Signaling in Leukocyte Transendothelial Migration

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Abstract—Under a variety of (patho) physiological conditions, leukocytes will leave the bloodstream by crossing the endothelial monolayer that lines the vessels and migrate into the underlying tissues. It is now clear that the process of extravasation involves a range of adhesion molecules on both leukocytes and endothelial cells, as well as extensive intracellular signaling that drives adhesion and chemotaxis on the one hand and controls a transient modulation of endothelial integrity on the other. We review here the current knowledge of the intracellular signaling pathways that are activated in the context of transendothelial migration in leukocytes and in endothelial cells. In leukocytes, polarization of receptors and of the signaling machinery is of key importance to drive adhesion and directional migration. Subsequent adhesion-induced signaling in endothelial cells, mediated by Rho-like GTPases and reactive oxygen species, induces a transient and focal loss of endothelial cell–cell adhesion to allow transmigration of the leukocyte. This review underscores the notion that we have just scratched the surface in revealing the complexity of the signaling that controls leukocyte transendothelial migration. (Arterioscler Thromb Vasc Biol. 2004;24:1-11.)

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The migratory properties of white blood cells are indispensable to drive immune responses throughout the body. To ensure migration to the proper locations, the trafficking of leukocytes is tightly regulated. The migration and extravasation of leukocytes across the endothelium that lines the vessel wall occurs in several distinct steps, referred to as the multi-step paradigm, originally introduced by Butcher1 and extended by Springer.2 The first step comprises the rolling of the leukocytes over the endothelial cells, mediated by transient weak interactions between adhesion molecules. Subsequently, loosely attached leukocytes are in such close proximity of the endothelium that they can be activated by chemotactic cytokines, presented on the apical surface of the endothelium. As a consequence, the activated leukocytes will spread and firmly adhere to the endothelium and finally migrate through the intercellular clefts between the endothelial cells to the underlying tissue. An additional level of complexity has been added by work that showed that in addition to chemokines, shear stress can also act as a transmigration-promoting stimulus.3 Figure 1 shows a schematic overview of the different stages that occur during transendothelial migration (TEM) of leukocytes.

The extravasation of leukocytes is essential for many (patho) physiological processes, including migration of T-lymphocytes for immune surveillance, recruitment of activated lymphocytes and granulocytes during acute and chronic inflammatory responses, and homing and mobilization of hematopoietic progenitor cells. In the past decade, knowledge of the molecules and the signaling events, both in the leukocytes and in the endothelial cells, that control transendothelial migration of leukocytes has increased significantly and is discussed here. We have divided this overview into 3 parts: the first part deals with chemokine-induced leukocyte activation; the second part describes the signaling events that occur in endothelial cells after leukocyte adhesion; and the final part discusses our current knowledge about the role of the endothelial cell–cell junctions in the final stage of the transmigration process.

Chemokine-Induced Leukocyte Activation

The migration of leukocytes across the endothelium is mainly driven by a large family of extracellular ligands: the chemotactic cytokines, better known as chemokines. Chemokines are small (8 to 14 kDa) structurally related molecules that interact with 7-transmembrane-spanning G-protein–coupled receptors and regulate leukocyte trafficking.4 Today, >40 different chemokines have been identified, and this number is still growing. The nomenclature of chemokines is based on the arrangement of the N-terminal cysteine residues. These residues can be adjacent (CC chemokines) or have one or more extra amino acids in between them (CXC or CX3C chemokines). Two chemokines have only one cysteine at the N-terminus, ie, lymphotactin/SCM-1α and SCM-1β. In the current nomenclature, an “L” (as in CXCL12) refers to a ligand, whereas an “R” (as in CXCR4) refers to a chemokine receptor.5 The main topics of this review are discussed with reference to stromal cell-derived factor-1 (SDF-1 or, according to the current nomenclature, CXCL12), because SDF-1/CXCL12 is involved in the migration of many, if not all,
different types of leukocytes and various types of tumor cells and is expressed in virtually all human tissues.

