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Endothelial signaling in paracellular and transcellular leukocyte transmigration

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Abstract

As the primary physical barrier between blood and tissue compartments within the body, blood vessel endothelial cells and integrity of the cell junctions connecting them must be carefully regulated to support leukocyte transendothelial migration only when necessary. Leukocytes utilize two independent routes across the endothelium: the paracellular route involves migration in-between adjacent endothelial cells and requires the transient disassembly of endothelial cell junctions, while the transcellular route occurs directly through an individual endothelial cell, likely requiring the formation of a channel or pore. In this review, I will first summarize the signaling events that are transduced by leukocyte engagement of endothelial cell-surface receptors like ICAM-1 and VCAM-1. Some of these signals include activation of GTPases, production of reactive oxygen species, and phosphorylation of target proteins. These signaling pathways converge to cause junctional disruption, cytoskeletal remodeling, and/or the membrane fusion events that are associated with leukocyte transendothelial migration. The review will conclude with a detailed discussion of the newly characterized transmigratory cup structure, and the recent advances made towards understanding the mechanisms of transcellular transendothelial migration.

Keywords

Endothelial cell; Transendothelial migration; Paracellular; Transcellular; Reactive oxygen species; GTPase; Cytoskeleton; Cell junctions; Transmigratory cup structures; Podosomes; Review

2. INTRODUCTION

Leukocyte transendothelial migration (TEM) is a vital physiological process that occurs during both the adaptive and innate immune response and during routine immune surveillance and homing. During TEM, leukocytes are stimulated to exit the bloodstream and enter lymphoid organs and infected tissues. Endothelial cells (ECs) lining the blood vessels are the primary barrier between the blood circulation and underlying tissue that leukocytes must navigate during TEM. Tight regulation of this process is critical, because if

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TEM occurs excessively or at inappropriate locations, it can cause inflammatory disorders such as atherosclerosis, chronic inflammation, multiple sclerosis, and rheumatoid arthritis. As such, research towards a better understanding of how this process is regulated will enable design of potential therapies for many of these pathological situations.

Leukocyte TEM has been described as a sequential set of events, each of which facilitates progression to the next stage. The original leukocyte adhesion multistep model comprised of three steps: 1. rolling adhesion, mediated by selectins, 2. activation, triggered by chemokine signals, and 3. firm adhesion/arrest, mediated by integrins (1, 2). TEM is added as the fourth and last step, concluding a complete inflammation cascade (Figure 1). During the initial rolling adhesion step, leukocytes become loosely tethered to the blood vessel wall due to transient weak interactions between selectins on activated ECs and leukocyte carbohydrate ligands. This step is thought to slow leukocyte passage through the blood vessel, allowing binding to ECs and exposure to a local environment that facilitates progression to the second step, activation. Local production of cytokines such as TNF- α by leukocytes serves a dual purpose: initiating a positive feedback loop resulting in further production of more cytokines, as well as causing upregulated expression of EC-leukocyte adhesion molecules such as ICAM and VCAM on the EC surface. Furthermore, production and display of specific chemokines (i.e. interleukin-8, MCP-1, SDF-1) by ECs attracts leukocytes and causes them to migrate towards the site of infection. Binding of these chemokines to leukocyte heterotrimeric G-protein coupled receptors results in rapid activation of beta-1 and beta-2 integrin activation. The third step in the adhesion cascade, firm adhesion, is promoted by this increased affinity of leukocyte integrins $\alpha 4\beta 1$ (VLA-4), $\alpha L\beta 2$ (LFA-2, CD11a/CD18), and $\alpha M\beta 2$ (Mac-1, CD11b/CD18) for EC Ig-family adhesion molecules such as the previously mentioned VCAM and ICAM. Engagement and clustering of these EC cell adhesion molecules is required in turn to initiate signals within the EC which ultimately trigger diapedesis. It is now widely appreciated that ECs are active participants during these later processes (3, 4). The focus of this review will be on the transendothelial migration stage, and how signaling within the ECs influences this process.

The multistage paradigm has undergone some additional refinements recently (5). For example, the TEM stage can be broken down into several more discrete steps occurring both before and during the actual diapedesis process. Additional steps prior to actual diapedesis include intraluminal crawling (6, 7), and podosome-mediated sensing by leukocytes interacting with the EC apical surface (8). Endothelial-dependent events include formation of transmigratory cup structures, followed by TEM, which can be further broken down into two distinct modes: paracellular and transcellular. “Paracellular” TEM requires transient junctional disruption as leukocytes migrate between adjacent cells. Migration thus occurs “alongside of” or “beside” adjacent ECs. Alternatively, some leukocyte subtypes such as neutrophils have been observed migrating through pre-existing gaps at cell borders where three ECs meet (tricellular junctions) (9). In contrast, “transcellular” TEM occurs when a leukocyte transmigrates directly “through” a single EC. Once across the EC barrier, the leukocyte must additionally traverse a second barrier which is the underlying perivascular basement membrane. *In vivo*, there is evidence for the existence of specific regions of low matrix protein deposition in the venular wall, and interestingly, leukocytes preferentially transmigrate at these sites (10).

This review will begin with an introduction of key EC junction and cell surface adhesion proteins, along with a description of key regulatory proteins involved in junction and cytoskeleton regulation (Section 3). Following this, Section 4 of the review will provide a broad overview of what is known about signaling events downstream from leukocyte engagement of EC adhesion proteins, touching on how this affects regulation of the actin cytoskeleton, EC junctions, and thus paracellular TEM. The last two sections of this review

will provide a more detailed discussion of the newly characterized EC apical cup structure, incorporating the most recent advances made towards learning how its assembly is regulated (Section 5). Finally, Section 6 of the review will conclude with an in-depth discussion of transcellular TEM; its renewed recognition as a bona fide pathway, its regulation, and hypotheses about mechanisms for transcellular pore formation.

