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Forum Review

The Signaling Pathways Induced by Neutrophil-Endothelial Cell Adhesion

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ABSTRACT

Adhesion of neutrophils to vascular endothelial cells (ECs), mediated by the interaction of CD11/CD18 and intercellular adhesion molecule-1 (ICAM-1), is often required for neutrophil transmigration across endothelium during most inflammatory responses. Induction of intracellular signaling in neutrophils as a result of adhesion has been recognized for many years. Recent studies demonstrated that neutrophil-endothelial adhesion also activates ECs. Examples of neutrophil adherence-induced changes in ECs include increases in intracellular Ca^{2+}, production of reactive oxygen species, and actin cytoskeleton changes. These changes result, in part, from ligation of EC adhesion molecules. This review article focuses on the signaling events that occur in ECs during neutrophil adhesion and the role of EC adhesion molecules, particularly ICAM-1, in the initiation of these signaling events in ECs. The evidence to date describing the molecular basis of ICAM-1-induced signaling will be summarized. Finally, the potential physiological roles of these signaling events induced by EC adhesion molecules in mediating neutrophil migration will be addressed. Antioxid. Redox Signal. 4, 39–47.

INTRODUCTION

NEUTROPHIL EMIGRATION underlies many acute inflammatory responses and represents a host defense mechanism against invading pathogens or other injury. Neutrophil emigration from blood to the tissues in response to an inflammatory stimulus is a complex process that is highly regulated. Events that happen during neutrophil adhesion to vascular endothelial cells (ECs), transmigration across endothelium, and migration through extracellular matrix toward the site of injury are all likely to regulate neutrophil emigration in response to an inflammatory stimulus.

The identification of adhesion molecules expressed by neutrophils and ECs that mediate the interactions between neutrophils and ECs, as well as extracellular matrix, has enhanced our understanding of the molecular and cellular mechanisms regulating neutrophil emigration during inflammation. The physiological roles of these adhesion molecules in mediating neutrophil emigration in *in vitro* and *in vivo* models of human diseases have been established during the past decade using functional blocking antibodies and adhesion molecule-deficient mice. The identification, expression, and roles of these adhesion molecules have been extensively described in previous review articles and will not be repeated in this review (2, 8, 14, 19).

Mechanisms regulating neutrophil emigration from blood to the tissues in response to an inflammatory stimulus and the roles of adhe-

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sion molecules are tissue- and stimulus-specific. For example, notable differences exist between the systemic circulation and the pulmonary circulation. Neutrophil emigration takes place in the capillaries of pulmonary circulation rather than in the postcapillary venules of the systemic circulation (4, 52). In the systemic circulation, most neutrophil emigration occurs through CD11/CD18-dependent adhesion, whereas in the pulmonary circulation, both CD18-dependent and -independent adhesion pathways are utilized, depending on the stimulus (12–15). This tissue and stimulus specificity may very likely be reflected *in vitro* when we examine the responses and the signaling mechanisms induced during neutrophil adhesion.

This review addresses the signaling events that occur in ECs during neutrophil-EC adhesion, in particular: (a) neutrophil-induced signaling in ECs during adhesion; (b) the role of EC adhesion molecules, particularly intercellular adhesion molecule-1 (ICAM-1), as signaling molecules; (c) how ICAM-1 may function as a signaling molecule; and (d) the potential role of ICAM-1-induced signaling in mediating neutrophil emigration during inflammatory processes.

NEUTROPHIL-INDUCED SIGNALING IN ECS DURING ADHESION

Neutrophils must interact with the postcapillary venular ECs in the systemic circulation or the pulmonary capillary ECs in the pulmonary circulation before they can reach the tissues. Firm adhesion, mediated by the interaction of neutrophil β2 integrins and EC ICAM-1, is often required for subsequent transmigration across endothelium. Neutrophil transmigration across ECs often occurs at EC junctions as observed *in vivo* and *in vitro,* although neutrophil migration through ECs in response to intradermal injection of formylmethionyl-leucyl-phenylalanine (fMLP) has also been demonstrated (7, 14, 20). Recent studies suggest that neutrophil adhesion results in signaling into both neutrophils and ECs, and that these signaling events may very likely influence subsequent events, including neutrophil crawling on the surface of ECs to the junctions and transmigration across ECs.