Chemokines and their receptors are one of the many levels that coordinate the migration of leukocytes and leukocyte subsets at various levels, resulting in a tightly controlled and very complex system of cell trafficking. Various studies have shown that leukocyte transendothelial migration is induced by soluble chemokine gradients in vitro. However, it is unlikely that soluble chemokine gradients persist in the bloodstream, because the gradient will be rapidly washed away. It is therefore generally accepted that leukocytes respond to chemokines that are immobilized on the surface of the endothelium, which is underscored by detailed studies showing that chemokines, such as IL-8/CXCL8 and SDF-1/CXCL12, are indeed present on the vascular endothelium in vivo. These immobilized chemokines are presented to nearby rolling leukocytes by heparin-sulfate proteoglycans, highly glycosylated proteins that are expressed on the surface of endothelial cells. Middleton et al have shown that IL-8, a potent chemokine for neutrophils, is presented on the microvillous-like extensions on the luminal surface of the endothelium. Moreover, these investigators showed that after injection of IL-8 into the skin of a rabbit, IL-8 is transported through the endothelial cells from the basolateral side to the luminal side.

For SDF-1/CXCL12, it has been shown extensively that immobilization of this chemokine leads to increased adhesion and TEM of leukocytes under physiological flow. Chemokine Receptors

Chemokines transmit their pro-migratory signals through G-protein–coupled, 7-times-spanning membrane receptors. These receptors initiate adhesion and motility via (pertussis toxin-sensitive) G-proteins, leading to integrin activation via inside-out signaling, followed by coordinated actin polymerization, spreading at the leading edge of the cell and contraction at the back. Directional migration of leukocytes is accompanied by polarization of the cell body, of the actin and tubulin cytoskeleton, and of a wide range of intracellular signaling proteins, such as PI-3K, PTEN, Rho-like GTPases, and Gβγ-subunits. Their differential distribution mediates the amplification of the chemokine gradient on the outside into a (steep) signaling gradient inside the cell. Concomitant polarization of the chemokine receptors at the cell surface is subject to conflicting reports. Studies with C5a-receptor–GFP fusion proteins in neutrophils or cAMP-receptor–GFP fusion proteins in Dictyostelium cells showed uniform distribution of these receptors over the plasma membrane in cells that migrate toward chemotactants. Other (chemokine) receptors, such as CCR2 and CCR5 on T-lymphocytes and the fMLP receptor on neutrophils, are distributed to the leading edge on exposure to their cognate ligands. In B-lymphocytes, SDF-1 induces polarization of CXCR4 to the leading edge of the cell. In addition, it has been reported that SDF-1/CXCL12 can bind to fibronectin and subsequently induces a polarized distribution of CXCR4 on adherent T-lymphocytes. We recently showed that immobilized SDF-1 on TNFα-activated endothelium rapidly induces polarization of CXCR4 to the leading edge of the migrating cell and that CXCR4 subsequently co-localizes with cholesterol lipid rafts (Figure 2A). Disruption of lipid rafts by cyclo-dextrin inhibited SDF-1/CXCL12-induced CXCR4 internalization and cell migration, indicating that internalization and proper SDF-1–mediated signaling requires the presence of lipid rafts.

Adhesion Molecules

In the first phase of extravasation, leukocytes roll over the endothelium, a process that is mediated by a family of adhesion molecules called selectins. Recently, it became

Figure 1. Schematic overview of the distinct steps that comprise leukocyte transendothelial migration. The first step (I) represents the rolling of leukocytes and involves adhesion molecules such as selectins on the leukocytes or endothelial cells and their ligands on the cellular counterparts. The second step (II) represents firm adhesion, mediated through immobilized chemoattractants on the endothelium and adhesion molecules such as integrins on the leukocytes and CAMs on the endothelium. The third step (III) represents the diapedesis of the leukocytes through the endothelial cell–cell junctions, in which homophilic binding molecules such as the JAMs, PECAM-1, CD99, and VE-cadherin are involved.
clear that stimulation of L-selectin by antibody-specific cross-linking induced CXCR4 expression in lymphocytes. Moreover, these authors showed that CXCR4 is stored in intracellular vesicles and found that L-selectin, just as CXCR4, localizes to lipid rafts. These findings suggest that selectin-mediated rolling of leukocytes over the endothelium already primes the leukocytes to target CXCR4 to lipid rafts. In addition, Shamri et al showed that the integrin VLA-4 (H9251 H9252 4/1) requires cholesterol rafts for adhesive activity but that leukocyte function-associated antigen-1 (LFA-1; H9251 H9252 L2) acts independent from lipid rafts to mediate adhesion. These data suggest the presence of independent clusters of adhesion molecules on the plasma membrane at the leading edge of migrating leukocytes, before chemokine stimulation (Figure 2A).