3. MOLECULAR PLAYERS

At the interface between blood and tissue, blood vessel ECs provide the primary physical barrier between these two distinct compartments within the body. The integrity of this barrier is maintained by junctional structures which seal the space between adjacent ECs (Figure 2). Junctions of ECs are morphologically heterogeneous (11), giving rise to different permeability and barrier characteristics between different vascular beds (12). At two extremes of the continuum are the relatively impermeable endothelium which comprises the blood brain barrier microvasculature, and the lymph node vessels known as high endothelial venules (HEVs) which support constitutive lymphocyte extravasation during homing and immune surveillance. It is important for EC junctions to be regulated appropriately in order to allow selective and specific passage of macromolecules and immune cells across this barrier only when necessary. Cell-cell junctions contain both transmembrane proteins with extracellular adhesive domains, and intracellular proteins which bind to the cytoplasmic domains of these transmembrane proteins. The cytoplasmic proteins often act as scaffolds, recruiting signaling molecules and providing an important link to the cell cytoskeleton which is critical for maintenance of barrier function. The transmembrane adhesion proteins contain extracellular adhesion domains to interact in a homo- or heterophilic manner to those on adjacent cells; they therefore constitute the actual physical barrier to a transmigrating leukocyte exiting the bloodstream via the paracellular route. ECs interact with neighboring cells using structures known as adherens junctions and tight junctions.

3.1. Tight junction proteins

In contrast to the well-developed and apically restricted tight junctions of epithelial cells, EC tight junctions are less structured and are found along the entire length of the paracellular cleft (13, 14). An exception to this is found in the blood-brain barrier; here ECs contain relatively impermeable tight junctions similar to those found in epithelial cells (15), and this is thought to be primarily due to differences in molecular composition with respect to transmembrane proteins (16, 17).

Occludin was the first transmembrane protein of the tight junction to be identified in epithelia (18), and later in endothelia (16). Indications that occludin plays a functional role in leukocyte TEM come from many sources. During experimentally induced inflammation, areas of neutrophil accumulation were often found adjacent to brain microvessels that had a disrupted staining pattern and lower expression of the tight junction proteins occludin and ZO-1 (19). This loss of occludin from cell junctions during TEM can be caused by matrix metalloprotease-mediated degradation (20). Furthermore, in a mouse model of HIV-1 encephalitis, sites of neuro-inflammation were not only correlated with diminished staining of total occludin, but there was an increase in occludin phosphorylation at those same locations; intriguingly, the phosphorylation was directly mediated by Rho kinase (21). However, occludin as a barrier protein in the paracellular pathway may not be the entire story; one intriguing study has suggested that at least in epithelial cells, certain domains of occludin may be required for efficient neutrophil trans-epithelial migration (22). It has even been reported that activated T-cells express occludin; raising the possibility that homophilic binding of leukocyte to EC occludin might help preserve barrier function as leukocytes migrate through EC junctions (23).

Claudin-5 is a vascular EC-specific member of the claudin family of transmembrane tight junction proteins, and this isoform is particularly enriched in the brain (24). Claudin-5 knockout mice exhibit impaired blood-brain barrier permeability to small molecules (25), and increased monocyte TEM has been correlated with local loss of claudin-5 expression in a neuro-AIDS model (21).

The junctional adhesion molecule (JAM) family proteins are another type of transmembrane protein found at the tight junction (26). JAM proteins can engage in homophilic interactions between molecules on adjacent cells (27-29), and when transfected into epithelial cells, JAM expression increases paracellular barrier sealing (30, 31). However, JAM proteins are unique junctional proteins in that they are also receptors for leukocyte integrins (32-35). This raised the possibility that JAM proteins aid adhesion and guidance of leukocyte transmigration across vessels (36). This hypothesis is supported by observations that neutralizing antibodies to JAM-A inhibit spontaneous and chemokine-induced neutrophil TEM (30, 37). Furthermore, intravital microscopy experiments with JAM-A knockout mice and adoptive cell transfer demonstrated decreased leukocyte TEM during IL-1 β -induced inflammation (38), and ischemia-reperfusion (39), although the two studies reported differences in whether EC-derived JAM-A (38) or neutrophil JAM-A (39) mediated the effect. In contrast, other groups report no inhibition of leukocyte TEM under flow using different JAM-A antibodies (40). The apparent differences have been attributed to the use of different inflammatory models, or the unique aspects of static versus flow-based assays. JAM-C involvement in positively mediating leukocyte TEM is less controversial; over-expression of JAM-C in ECs promoted lymphocyte TEM (41), while neutralizing antibodies against JAM-C partially blocked TEM during pulmonary inflammation (42), and in a mouse model of acute thioglycollate-induced peritonitis (43). Interestingly, this is not due to a decrease in TEM out of the blood vessel but instead represents an increase in "reverse" TEM from the interstitial tissues back into the blood circulation, indicating that JAM-C plays a key role in egressed leukocyte retention (44).