By using cultured ECs derived from different vascular beds, recent studies demonstrated that neutrophil adhesion indeed induces signaling into ECs. The changes in ECs induced by neutrophil adhesion include increases in intracellular Ca^{2+}, cytoskeletal changes, and oxidant production. Huang and colleagues demonstrated that adhesion of fMLP-activated neutrophils to human umbilical vein ECs (HUVECs) induces an increase in intracellular Ca^{2+} in ECs (30). Similarly, neutrophil adherence to cytokine-stimulated ECs induces an increase in intracellular Ca^{2+} in ECs (36, 60). Chelation of intracellular Ca^{2+} inhibits neutrophil transmigration across ECs without inhibiting neutrophil adhesion, suggesting that Ca^{2+}-dependent events in ECs induced by adhesion are required for subsequent transmigration (30). Indeed, fMLP-stimulated neutrophils induce myosin light chain phosphorylation and isometric tension generation in cultured HUVECs or pulmonary arterial ECs, and inhibition of myosin light chain kinase in ECs prevents neutrophil transmigration across ECs (21, 28, 44). These studies suggest that neutrophil adherence-induced Ca^{2+} increases, activation of myosin light chain kinase, and increased interactions between actin and myosin light chain molecules in large-vessel ECs may be essential for subsequent neutrophil transmigration across ECs.

We have recently demonstrated that neutrophil adherence to tumor necrosis factor-α-pretreated human pulmonary microvascular ECs induces changes in the biomechanical properties and F-actin cytoskeleton of ECs within 2 min (53, 55). These cytoskeletal changes in ECs consist of increased thickness of microfilaments and focal F-actin aggregates. These changes require actin rearrangement because they are prevented by agents that either disrupt or stabilize F-actin (55). These cytoskeletal changes, however, do not appear to require Ca^{2+} or myosin light chain phosphorylation, but occur through a phosphatidylinositol-dependent mechanism (55). This cytoskeletal remodeling in ECs is accompanied by an increase in neutrophil migration toward EC borders (55). These studies demonstrating that neutrophil adhesion induces cytoskeletal changes in pulmonary microvascular ECs during adhesion that do not require calcium or myosin light chain kinase are in contrast to the previously described studies in large-vessel ECs (21, 28, 36, 44, 60). These apparent discrepancies likely reflect unappreciated and exciting differences in mechanisms underlying the migratory process or in cell types. First, the changes in ECs induced by neutrophil adhesion may be differentially regulated compared with those occurring during transmigration. Our studies were performed in the absence of an exogenously applied chemoattractant, and neutrophil transmigration did not occur. In a recent study by Su and colleagues, Ca^{2+} responses at the single-cell level were examined in HUVECs during neutrophil transmigration induced by fMLP (49). Interestingly, increases in Ca^{2+} in HUVECs are associated with neutrophil transmigration, but not adhesion (49). Second, the responses in ECs induced by neutrophil adhesion may depend on the type of ECs. There may be different mechanisms regulating neutrophil-induced cytoskeletal changes in ECs derived from different vascular beds or from large vessels compared with microvessels of the same tissue.

Neutrophil adhesion to ECs also induces intracellular oxidant production in ECs. Adhesion of activated neutrophils to arterial ECs induces conversion of xanthine dehydrogenase to its active form, xanthine oxidase (41, 42, 51). As activation of xanthine oxidase represents an important mechanism for superoxide production in ECs (25, 26), this conversion to xanthine oxidase likely results in increases in the production of superoxide and other reactive oxygen species (ROS) in ECs. The conversion of xanthine dehydrogenase to xanthine oxidase is inhibited by an anti-CD18 antibody, suggesting that neutrophil adhesion is required (42, 51). This conversion to xanthine oxidase may mediate neutrophil-induced EC injury during inflammatory processes (42, 51).

We have recently shown that neutrophil adhesion to tumor necrosis factor-α-pretreated pulmonary microvascular ECs rapidly induces oxidant production in ECs, but not in neutrophils (53). This increase in oxidant production in ECs is partially inhibited by allopurinol, a xanthine oxidase inhibitor, suggesting that xanthine oxidase contributes to oxidant production in ECs induced by neutrophil adherence. This increase in oxidant production is required for the EC stiffening response induced by neutrophil adherence, suggesting that oxidants produced soon after neutrophil adhesion may also serve as signaling molecules in ECs and result in subsequent cytoskeletal changes (53).