Integrins play a major role in TEM of leukocytes and are, in fact, indispensable for proper extravasation. The main integrins that regulate SDF-1-induced adhesion and spreading of leukocytes are LFA-1 (H9251 H9252 α1β2), Mac-1 (H9251 H9252 α6β3), VLA-4 (H9251 H9252 α2β1), and VLA-5 (H9251 H9252 α5β1).37,38 LFA-1, Mac-1, and VLA-4 bind to the adhesion molecules intercellular cell adhesion molecule-1 (ICAM-1) and ICAM-2 (for LFA-1 and Mac-1), and vascular cell adhesion molecule-1 (VCAM-1) (for VLA-4), present on the apical surface of the endothelium. For the integrins VLA-4 and VLA-5, it has been shown that they bind to fibronectin. Studies in mice that lack β2-integrins or studies that used blocking antibodies against β2-integrins showed that neutrophil traffic was dependent on this integrin. Similarly, mice that lacked the α4-integrin showed impaired T-lymphocyte homing to Peyer patches, although not to other secondary lymphoid organs. In vivo studies with blocking antibodies to the α4-integrin VLA-4 also showed that this integrin was involved in migration of CD34+ cells. These data point to a prominent role for these integrins in the control of trafficking of leukocytes in general.

Figure 2. A. Schematic overview of the signaling pathways at the leading edge of a migrating cell. A leukocyte adheres through L-selectin to cellular adhesion molecules (CAM) or glycoproteins such as CD34 on the endothelial cells. L-selectin induces CXCR4 upregulation from intracellular granules on the leukocyte surface, resulting in increased activation of Rap1. Rap1 induces α-integrin activation, possibly through phosphorylation. These actions on the leukocyte surface appear to take place in a lipid raft. B. Schematic overview of the signaling pathways at the trailing edge of a migrating cell. An adhered leukocyte moves forward by leading-edge protrusion and retraction of its tail. Therefore, αβ2-integrin activates through Vav1, RhoA, which subsequently activates the kinase p160ROCK. ROCK activation leads, through MLCK activation, to MLC phosphorylation, resulting in retraction of the actin cytoskeleton.

Binding of the integrins to their ligands induces multiple signaling cascades in the leukocytes. This outside-in signaling is translated by the leukocyte into cell polarization and forward movement. This movement is generally assumed to be driven by the combination of leading edge protrusion and contractility at both the front and the back of the cell, which allows displacement of the cell body. Of these studies have used Dictyostelium discoidea amoebae as a model, because the ability to sense and to respond to extracellular signals is remarkably similar to that of mammalian leukocytes. A large number of structural and regulatory proteins are located at the leading edge of the migrating cell, including proteins that regulate the dynamics of the actin cytoskeleton, such as the small GTPase Rac1, its effector PAK1, and the Arp2/3 complex. Also, focal adhesion and focal contact proteins such as adapter proteins, tyrosine kinases, and integrins are found at the leading edge. These studies mainly focused on fibroblasts; therefore, one should take in account that different cell types might use different signaling pathways to achieve the same goals.
At the back of the migrating cell, proteins such as ICAM-3, PSGL-1, CD43, and CD44 are localized. ERM proteins act as adapter proteins and link these transmembrane proteins to the actin cytoskeleton. To move forward, cells have to form new adhesion sites and break-down old ones, both in the protruding and in the retracting part of the cell. The turnover of focal adhesions is very complex and involves integrins and microtubules. Because of space limitations, we cannot discuss the complex mechanisms that drive of focal adhesion turnover. Recently, it has been shown that the $\alpha_l$-integrin is phosphorylated on serine residues at the leading edge of polarized cells and is required for lamellipodia stability, although $\alpha_l$ is also present at the trailing edge. In addition, these authors showed that paxillin, a protein present in focal adhesions, co-localizes with $\alpha_l$ at the trailing edge but not at the leading edge with the phosphorylated $\alpha_l$. Moreover, enforced association of paxillin with $\alpha_4$ inhibits migration, indicating that the $\alpha_l$-paxillin interaction mediates attachment and detachment of membrane protrusions, which is required for migrating cells to move forward.