ESAM, or endothelial-specific adhesion molecule (45), is another tight junction transmembrane protein that contains extracellular immunoglobulin domains, similar to JAM proteins. ESAM knockout mice display inhibition of neutrophil but not lymphocyte TEM using *in vivo* inflammation models (46). Thus, both ESAM and JAM protein functions correlate positively with leukocyte TEM, reflecting their role as leukocyte counter-receptors. Additionally, knockdown of ESAM protein expression in cultured ECs reduced Rho activity (46), which as I will discuss later, is required for leukocyte TEM.

In addition to these transmembrane proteins, there are also a number of intracellular proteins which form a complex on the cytoplasmic face of the plasma membrane immediately underlying the tight junction. The predominant cytoplasmic TJ proteins in endothelia are the zonula occludens family proteins ZO-1 and ZO-2 (47, 48). These play a scaffolding role, linking the cytoplasmic domain of occludin to the actin cytoskeleton (49, 50), and also recruiting other signaling molecules that function during junctional regulation. Some of these proteins include AF-6, a putative GTPase effector, exchange factors for GTPases (GEF-H1), kinases such as aPKC, and phosphatases (RPTP β , PP2A) (51).

3.2. Adherens junction proteins

Adherens junctions present along the paracellular cleft form the other major barrier to leukocyte TEM, and the predominant transmembrane protein forming contacts between adjacent cells is VE-cadherin. The extracellular domain interacts with other VE-cadherin molecules on adjacent ECs in a calcium-dependent manner (52). That VE-cadherin is relevant during leukocyte TEM is highlighted by the observation that injection of function-blocking antibodies cause increased leukocyte TEM in several mouse models of

inflammation (53, 54), as well as in *in vitro* studies (55, 56). Real-time video microscopy has revealed that during TEM, VE-cadherin is physically displaced from adherens junctions during TEM and then relocalizes within minutes once the leukocyte has completed transmigration (57). Thus, regulation of VE-cadherin at the adherens junction is an important aspect of regulating leukocyte TEM. Further discussion of VE-cadherin-mediated signal transduction as it occurs during TEM will be elaborated in later sections of this review.

At the cytoplasmic face of the adherens junction, VE-cadherin forms a complex with alpha-, beta-, and p120-catenin. Through these interactions, VE-cadherin is connected to the actin cytoskeleton and this connection is required for maintenance of EC barrier function (55). The dogma has been that alpha-catenin acts as an intermediate binding protein linking F-actin with the beta-catenin/VE-cadherin complex (58). However, the idea of a static VE-cadherin/beta-catenin/alpha-catenin/F-actin complex has been challenged recently by reconstitution experiments showing that alpha-catenin does not interact simultaneously with the cadherin/beta-catenin complex and F-actin (59). Instead, the interaction between these proteins may be too weak or transient to be readily detected *in vitro*, or there may be unidentified components or physical properties such as mechanical tension present in a cellular context that are absent *in vitro* (60). Recent data show that the actin-binding protein EPLIN (epithelial protein lost in neoplasm) (61) may be one of these unidentified components linking the cadherin-catenin complex to F-actin (62). p120 catenin does not participate in connecting the cadherin complex to the cytoskeleton, but it does contribute to barrier function by regulating the stability of the complex (63). Junctional proteins like VE-cadherin and the catenins are often targeted for phosphorylation; high level of phosphorylation is an indicator of junctional immaturity, junctional disassembly, and increased TEM events (64-66).

3.3. PECAM and CD99

PECAM-1 (CD31) is a member of the Ig superfamily of transmembrane proteins and is expressed on ECs, platelets, and subsets of leukocytes (67). In numerous studies, PECAM-1 has been described as being present along the full length of EC lateral membranes, with particular concentration at cell-cell junctions (68, 69). PECAM-1 has also been found evenly distributed over the entire apical surface (70); this differential localization was attributed to accessibility of different antibodies used in these studies. Interestingly, localization of cell surface PECAM-1 is regulated by various angiogenic (angiopoietin-1) and inflammatory agents (TNF-alpha, IFN-gamma) (71, 72). The junctional pool of PECAM-1 plays a critical role in cell-cell adhesion, owing to its ability to engage in homotypic binding interactions with PECAM molecules on the adjacent endothelial cells (73, 74). In addition to its homophilic binding capabilities, the intracellular domain of PECAM-1 also acts as a molecular scaffold, recruiting and binding other junctional proteins like beta- and gamma-catenin (75), as well as a host of signaling proteins including phosphatases and kinases (reviewed in (76)). The importance of this scaffolding function is underscored by the recent observation that upon histamine treatment of ECs, phosphorylated PECAM-1 recruits both the phosphatase SHP-2 and phosphorylated beta-catenin to its cytoplasmic domain. This facilitates the subsequent dephosphorylation of beta-catenin, allowing it to bind to VE-cadherin and thus promotes adherens junction reassembly (77). A unique PECAM-containing membranous compartment underlying EC junctions has also been described (78). During TEM, PECAM-1 is specifically targeted to sites of monocyte migration, and blocking antibodies inhibit both this targeting (78). PECAM-1 has long been implicated in the process of leukocyte TEM. Early studies demonstrated that pre-treatment of either monocytes or ECs with PECAM antibodies blocks TEM (79), and this also holds true in models of acute inflammation *in vivo* (80-82). Furthermore, PECAM-1 knockout mice

exhibit increased vascular permeability (83), and reduced leukocyte TEM in the majority of mouse strains (80). PECAM-1 engagement and subsequent upregulation of alpha-6/beta-1 integrin expression on extravasating neutrophils may also aid their migration through the basement membrane (84).