These changes in ECs induced by neutrophil adhesion may occur through two mechanisms (Fig. 1). In the first mechanism, neutrophil-derived mediators may initiate these changes. In response to adhesion and ligation of β2 integrin,

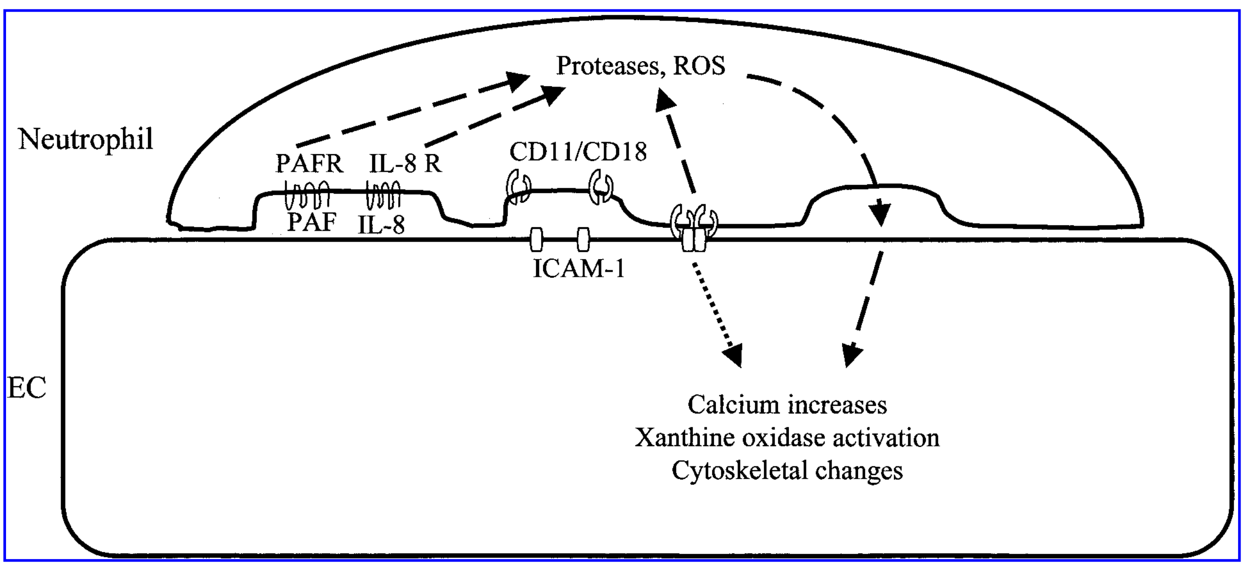

FIG. 1. Postulated mechanisms through which neutrophil adhesion may induce signaling events in ECs. In the first mechanism, during adhesion, ligation of β2 integrins and/or exposure to interleukin-8 (IL-8) or platelet-activating factor (PAF) expressed on EC surface induces release of proteases or ROS from neutrophils. These mediators in turn act on ECs and induce signaling into ECs (-------). In the second mechanism, during adhesion, ligation of EC adhesion molecules may directly initiate signaling events into ECs (. . . . . .). IL-8 R, IL-8 receptor; PAFR, PAF receptor.

neutrophils are capable of releasing ROS and elastase (6, 38, 39, 46, 59). These mediators may act on ECs and induce subsequent responses. For example, in pulmonary arterial ECs, Phan and colleagues identified a role for neutrophil elastase in mediating conversion of xanthine dehydrogenase to xanthine oxidase in response to adhesion by phorbol myristate acetate-activated neutrophils (42). This requirement for elastase seems also to depend on EC type, because Wakabayashi and colleagues could not demonstrate a similar role for elastase in carotid arterial ECs (51). Nevertheless, neutrophil-derived mediators are very likely to play important roles in mediating the EC responses during neutrophil adhesion, and high concentrations of these mediators may accumulate at the site of adhesion. In this context, EC adhesion molecules may play a role in neutrophil activation as ligands for neutrophil β2 integrins. In the second mechanism, ligation of EC adhesion molecules during neutrophil adhesion may directly initiate signaling events into ECs.

THE ROLE OF EC ADHESION MOLECULES AS SIGNALING MOLECULES

EC adhesion molecules, upon ligation, initiate signaling events into ECs. Ligation of E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), or ICAM-1 can induce a cascade of signaling events (Fig. 2).