Next to the $\alpha_l$-integrin, LFA-1 ($\alpha_l\beta_2$) signaling is required for proper leukocyte migration. Whereas the $\alpha_l$-integrin uses paxillin to regulate the attachment and detachment of the cells’ leading edge, LFA-1 seems to signal through myosin light chain kinase and through p160ROCK, which regulates cell contraction and detachment from the matrix (Figure 2B). Moreover, LFA-1 engagement induces actin polymerization at the leading edge of the cell, possibly through the Rho family exchange factor Vav1. Thus, both $\alpha_4$ integrins and LFA-1 are required for leukocyte migration, acting through distinct signaling cascades to drive efficient chemotaxis.

### Small GTPases

As is well known from studies in fibroblasts as well as in leukocytes, Rho-like small GTPases (in particular Rho, Rac, and Cdc42) are crucial for coordinated cell spreading, migration, and signaling. Rac1, being a critical regulator of cell spreading, actin polymerization, and membrane ruffling in any type of cell, is found throughout the cytoplasm under resting conditions. However, in its active, GTP-bound form, Rac1 localizes to growth factor-induced membrane ruffles and to the leading edge of migrating cells. These authors showed additionally that $\beta_2$-integrins induce the interaction of active Rac1 with its downstream effectors, such as PAK1. Using C-terminal cell-permeable peptides as selective inhibitors, we recently showed that Rac1 (and not Rac2 or CDC42) plays an important role in SDF-1-induced actin polymerization in HL-60 cells.

Next to the involvement of Rac1 in driving membrane protrusion, the small GTPase Rap1 was shown to be involved in the control of the adhesive capacities of LFA-1 and VLA-4. Moreover, Rap1 can be activated by SDF-1, and in its active form induces cell polarization. In addition, active Rap1 promotes transendothelial migration of T-lymphocytes. Recently, RAPL, a Rap1-binding protein, was shown to associate with Rap1 after SDF-1 stimulation. RAPL induces polarization of T-lymphocytes and distribution of LFA-1 to the leading edge of the cell. These data suggest that Rap1 can signal between chemokine-activated G-protein–coupled receptors, such as CXCR4, and integrin activation (Figure 2A).

As described, lamellipodia and membrane protrusions at the front of the cell are required to move the cell forward. Consequently, the rear of the cell has to contract and to detach from the underlying matrix, ie, from the endothelial monolayer. RhoA and one of its effectors, the serine/threonine kinase p160ROCK, have been found to mediate tail retraction of migrating leukocytes. Inhibition of RhoA activity by C3 transferase in monocytes prevents migration through an endothelial monolayer, although overall attachment of monocytes to activated endothelial monolayers was not affected. Interestingly, the adhesion to either ICAM-1 or VCAM-1 was largely promoted on inhibition of p160ROCK. In addition, the $\beta_2$-integrin mislocalized in these cells from the leading edge to the trailing edge, indicating that RhoA and p160ROCK signaling mediates integrin localization at the leading edge. The results suggest a model in which RhoA activity is inhibited at the front of the cell to allow the induction of protrusions, in concert with enhanced Rac1 activity at the leading edge. Conversely, RhoA is activated at the rear of the cell to retract and detach the uropod and Rac1 may become inhibited at sites of tail retraction. The events that occur in chemokine-mediated leukocytes and are involved in migration are summarized in Figure 2A and 2B.