CD99 is a heavily glycosylated protein expressed by most leukocytes and ECs (reviewed in (85)). Addition of blocking antibodies inhibited *in vitro* TEM by >90% (86). Confocal microscopy has shown that CD99 acts at a later stage of diapedesis than PECAM-1; while PECAM antibody treatment arrests leukocytes on the apical surface of endothelium (79), CD99 antibody-blocked neutrophils are seen lodged part-way through adjacent ECs in the area of cell junctions (87). This, together with the observation that antibody blockade of both PECAM-1 and CD99 together have additive effects on leukocyte TEM (87), indicates a non-overlapping role for these two proteins. Recently a homolog called CD99L2 was identified, and it was shown to be required for monocyte and neutrophil, but not lymphocyte TEM *in vivo* (88).

3.4. EC-leukocyte adhesion molecules

Other EC adhesion proteins are involved in leukocyte TEM not as part of a junctional structure per se, but as receptors for leukocyte adhesion (Figure 2). When they are engaged and clustered by leukocyte binding, they transduce intracellular signals which we will see later on in this review, can lead to the weakening of cell-cell junctions, cytoskeleton remodeling, and/or membrane fusion events that all contribute to leukocyte TEM.

3.4.1. ICAM-1—Intercellular adhesion molecule 1 (ICAM-1) is an Ig-like adhesion receptor that binds the leukocyte integrin LFA-1 (89, 90). The importance of ICAM-1 for TEM is underscored by the fact that transfection of ICAM-1 alone is completely sufficient to reconstitute neutrophil transmigration across a non-endothelial cell type (CHO, epithelial) that lacks any other leukocyte adhesion receptors (91). The crucial nature of ICAM-1 and also its close relative ICAM-2, is further supported by the observation that knockout mice exhibit deficiencies in the immune response stimulated by chemically-induced peritonitis *in vivo* (92, 93). Antibody blockade of ICAM-1 also inhibits leukocyte TEM; despite being able to adhere properly, monocytes are not able to direct their lateral movement across the apical surface of the ECs to search out permissible sites for TEM (6). Using real-time imaging of neutrophils migrating across HUVEC monolayers, Shaw and colleagues found that neutrophil integrins rapidly redistributed to form a ring structure which co-clustered with endothelial ICAM-1 close to endothelial cell junctions, and this ring structure was the origin of pseudopods which extended from the leukocyte through EC junctions (94).

3.4.2. VCAM-1—VCAM-1 also mediates adhesion of leukocytes expressing VLA-4 to the endothelium; however it is probably not the principle player during the transmigration stage. This is because antibody blockade of VCAM-1 alone does not significantly reduce monocyte TEM, but blocking both ICAM-1 and VCAM-1 simultaneously has an additive effect (95). Recently, an unbiased screen for VCAM-interacting proteins identified SPARC (secreted protein acidic and rich in cysteine) as a non-integrin receptor for VCAM-1 that is necessary for efficient TEM (96). During leukocyte TEM, the major role of VCAM seems to be more as a signal transducer, whereby engagement of VCAM-1 on the EC surface generates intracellular signals resulting in junctional disruption (97). The signaling mechanisms downstream of both VCAM-1 and ICAM-1 engagement will be discussed in detail in Section 4.

3.5. GTPases and the cytoskeleton

Linking transmembrane junctional proteins to the actin cytoskeleton is critical for the dynamic regulation of EC junctions during permeability fluctuations and leukocyte TEM (98). Indeed, the process of TEM requires the ability of ECs to activate GTPases and rearrange the cytoskeleton (99).

3.5.1. Cytoskeleton—Cortical actin bundles in the cell periphery promote junctional stability and function (13). However, during junctional disruption, for example after acute exposure to shear flow, the distribution of F-actin changes from a peripheral localization to an organization exhibiting cytoplasmic stress fibers (100). Insertion of these stress fibers into junctional sites and the resulting generation of isometric tension are proposed to be major mechanisms of junctional breakdown and induction of permeability. Generation of actomyosin contractility can be mediated either by intracellular calcium signaling or activation of the Rho/Rho kinase pathway (reviewed in (101, 102)). In the first scenario, intracellular calcium leads to Ca^{2+} /calmodulin-dependent myosin light chain kinase (MLCK)-mediated phosphorylation of myosin light chain (MLC). In the second case, Rho kinase activity also results in increased phosphorylation of MLC, both by inhibiting myosin light chain phosphatase (MLCP) (103, 104), and by phosphorylating MLC directly (105). In either situation, the resulting actin-myosin interactions allow contractile force generation (106) which promotes the cell shape changes and junctional breakdown necessary for paracellular TEM. Indeed, pre-treating ECs with specific MLCK inhibitors significantly reduced neutrophil transmigration (107, 108), and furthermore, the adhesion of neutrophils to ECs directly increases MLC phosphorylation (107, 109). Collectively these results confirm that tension-generation by the actomyosin system is used to facilitate the actual paracellular TEM of leukocytes across the endothelium.

3.5.2. Rho family GTPases—When discussing regulation of the cytoskeleton, low molecular weight GTPases are usually implicated as central players (110, 111). These include the familiar members of the Rho family, RhoA, Rac1, and Cdc42, but also include the less-studied RhoG, and GTPases like Rap1, which is actually a member of the Ras subfamily (112). RhoG and Rap1 will be discussed in detail in later sections.