E-selectin and P-selectin

E-selectin and P-selectin play important roles in mediating neutrophil rolling on postcapillary venules in the systemic circulation. Neutrophil adherence to HUVECs induces association of E-selectin with the actin cytoskeleton (57), and monocyte adhesion to ECs induces E-selectin clustering (56). These studies suggest that E-selectin may be able to signal into ECs. Indeed, both E-selectin and P-selectin function as signal transducers (for review, see 3). Ligation of E-selectin and P-selectin with monoclonal antibodies results in increases in intracellular Ca^{2+}, stress fiber formation, shape changes, as well as dephosphorylation of E-selectin in its cytoplasmic tail in HUVECs (32, 36, 58). E-selectin-induced activation of extracellular signal-regulated kinase (ERK) and up-regulation of *c-fos* mRNA have also been demonstrated in HUVECs (29).

VCAM-1

VCAM-1 is a ligand for VLA-4. Monocyte adhesion on ECs induces VCAM-1 clustering

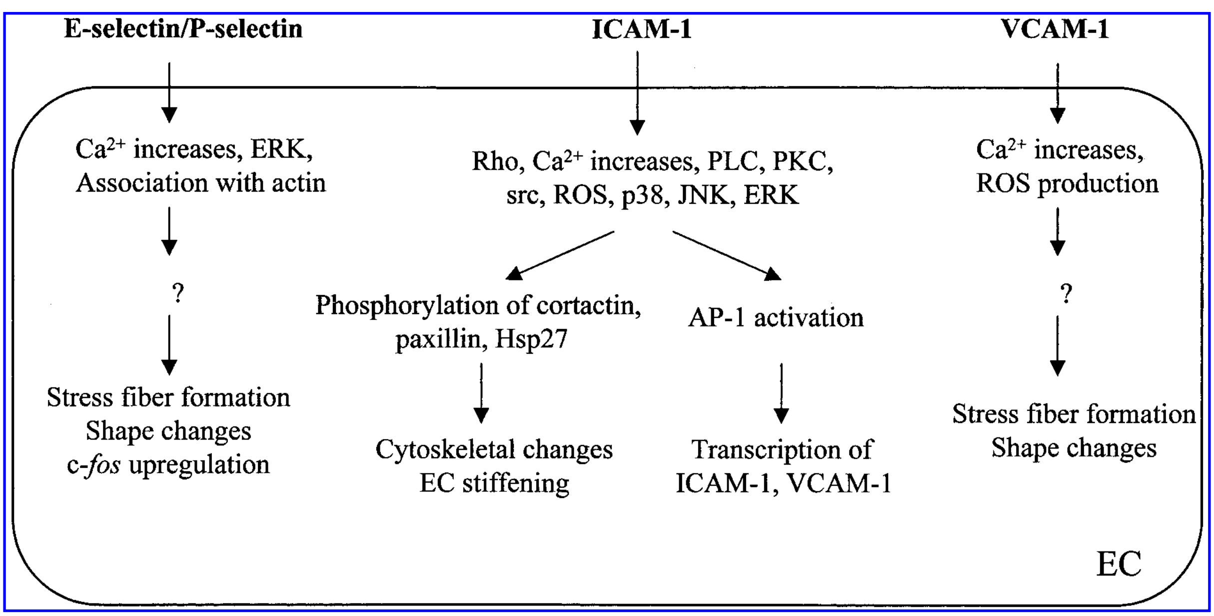

FIG. 2. Signaling pathways induced through ligation of E-selectin/P-selectin, ICAM-1, or VCAM-1. JNK, c-Jun NH2-terminal kinase; PKC, protein kinase C; PLC, phospholipase C.

(56). Ligation of VCAM-1 induces intracellular Ca^{2+} increases in ECs (36). Ligation of VCAM-1 by lymphocytes or antibodies induces activation of NADPH oxidase and production of ROS in ECs, which is required for actin cytoskeletal changes in ECs and lymphocyte transmigration across ECs (37).