### Consequences of Leukocyte–Endothelium Interactions

Huang et al were the first to show that intracellular calcium levels in endothelial cells were increased on adhesion and transmigration of neutrophils. Moreover, they showed that this was also required for efficient TEM. In addition, Hixenbaugh et al showed that chemoattractant-stimulated neutrophils induce an increase in the phosphorylation of myosin light chain (MLC) in endothelial cells. Increased MLC phosphorylation accompanies RhoA activation, subsequent cell contraction, and a loss of endothelial cell–cell junctions, which then leads to increased permeability of the endothelial barrier. Using an inhibitor to MLC kinase (MLCK) on endothelial cells, Saito et al showed that transmigration of neutrophils was blocked and MLC phosphorylation was inhibited. Adhesion molecules such as P-selectin and E-selectin and VCAM-1 on the endothelium might be responsible for transmitting the signals, induced by adhered neutrophils into the endothelium, because activation of these adhesion molecules through antibody-specific cross-linking results in a transient increase of intracellular calcium. These studies put forward the notion that leukocyte adhesion affects the endothelial cells in a specific fashion and that endothelial cell function contributes importantly to TEM. Today, the involvement of endothelial signaling in transmigration of leukocytes is an accepted phenomenon, and various signaling events in endothelial cells have been implicated in the process of leukocyte TEM, although the exact mechanisms by which the endothelial cells facilitate the passage of leukocytes is still not entirely clear.

As noted, leukocytes use mainly $\beta_1$ (VLA-4/$\alpha_4\beta_1$) and $\beta_2$ (LFA-1/$\alpha_l\beta_2$) integrins to firmly adhere to the endothelium.
The ligands for these integrins on the endothelium are VCAM-1 and ICAM-1, respectively. More recently, a novel family of integrin ligands, called junctional adhesion molecules (JAM), has been discovered.83 The role of JAMs in transendothelial migration will be discussed. Signaling through ICAM-1, induced by antibody-mediated cross-linking, activates RhoA.84 Moreover, other proteins that regulate the actin cytoskeleton, such as FAK, Cas, and cortactin, are phosphorylated on tyrosine on antibody-induced cross-linking of ICAM-1.85 Thus, engagement of ICAM-1 induces signaling in endothelial cells. However, one should take into account that receptor cross-linking is a rather global stimulus compared with leukocyte binding to the endothelium. The short (28 amino acids) intracellular tail of ICAM-1 interacts with ezrin, an adapter protein that links ICAM-1 with the actin cytoskeleton.86 Ezrin is a member of the ERM protein family and plays an important role in lamellipodia induction at the leading edge of fibroblasts.87,88 Moreover, it appears that ezrin links the proteoglycan syndecan-2, which might possibly bind SDF-1, to the actin cytoskeleton.89 Detailed analysis of monocytes adherent to activated endothelium or cross-linking of the adhesion receptors ICAM-1 and VCAM-1 showed clustering of the ERM proteins near those sites of activation.90 Next to its function as an adapter protein, there is evidence that shows ezrin to act as a signaling molecule, because active ezrin is reported to be responsible for the formation of lamellipodia.91 In addition, these authors showed that Rac1, but not RhoA or Cdc42, is activated in cells that express the active form of ezrin. Other previous work suggested that the ERM proteins are rapidly phosphorylated in a Rho-dependent manner.92 Barreiro et al recently showed that the endothelial cells form a so-called docking structure to “catch” the migrating leukocyte, and they subsequently showed that moesin and ezrin localized to those docking structures, together with ICAM-1 and VCAM-1.93

We recently showed that clustering of VCAM-1 activates Rac1,94 Inhibition of downstream signaling by Rac1 with an inhibitory peptide, encoding the effector loop of Rac1 (aa17-32,95), reduced the migration of leukocytes across endothelial monolayers.94 Because transduction of an active mutant of Rac1, RacV12, reduces endothelial cell–cell contacts,96–98 blocking Rac activity might prevent opening of endothelial cell–cell contacts, thereby preventing leukocytes from migrating. Moreover, ligation of VCAM-1 activates phosphatidylinositol 3-kinase (PI3-K), implicated as an activator of Rac1.99,100