Low molecular weight GTPases are proteins that act as molecular switches which cycle between an active (GTP-bound) and inactive (GDP-bound) form. GTP-binding and subsequent activation of Rho proteins is facilitated by guanine nucleotide exchange factors (GEFs), while inactivation of Rho proteins by hydrolysis of GTP to GDP is catalyzed by GTPase-activating proteins (GAPs) (Figure 3A). Extracellular stimuli are transduced by EC-leukocyte receptors, activating the GTPases, which now bind downstream effectors that lead to multiple downstream signaling cascades (Figure 3B). In ECs these signaling pathways in turn influence cell-cell junctions, actin cytoskeleton remodeling, and membrane dynamics, putting them in a central position to be important regulators of leukocyte TEM events (113).

In particular, RhoA and Rac1 were the first small GTPases implicated in modulating the cytoskeleton and cell junctions in ECs. Microinjection of a constitutively active RhoA mutant increased the amount of F-actin stress fibers in ECs, ultimately inducing loss of VE-cadherin from cell junctions and causing EC contraction and gap formation (114). These were qualitatively similar effects to that seen during neutrophil transmigration across a HUVEC monolayer (108), thrombin-induced permeability (115), and TNF- α stimulation (114). In agreement with the idea that EC Rho activity is required for TEM is the observation that inhibiting Rho/Rho kinase with C3 toxin or Y-27632 significantly inhibits MLC phosphorylation and the associated leukocyte TEM (116, 117).

On the other hand, Rac involvement in regulating EC junctions is much more controversial. Some experiments, involving over-expression of constitutively active Rac or using sphingosine 1-phosphate to activate Rac have indicated that Rac activity promotes junction formation and reduces EC permeability (118, 119). However, other experiments indicate that Rac activation disrupts EC junctions and increases permeability by a ROS-mediated mechanism (120). In the latter study, the authors showed that cells transduced with constitutively active Rac had a greater permeability increase than those expressing constitutively active Rho (120). A second mechanism by which Rac activation may induce junctional disruption is via its downstream effector, PAK (121). Stockton *et al.* have shown that EC fluid permeability is regulated by direct phosphorylation of MLC by PAK family kinase activity downstream from Rac (121, 122). PAK activation downstream of Rac can also induce VE-cadherin phosphorylation, subsequent β -arrestin-mediated internalization, and resulting junctional disassembly (123). These discrepancies highlight the need to strictly control GTPase activity for optimal junction integrity because either excessive, or deficient, activity can negatively impact the formation and maintenance of cell junctions (124).

3.5.3. Rap1 GTPase—Rap1 is another member of the small G-protein superfamily, but unlike Rho, Rac, and cdc42, it is a member of the Ras subfamily (125). Previously, Rap1 had been well-studied in the context of leukocyte integrin activation (126). With regard to leukocyte TEM, this is critical during the transition to firm adhesion onto ECs as well as aiding polarization during directed migration on the apical surface of ECs before the transmigration step (127). It is now appreciated that Rap1 has important integrin-independent functions within the EC. For example, Rap1 activation downstream of tissue inhibitors of metalloproteinase (TIMP)-signaling has been implicated as a mechanism regulating EC adhesion and migration during processes such as angiogenesis (128, 129). A number of groups have also documented a role for Rap1 and its cAMP-responsive GEF Epac-1, in promoting EC junctional integrity and barrier function (4, 130-132). Rap activation and subsequent EC junctional strengthening has also been implicated as a mechanism for inhibiting leukocyte TEM (133). Calcium switch experiments have shown that endogenous Rap1 is activated at early time points of junctional re-assembly (133), while biosensor experiments have confirmed that Rap1 is specifically localized to, and activated at, sites of junctional contact (134). The exact mechanism of how Rap1 promotes junctional stability is still elusive, although it likely depends on cytoskeletal rearrangements, in particular, the loss of actin stress fibers and increased cortical actin that is seen upon Rap1 activation (130-132). Besides the actin-based cytoskeleton, an intact microtubule cytoskeleton is also required for the dynamic regulation of EC junctions (135, 136). The Rap GEF Epac1 may perform another function that aids in junction stability independent of Rap1 activation, via its effects on these microtubules. Epac-1 co-localizes with microtubules, promoting their growth and stabilization (137). The authors propose that Epac-1 promotes barrier function in two ways: activating Rap which induces cortical actin remodeling, as well as a more direct effect on stabilizing the microtubule network; it is the coordinate regulation of both of these cytoskeletal structures that is critical for EC junction integrity (137).

Recently, the tight junction protein JAM-C was shown to be a key regulator of Rap1 activity in ECs, with JAM-C downregulating Rap1 activity and subsequently destabilizing adherens junctions (138). Intriguingly, this is opposite to the case for JAM-A in epithelial cells, in which JAM-A engagement improved junctional integrity along with Rap1 activation (139). An interesting possibility is that JAM protein regulation of Rap1 activity and thus VE-cadherin-based adhesion is in analogy to the role of nectins in epithelial cells, in which the stability of E-cadherin cell contacts is also mediated through effects on Rap1 (140). Thus Rap1 may be a central player linking regulation of both adherens and tight junctions in endothelia (141).