ICAM-1

ICAM-1-initiated signaling events can be induced through ligation of ICAM-1 by neutrophils, antibodies, or fibrinogen in different cell types, resulting in Ca^{2+} increases, cytoskeletal changes, and gene transcription. In pulmonary microvascular ECs, neutrophil adherence-induced cytoskeletal changes are completely inhibited by an anti-ICAM-1 antibody and can be mimicked by crosslinking ICAM-1 with antibodies, suggesting that ICAM-1 is required for transducing signaling events into ECs (53). In addition, neutrophil adherence-induced production of ROS is inhibited by an anti-ICAM-1 antibody in pulmonary microvascular ECs and in carotid arterial ECs, suggesting that ROS production may be an integral part of ICAM-1 signaling events (51, 53).

The role of ROS in ICAM-1 signaling in pulmonary microvascular ECs is further demonstrated by the following series of experiments. Crosslinking ICAM-1 in pulmonary microvascular ECs induces activation of p38 mitogen-activated protein kinase (MAPK) that is inhibited by allopurinol, a xanthine oxidase inhibitor, whereas inhibition of p38 MAPK has no effect on ROS production, indicating that ICAM-1-induced ROS production occurs upstream of p38 activation (54). Activation of p38 MAPK in turn induces phosphorylation of heat shock protein 27 (Hsp27), an actin-binding protein that may induce actin polymerization when phosphorylated and is required for the cytoskeletal rearrangement induced by neutrophil adherence or ICAM-1 crosslinking (54). These studies demonstrated that ligation of ICAM-1 in pulmonary microvascular ECs induces a sequence of signaling events, including production of ROS, activation of p38 MAPK, and phosphorylation of Hsp27, and that these signaling events play important roles in mediating the cytoskeletal changes in ECs induced by neutrophil adherence.

Signaling through ICAM-1 initiated by crosslinking antibodies has also been reported in other ECs. Crosslinking ICAM-1 with antibodies in brain EC lines or venular ECs induces increases in intracellular Ca^{2+} and activation of $pp60^{src}$, Rho, and protein kinase C (10, 16, 18, 56). These signaling pathways act upon several actin-associated proteins, including cortactin, FAK, paxillin, and p^{130} Cas, which in turn may induce changes in the actin cytoskeleton of these ECs (1, 16, 17). In addition, crosslinking ICAM-1 induces transcription of VCAM-1 and ICAM-1 through activation of ERK-1 and AP-1 (10, 34). These studies demonstrate that ICAM-1-induced signaling events result in changes in ECs, including cytoskeletal rearrangement and gene transcription, that are likely to modulate leukocyte migration during inflammatory responses.

ICAM-1 signaling also occurs in other cell types than ECs. In astrocytes, ligation of ICAM-1 induces expression of proinflammatory cytokines that requires activation of ERK and p38 MAPK (35). Signaling through ICAM-1 in B and T lymphocytes and fibroblasts has also been reported, and is described in a recent review article by Hubbard and Rothlein (31). In addition, ICAM-1 signaling can also be initiated through ligation by fibrinogen. Fibrinogen-induced activation of ERK-1, $pp60^{src}$, and cell proliferation is mediated through ICAM-1 (22, 23, 43). Whether different ligands for ICAM-1 initiate a different set of signaling pathways remains to be defined, as well as differences in ICAM-1 signaling depending on the cell type.

SPECULATIONS ABOUT HOW ICAM-1 MAY INDUCE SIGNALING IN ECS

It is not apparent how ICAM-1 ligation initiates oxidant production and downstream signaling events in ECs. ICAM-1 is a glycosylated protein that belongs to the superfamily of immunoglobulin-like proteins (48). ICAM-1 has five extracellular immunoglobulin domains, a transmembrane domain, and a short cytoplasmic domain. The signaling events induced by antibodies often require crosslinking by a secondary antibody, suggesting that ICAM-1 clustering may be required for ICAM-1 signaling. Indeed, ICAM-1 crosslinking induces formation of ICAM-1 clusters and aggregates, which is regulated by Rho family GTPases, and ICAM-1 clustering is observed at the site of monocyte adhesion (56). The cytoplasmic domain of ICAM-1 is composed of 28 amino acids (478–505): RQRKIKKYRLQQAQKGTPMKPNTQATPP (9, 47). This domain does not have intrinsic kinase activity or Src homology domains that can recruit tyrosine-phosphorylated proteins (47). However, this domain does interact with other molecules, including actin-binding proteins, suggesting that ICAM-1-induced signaling may be initiated at the membrane–cytoskeletal interface. The intracellular domain of ICAM-1 is linked to an actin-binding protein, α-actinin (9, 50). In addition, this domain can bind phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], a molecule implicated in various signaling cascades (27). This domain can also bind ezrin/radixin/moesin (ERM) proteins, and this interaction is facilitated by the presence of PtdIns(4,5)P_2 (27). ERM proteins function as plasma membrane–actin cytoskeleton linkers and may also regulate signal propagation. ERM proteins are tyrosine-phosphorylated in response to stimulation by growth factors, and tyrosine-phosphorylated ERM proteins recruit and activate Src homology 2 (SH2)-containing kinases such as phosphatidylinositol 3-kinase by binding to their SH2 domain (24). Thus, the interaction of ICAM-1 with PtdIns(4,5)P_2 may result in binding to the ERM proteins, which in turn recruit additional signaling molecules. Upon ICAM-1 crosslinking, ICAM-1 clusters indeed colocalize with the ERM proteins (56).