Studies performed by our group already showed that inhibition of endothelial RhoA by C3 transferase decreases monocyte migration across human umbilical vein endothelial cells, although the C3 pretreatment had little or no effect on the migration of primary CD34+ cells across bone marrow endothelial cells.12,94 Also, inhibition of p160ROCK signaling in endothelial cells by Y-27632 decreases not only migration of lymphocytes across endothelium but also adhesion.93 The latter results indicate that RhoA activity is linked to adhesion molecules on the endothelium and that blockade of RhoA activity or downstream effectors prevents leukocyte adhesion as well as endothelial cell contraction and subsequent loss of cell–cell contacts. Thus, blocking signaling through Rac1 or RhoA in endothelial cells inhibits transendothelial migration of monocytes. It is therefore likely that both RhoA and Rac1 in endothelial cells are involved in leukocyte-induced endothelial signaling: RhoA through ligation of ICAM-1 and Rac1 through the ligation of VCAM-1.

For many years, it has been known that reactive oxygen species (ROS) play an important role in the maintenance of the endothelial monolayer integrity.101,102 The generated ROS are spontaneously or enzymatically converted to hydrogen peroxide (H2O2), which may disrupt the integrity of the endothelial monolayer, possibly through inhibition of tyrosine phosphatases.103,104 Some studies already reported the presence of components of the neutrophil-NADPH-oxidase (gp91) complex in endothelial cells.105,106 Other studies suggested the expression of various NADPH-oxidase homologues in endothelial cells.107 Upregulation of Nox-based NADPH-oxidases after vascular damage have been reported.108,109 Nevertheless, how the activity or intracellular localization of these protein complexes is regulated remains unclear and warrants further study. Our group has previously shown that active Rac1 induces ROS production in endothelial cells and that endothelial ROS are actively involved in TEM, because leukocyte migration across endothelium is inhibited when the endothelial monolayer is pretreated with oxygen-radical scavengers.97 Similar findings have been reported by Wang and Doerschuk, who showed that engagement of ICAM-1 induces ROS production in endothelial cells.110 In line with these findings, we and others have found that activation of VCAM-1 and PECAM-1, by antibody-mediated cross-linking, results in increased levels of ROS production.12,111,112 These findings suggest that leukocyte adhesion to endothelium induces ROS production, which in turn might transmit signals into the endothelium to facilitate leukocyte passage.

Activation of ICAM-1, by cross-linking, remodels the actin cytoskeleton through activation of p38 mitogen-activated protein kinase.113 Also, VCAM-1 is able to activate p38 mitogen-activated protein kinase in a ROS-dependent manner.93 Extracellular signal-regulated kinase-2, which is also involved in motility and in proliferation, is also activated by VCAM-1.99 Lorenzon et al showed previously that VCAM-1 can act as a signaling receptor.80 In their studies, VCAM-1 cross-linking was found to induce a transient increase in endothelial calcium concentration, which is required for efficient TEM.

These data suggest that engagement of ICAM-1 and VCAM-1, through the integrin-mediated adhesion of leukocytes, induces intracellular signaling in the endothelial cells that finally results in the opening of the endothelial cell-to-cell junctions. This hypothesis is supported by studies that showed that active Rac1 induces loss of cell-to-cell adhesion in endothelial cells.96–98 This contrasts with epithelial cells, in which active Rac1 promotes cadherin-based cell-to-cell adhesion.114 Although a lot is known about epithelial regulation of cell-to-cell junctions, relatively little is known about the control of endothelial cell-to-cell junctions with respect to the opening and resealing of these junctions during the passage of
leukocytes. The last part of this review discusses our current knowledge about this topic.

**Endothelial Cell-to-Cell Junctions in Leukocyte Transendothelial Migration**

Leukocytes need to migrate through the endothelial cell–cell junctions to cross the endothelial monolayer in the final stage of extravasation.\(^2\) To successfully cross the junctions, the leukocytes have to bypass or block the adhesion, which is mediated by junctional proteins. The endothelial cell-to-cell junctions contain a large number of (adhesion) proteins, including vascular endothelial (VE)-cadherin (CD144, cadherin-5), a homophilic calcium-dependent transmembrane adhesion molecule, which is localized at the adherens junctions. In addition, the endothelial junctions comprise tight junction proteins, eg, occludin, JAM family members, and claudins, as well as proteins such as CD99 and PECAM-1 (CD31), which are more diffusely distributed at the cell–cell junctions.

