Statistics. All experiments were performed at least three times. The data were analyzed using either an unpaired Student's *t* test or analysis of variance. Nonparametric tests were used when appropriate. $P < 0.05$ was considered significant.

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