Progress is also being made in identification of downstream Rap effectors that may play a role in EC junctional regulation. One of these, KRIT-1 (protein mutated in cerebral cavernous malformation, a disease of vascular impairment) localizes to EC junctions in a Rap-GTP dependent manner, possibly by binding to beta-or p120-catenin (142). Using an siRNA approach, KRIT-1 was shown to be necessary for such Rap1-mediated junctional effects like decreased stress fibers, increased cortical actin, and decreased permeability. Another putative Rap effector at cell-cell junctions is AF-6 (afadin), which is a multidomain scaffolding protein that participates in linking transmembrane junctional components like cadherins, nectins, and JAM proteins to the actin cytoskeleton (143-145). AF-6 also selectively binds active Rap1-GTP, along with its GAPs Spa-1 and RapGAP-1, suggesting that AF-6 could be a central player in regulating EC junctions by controlling local Rap1 activation status through the recruitment of both Rap and its GAPs (146). However, besides negatively regulating local Rap1 activity, AF-6 might also be involved in the cytoskeleton remodeling effects seen upon Rap1 activation at cell-cell junctions, since it also binds profilin, which has been shown to promote cortical actin assembly (147). MAGI-1 (Membrane-Associated Guanylate kinase with Inverted orientation), yet another multi-domain scaffolding protein that localizes to cell-cell junctions by binding to ESAM and/or beta-catenin (134, 148), also binds a Rap GEF, PDZ-GEF1 (149). Homophilic engagement of VE-cadherin activates Rap1 in a MAGI-1-dependent manner, owing to the recruitment of PDZ-GEF1 by MAGI-1 (134). In conclusion, the existence of multiple junctional proteins that recruit both positive and negative regulators of Rap GTPase activity points toward a role for Rap1 in fine-tuning dynamic junction assembly/disassembly events such as those that occur during leukocyte TEM.

4. SIGNALING EVENTS DURING TEM

During the early stages of leukocyte TEM, firm adhesion of leukocytes induces clustering of EC adhesion molecules like ICAM-1 and VCAM-1 (150-152). This engagement triggers activation of many intracellular signaling pathways which induce EC actin cytoskeleton and cell junction remodeling. This sets the stage for weakening of EC junctions, allowing paracellular TEM to occur. Next, I will summarize the diverse signaling mechanisms downstream of leukocyte engagement of EC adhesion proteins and how this influences TEM events.

4.1. Signaling downstream of ICAM

ICAM-1 is perhaps the most prominent upstream adhesion molecule responsible for transducing outside-in signals that aid leukocyte TEM. The observation that deletion of the intracellular region of ICAM-1 completely abolished neutrophil TEM provided a convincing demonstration that ICAM-1-mediated signals generated by the EC were required for TEM, and that ECs were active participants in the leukocyte TEM process (91). ICAM-1 involvement in some of the key pathways is described in the following sections and is illustrated schematically in Figure 4.

4.1.1. Rho GTPase signaling—Subsequently, it was discovered through the use of bacterial toxins that inhibit Rho activity, that Rho GTPase signaling and its effects on the actin cytoskeleton were a critical aspect of this ICAM-1-mediated signal (153, 154). Artificially simulating leukocyte adhesion to ECs by cross-linking ICAM-1 with antibodies revealed that RhoA activation was an early event occurring downstream of ICAM-1, but not ICAM-2 receptor clustering (155). Rho activation correlated with stress fiber formation as shown previously (99, 155), and furthermore, this cytoskeletal remodeling occurred in conjunction with recruitment of ERM proteins to sites of ICAM-1 clustering (150, 155). The ERM (ezrin/radixin/moesin) family of proteins functions to link cytoskeletal elements to

transmembrane proteins and therefore they play an important role during cytoskeletal dynamics and regulation of signaling pathways downstream from proteins like ICAM-1 (156). The recruitment and activation of ERM proteins is a potential mechanism for connecting Rho activation to localized cytoskeleton rearrangements such as formation of stress fibers (157) and actin-rich cell surface membrane structures such as microvilli and transmigratory cups (158) (discussed in Section 5.) Upon ICAM-induced Rho activation, ERM proteins participate to further increase Rho activity in two ways: by binding to and recruiting the Rho GEF Dbl (159), and by binding to RhoGDI which releases its inhibition of Rho (160).

4.1.2. Calcium signaling—Another signaling event downstream from ICAM-1 engagement is increased intracellular calcium levels. Calcium-dependent activation of PKC activates Src and mediates tyrosine phosphorylation of actin-associated proteins like FAK and paxillin (161). Furthermore, the same group showed that chelating intracellular calcium or treating ECs with PKC inhibitors significantly decreased T-cell TEM, highlighting the importance of calcium-mediated signaling. Another outcome of calcium release that impacts TEM events is calcium/calmodulin-driven MLCK activation which as mentioned previously, generates actomyosin-based cell contractility and junctional disruption (101).

4.1.3. Phosphorylation—In addition to activating RhoA, ICAM-1 crosslinking also induces the tyrosine phosphorylation of many EC proteins. Some of these are cytoskeleton-associated proteins, like FAK, paxillin, p130Cas, ezrin, and cortactin (99, 151, 162-164). In addition, kinases like Src and p38MAPK have been identified as being phosphorylated and/or activated after ICAM-1 crosslinking (165, 166). Activation of the tyrosine kinase Src is a key step downstream of ICAM-1 engagement, responsible for many of these phosphorylation events. In particular, ICAM-1-mediated phosphorylation of cortactin by Src is clearly involved in leukocyte TEM. Cortactin is a protein scaffold that is well-studied for its role in Arp2/3-mediated actin remodeling events especially in the cell cortex (167). Thus cortactin is an ideal candidate for linking ICAM-1 engagement during leukocyte adhesion to the downstream clustering of adhesion molecules required during TEM. Src-mediated phosphorylation of cortactin was required for both E-selectin and ICAM-1 clustering on the EC surface (151). Furthermore, Src phosphorylation of Y421, Y466, and Y482 of cortactin was necessary for its dynamic redistribution to junctional regions where neutrophils were actively transmigrating, and inhibition of Src or knockdown of cortactin inhibited neutrophil TEM (164). Collectively, these results imply a role for cortactin in TEM, not only for its association with the actin polymerization machinery at the cell cortex, but also for stabilizing the association of actin with adhesion molecules such as ICAM-1.