Many studies have described PECAM-1 as an important player in the process of transendothelial migration based on the effects of blocking PECAM-1 in vitro\(^115–118\) and in vivo.\(^119–127\) However, PECAM-1 knockout mice do not show a significant loss of inflammatory response, demonstrating that PECAM-1 is not indispensable for transmigration.\(^128\) The mechanisms that compensate for the loss of PECAM-1 are not clear; however, Mamdouh et al recently found that in the absence of leukocytes, PECAM-1 is present in a compartment directly under the junctional border membrane.\(^129\) These investigators suggest a new mechanism in which passing leukocytes trigger these compartments to fuse with the junctional border membrane. Because PECAM-1 is present on the leukocyte as well as on the endothelium, this mechanism might facilitate leukocytes to cross the endothelial cell-to-cell junction through homophilic PECAM-1 binding.\(^129\) This mechanism also implies that leukocytes could be engulfed by endothelial cells, for instance, through the aforementioned docking structure.\(^93\) Intriguingly, it has been described that neutrophils are also able to cross the endothelial layer by a transcellular route, particularly in vivo.\(^130\) How this process is controlled is unknown.

PECAM-1 is phosphorylated on tyrosine residues after endothelial cell stimulation by mechanical force and shear stress by means of the Fer tyrosine kinase, which is localized on microtubules.\(^131,132\) Moreover, Ilan et al showed that PECAM-1 can function as a reservoir for tyrosine-phosphorylated β-catenin.\(^133\) These data suggest that PECAM-1 binding to β-catenin prevents cytosolic β-catenin from degradation by the proteosomes. Subsequently, PECAM-1 engagement induces SHP-2 phosphorylation through Fer, which then dephosphorylates PECAM-1–bound β-catenin. It has been shown that increased tyrosine phosphorylation of junctional proteins leads to loss of cell-to-cell adhesion and that subconfluent endothelial monolayers have increased levels of tyrosine phosphorylation of the junctional proteins VE-cadherin, β-catenin, and γ-catenin.\(^134,135\) Moreover, SHP-2 and β-catenin are both present in the VE-cadherin complex at cell-to-cell junctions.\(^136\) In addition, inhibition of protein tyrosine phosphatase activity promotes the flux of albumin across an endothelial monolayer and the extravasation of neutrophils, indicating that phosphorylation plays an essential role in the control of endothelial integrity and of the process of TEM.\(^137\) Phosphatase activity can be inhibited by H\(_2\)O\(_2\), because H\(_2\)O\(_2\) mediates the oxidation of critical cysteine residues in tyrosine phosphatases.\(^138\) As a result, tyrosine phosphorylation levels are increased, triggering loss of endothelial cell-to-cell junctions. This suggestion links VCAM-1–induced ROS production and phosphatase/kinase activity to the transendothelial migration of leukocytes.

Another family of junctional proteins that has attracted a lot of attention are the JAMs. Recently, new nomenclature for the JAM family has been put forward by Muller and is used in this review.\(^139\) JAM-A has been cloned and identified recently and appears to mediate homophilic and heterophilic interactions.\(^140\) JAM localizes at the apical regions of cell–cell junctions, not only of endothelial cells but also of epithelial cells, and regulates the electrical resistance across monolayers of these cells.\(^141,142\) Antibodies to JAM cause decreased transendothelial migration of leukocytes in vitro and in vivo, strongly suggesting that this molecule might be involved in TEM.\(^140,143\) Ostermann et al have shown that JAM-A on endothelial cells is a ligand for the β2-integrin LFA-1, and as such is involved in transendothelial migration of leukocytes.\(^144\) The JAM protein family now comprises 3 members: JAM-A (approximately 32 kDa), present on endothelial cells, epithelial cells, and on all leukocytes, functions as a regulator of TEM and of electrical resistance. JAM-B (or VE-JAM), which is ~40 kDa in size, is expressed on vascular and lymphatic endothelium and has been proposed to play a role in lymphocyte homing.\(^145,146\) JAM-B also interacts with the β1-integrin receptor VLA-4.\(^147\) Most recently, a third family member has been identified as JAM-C (~43 kDa), present on endothelial cells, T-lymphocytes, platelets, and NK-cells.\(^147,148\) JAM-C also functions as a regulator of TEM of leukocytes and electrical resistance. It interacts with JAM-B and Mac-1 (αvβ3-integrin, present on leukocytes) but not with LFA-1 or VLA-4.\(^149,150\) These findings indicate that all 3 JAM family members could potentially be involved in leukocyte transendothelial migration but that they are independently involved in specific parts of the TEM process.\(^83\)