In addition, in response to ligation by fibrinogen, ICAM-1 becomes tyrosine-phosphorylated (most likely at Y^{485} in the cytoplasmic domain), possibly through activation of $pp60^{Src}$, and tyrosine-phosphorylated ICAM-1 binds to the SH2-containing tyrosine phosphatase-2 (SHP-2) (43). A dominant negative form of SHP-2 inhibits activation of Ras and MAPK induced by growth factors (5, 11). Thus, tyrosine phosphorylation of ICAM-1 and recruitment of SHP-2 may be yet another mechanism through which ICAM-1-induced signaling events are initiated.

THE PHYSIOLOGICAL SIGNIFICANCE OF ICAM-1 SIGNALING

What is the physiological role of ICAM-1-dependent outside-in signaling in mediating neutrophil migration on EC surface and transmigration across ECs? A recent study by Sans *et al.* demonstrates that deletion of the ICAM-1 cytoplasmic domain completely inhibits neutrophil transmigration, but not adhesion, in a reconstituted cell line, suggesting a role for ICAM-1 signaling in neutrophil migration (45).

The role of ICAM-1 signaling is also supported by studies examining neutrophil migration on EC surface. ICAM-1-dependent changes in ECs induced by neutrophil adhesion are accompanied by the crawling of neutrophils to EC borders (54). This migration is reduced when ECs are pretreated with SB203580, a p38 inhibitor that also inhibits the cytoskeletal changes and the stiffening of ECs (54). These studies suggest that ICAM-1-dependent activation of p38 MAPK and its downstream events may regulate neutrophil migration on EC surface toward the junctions, where transmigration occurs. How neutrophil-induced signaling pathways in ECs, including p38 activation, may influence neutrophil migration on EC surface is unknown. These signaling events may: (a) induce redistribution of ICAM-1 on EC surface and association of ICAM-1 with cytoskeletal proteins such as the ERM proteins, which in turn affect neutrophil adhesion and/or migration; (b) influence the characteristics of EC surface on which neutrophils crawl; (c) induce increases in EC stiffness, which may enhance neutrophil migration to EC borders, because changes in the substrate rigidity alone are sufficient to alter cell adherence and locomotion as demonstrated by a study using cultured fibroblasts (40); and (d) alter the junctional functions in ECs that may regulate neutrophil emigration during inflammation.

CONCLUSIONS

Accumulating evidence demonstrates that neutrophil-EC adhesion induces signaling events in both neutrophils and ECs. In ECs, these signaling events occur, at least in part, as a result of ligation of EC adhesion molecules, which function as signal transducers. As a result, oxidant production, changes in the EC actin cytoskeleton, and transcription of genes occur. We are beginning to understand the signaling events induced during adhesion and the physiological significance of these events. Understanding how these signaling events may influence neutrophil crawling on EC surface and transmigration across ECs will further our understanding of the mechanisms regulating neutrophil emigration during inflammatory responses.

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ABBREVIATIONS

ECs, endothelial cells; ERK, extracellular signal-regulated kinase; ERM, ezrin/radixin/moesin; fMLP, formyl-methionyl-leucyl-phenylalanine; Hsp27, heat shock protein 27; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; MAPK, mitogen-activated protein kinase; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; ROS, reactive oxygen species; SH2, Src homology 2; SHP-2, SH2-containing tyrosine phosphatase; VCAM-1, vascular cell adhesion molecule-1.

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