4.1.4. VE-cadherin—Most recently, the adherens junction protein VE-cadherin has been identified as target for Src and/or pyk2-mediated tyrosine phosphorylation downstream of ICAM-1 engagement (168). As mentioned previously, VE-cadherin is one of the main transmembrane proteins that act as a barrier to leukocyte TEM via the paracellular route, and tyrosine phosphorylation of both cadherins and catenins has been implicated as a mechanism for inducing junctional disassembly (64, 65, 169, 170). Furthermore, increased tyrosine phosphorylation of the VE-cadherin-catenin complex is seen upon treatment of ECs with permeability-inducing compounds such as histamine and VEGF (171, 172). Previous experiments indicated that VE-cadherin was a target for Src kinase and that tyrosine phosphorylation of residues Y658 and Y731 inhibited the binding of beta-catenin and p120 catenin, thus negatively regulating adherens junction function and stability (173). Allingham *et al.* (168) showed that this phosphorylation of VE-cadherin occurred downstream of ICAM-1 cross-linking, and that both Src and pyk2 tyrosine kinases are specifically recruited to sites of ICAM-1 engagement. Moreover, inhibition of either kinase or expression of

nonphosphorylatable VE-cadherin mutants significantly attenuated neutrophil TEM (168). These findings are corroborated by a recent report where ICAM-mediated VE-cadherin phosphorylation in brain microvascular cells also increased paracellular TEM of lymphocytes (174).

4.1.5. Reactive oxygen species—Another EC-generated signal downstream of ICAM-1 engagement is production of intracellular reactive oxygen species (ROS). In one study, neutrophil adherence to ECs triggered production of xanthine oxidase-generated ROS and this could be inhibited with anti-ICAM-1 antibodies (175). The production of ROS is now known to be downstream of other EC-leukocyte receptors such as ICAM-3 and VCAM-1, and ROS has been implicated during conditions of increased permeability and leukocyte TEM (97, 176). Subsequent work placed ICAM-1-induced ROS production upstream of both Src and p38MAPK activation, and showed that this entire pathway was required for ICAM-1-induced cytoskeletal remodeling such as the generation of actin stress fibers (165, 166). EC-derived ROS may mediate leukocyte TEM in several ways, one of which is by promoting the necessary EC actin cytoskeleton rearrangement to facilitate TEM. Indeed, one of the proteins that is activated upon ROS production in ECs is hsp27 (177). Hsp27 is a substrate of p38MAPK, and is thought to be an important effector protein regulating actin polymerization (178); whether this has relevance for mediating leukocyte TEM remains to be tested. ROS-induced cytoskeleton rearrangements might be necessary for efficient leukocyte migration towards EC junctions as hypothesized by Wang *et al.* (179), or alternatively, the ROS-mediated generation of actin-based contractile elements within the EC may have a more direct effect on junction instability. A second way EC-derived ROS may mediate leukocyte TEM is via effects on tyrosine phosphatases. ROS can inhibit phosphatase activity by reversible oxidation of the catalytic cysteine residue (180), and the resulting increase in tyrosine phosphorylated junctional proteins might cause junctional disruption and concomitantly increased TEM.

4.2. Signaling downstream of VCAM

VCAM-1, the other major EC leukocyte-adhesion molecule, also acts as a signal transducer during leukocyte TEM. Engagement of VCAM-1 by adhering leukocytes or artificial stimulation with VCAM-1 antibody-coated beads generates two major intracellular signals: activation of the GTPase Rac1 and production of ROS, these pathways are shown in Figure 5.

4.2.1. Rac activation and ROS generation—It is now appreciated that non-phagocytic cells also produce ROS, which have been implicated as novel second messengers that help regulate cellular events like angiogenesis and inflammation (181). Interestingly, Rac1 is a component of the vascular NADPH oxidase complex, and active Rac promotes the assembly and activity of the complex (182). Several key observations linked ROS production and Rac activity to increased EC permeability and leukocyte TEM. For example, introduction of constitutively active Rac caused loss of VE-cadherin-mediated adhesion, and blocking ROS production with free radical scavengers protected against this loss of cell-cell adhesion (120). Treating ECs with inhibitors of NADPH oxidase and ROS scavengers was also found to block lymphocyte TEM (183). Subsequently, it was discovered that cross-linking of VCAM leads to a similar increase in permeability, while preventing engagement of VCAM with blocking antibodies inhibited T-cell TEM; these effects also required the production of EC-derived ROS (56). Together, this leads to a pathway whereby VCAM-1 cross-linking results in Rac activation, followed by the production of ROS via Rac-dependent activation of the NADPH oxidase, and Rho-dependent induction of stress fibers, all of which result in the gap formation or at least junctional weakening, that facilitates paracellular TEM (97).

ROS may also in turn influence the activity of Rho and Rac, providing a feedback loop of cytoskeleton regulation.