The integral transmembrane protein CD99 regulates VLA-4–dependent adhesion of T-lymphocytes to endothelial cells under physiological shear stress.\(^151\) In contrast to VLA-4, β3-integrins (ie, LFA-1) were not influenced by CD99 stimulation. Recently, CD99 was found to be present on endothelial cells as a homophilic binding molecule at cell-to-cell junctions.\(^152\) Antibodies to CD99 were found to block transmigration of monocytes, particularly during diapedesis. Blocking antibodies to PECAM-1 inhibit monocyte migration before diapedesis started, indicative for a sequential engagement of these proteins during transmigration across the junction.\(^152\) Thus, whereas JAM and PECAM-1 are involved in the first stages of transmigration across endothelium, CD99 seems to regulate further passage through the endothelial junctions. A note should be made that, as mentioned, PECAM-1 is required for migration through the basement membrane but is apparently also involved in early steps of diapedesis.
Previous reports have shown that adhesion of neutrophils to endothelium disrupts the VE-cadherin complex at cell–cell junctions. However, it was subsequently shown that preparation of cell lysates under relatively mild conditions allows neutrophil proteases to cause breakdown of the VE-cadherin-catenin complex. However, the latter study by Moll et al did not indicate whether proteases are involved in the physiological process; it only clarified that such experiments could not demonstrate regulated proteolysis of VE-cadherin on neutrophil attachment. Nevertheless, it has been observed with GFP fusion proteins and live-cell imaging that VE-cadherin and the associated catenins are indeed not localized from cell–cell junctions on passage of monocytes or CD34/H11001 cells through the endothelium. Recent evidence showed that elastase, a protease present in neutrophil granules and released on activation, is able to degrade the extracellular part of VE-cadherin, keeping the hypothesis alive that neutrophils disrupt endothelial cell–cell junctions by releasing elastase and possibly other proteases to migrate through the endothelial cleft. However, studies with neutrophils from elastase-deficient or MMP-9–deficient mice have shown that these proteases are not required for rolling over, adhesion to, or migration across endothelium, although these neutrophils were still able to disrupt VE-cadherin function. Together, these studies indicate that proteases might be involved in transendothelial migration, but that the role for elastase and MMP-9 seems to be limited. Figure 3 summarizes schematically the endothelial signaling that accompanies and drives TEM.

**Conclusions**

Little more than one decade since the seminal studies that started this field of research, it has become increasingly clear that the emigration of leukocytes from the bloodstream involves complex signaling in the migrating leukocyte and in the endothelium. This signaling needs to be carefully controlled, not only to ensure proper and efficient extravasation but also to limit leukocyte transendothelial migration to those sites where the cells are required. Mislocalized or excessive leukocyte exit damages the endothelium and underlying tissues. Therefore, leukocyte migration and adhesion represent important areas for drug targeting. This is even more underscored by the realization that tumor cell metastasis resembles leukocyte TEM and also that some tumor cells even rely on specific chemokines, such as SDF-1, for their spreading through the body. The events that take place in the endothelium and accompany or even allow leukocyte diapedesis have everything to do with control of endothelial integrity. Given the essential role of the endothelium and its barrier function for normal physiology, detailed knowledge of the intracellular signaling and adhesion molecules involved is of key importance. The unique interactions between activated leukocytes and endothelial cells will likely, despite their complexity, help us to understand the interplay between cell–surface molecules, intracellular signaling cascades, and the control of motility and cell–cell adhesion.

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