4.2.2. Phosphorylation—Rac-mediated ROS production downstream of VCAM also influences other signaling pathways that are implicated in regulation of leukocyte TEM. As mentioned earlier, phosphorylation of junctional proteins is a predominant mechanism for regulating their stability and localization at the junctional complex (180). In this regard, ROS production has been correlated with increased tyrosine phosphorylation of alpha-catenin (120). In addition, ROS can activate a redox-sensitive kinase pyk2, which phosphorylates beta-catenin, leading to its dissociation from VE-cadherin and the dissolution of the cadherin/catenin complex (170). PKC has also been shown to be activated downstream of VCAM-1 by direct oxidation, and this transient activation is required for efficient TEM (184). Activation of PKC-alpha in this manner can also phosphorylate PTP1b, a phosphatase (185). While it was shown that PTP1b activity is required for TEM, the exact mechanism of how its activity promotes lymphocyte TEM remains to be determined. Another potential signaling target of Rac-induced ROS produced downstream of VCAM engagement is the activation of cell-associated proteases. Experiments by Deem *et al.* showed that lymphocyte binding to VCAM rapidly activates matrix metalloproteases (MMPs); this activation was accompanied by production of ROS, and scavenging that ROS prevented VCAM-1-dependent lymphocyte TEM (186).

5. EC APICAL CUP STRUCTURES

As discussed in the previous section, it is evident that ECs play an active role during the process of leukocyte TEM. This can occur either via activation of signaling events which alter the EC cytoskeleton, or through opening of intercellular junctions. Recently however, another dramatic example of EC participation in TEM was also described. Upon leukocyte adhesion, a novel endothelial “docking structure” was induced that anchored and partially embraced the adherent leukocyte (158). In fact, earlier *in vivo* ultrastructural studies also described ECs “embracing” migrating leukocytes (187-189). Barreiro *et al.* were the first group to describe the structure in terms of its protein components as actin-rich, containing α -actinin, vinculin, VASP, the ERM proteins ezrin and moesin, and the EC adhesion molecules ICAM-1 and VCAM-1 (158). In their model, leukocyte attachment induced clustering of VCAM-1 and ICAM-1 on the EC surface, activating bound ERM proteins and causing local actin remodeling and formation of the membrane protrusions around the attached leukocyte (Figure 6). It was hypothesized that the formation of these docking structures was necessary for efficient capture of leukocytes by the EC, especially under conditions of shear flow, and that disassembly of these structures must presumably occur before diapedesis. Expanding on these findings, Carman *et al.* determined that leukocyte LFA-1 binding to ICAM-1 was necessary for formation of these “microvilli-like” endothelial membrane projections (190). Further characterization revealed that “cup” structure formation around adherent leukocytes required an intact actin and microtubule cytoskeleton as indicated by the inhibitory effect of cytochalasin D and colchicine treatment. In contrast to Barreiro *et al.* (158), formation of cup structures did not significantly increase adhesion under flow. Thus their hypothesis was that the function was not adhesion-strengthening, but instead could be required for the actual TEM process. This idea was validated by further data that ICAM-1-containing projections are highly associated with leukocytes that are actively transmigrating (191). Cumulatively, these findings provided more evidence for “transmigratory cup structures” as directional guides for leukocytes to traverse the endothelium.

5.1. Mechanisms of assembly

These seminal papers described the morphology and molecular components of cup structures, and began to characterize their putative function in TEM. However, much work remains to be done to describe the signaling mechanism(s) required for assembly. One recent report implicated cortactin as a possible mechanistic link between adhesion molecule engagement/redistribution and the subsequent association with the underlying actin cytoskeleton that ultimately leads to cup formation and enhanced TEM (152). A scheme has been envisioned whereby leukocyte engagement of ICAM-1 leads to recruitment of Src and its phosphorylated substrate cortactin, which then leads to temporal and spatial actin remodeling. Further clustering and adhesive strengthening between the leukocyte integrins and EC adhesion molecules such as ICAM-1 may feedback to amplify signals leading to more efficient leukocyte TEM (152). Whether the ring-like docking structures observed in these studies are transitional, leading to transmigratory cup formation was left unclear. However, the role for cortactin in assembly of either of these structures leading to TEM was certainly established.

Apical cup formation has also been reported to require localized polymerization of actin filaments (158, 190), an intact microtubule cytoskeleton (190), and also vimentin intermediate filament networks (192). Rho family GTPases are well-known regulators of such cytoskeletal dynamics (193). One of the most recent studies to delve into the molecular mechanisms of cup formation and the signaling pathways that regulate their assembly focused on one of these small GTPases, RhoG, and its upstream regulator SGEF (SH3-containing GEF). SGEF is a RhoG-specific GEF that when expressed in fibroblasts, induces the formation of dorsal membrane ruffles and enhances macropinocytosis (194). Since dorsal ruffles induced by SGEF superficially resemble endothelial cup structures induced by leukocyte adhesion, the rationale was that they may be regulated by similar mechanisms. Van Buul *et al.* (195) discovered that engagement of ICAM-1 rapidly activates RhoG, and both RhoG and SGEF become colocalized with clustered ICAM-1 in the apical membrane protrusions surrounding the adherent leukocyte. SGEF/RhoG involvement in apical cup formation was confirmed by siRNA-mediated knockdown of both proteins, and while leukocyte adhesion was not affected, cup formation and subsequent leukocyte TEM was inhibited. The exact mechanism of SGEF-mediated RhoG activation downstream of ICAM engagement remains unclear. The SGEF SH3 domain binds to the ICAM-1 cytoplasmic region; however, this interaction is independent of SGEF activation state since binding occurs even when SGEF is mutationally inactivated. Presumably ICAM engagement by leukocyte adhesion must trigger activation of the already-bound SGEF, possibly through phosphorylation by a kinase such as Src (195), which is known to target multiple substrates downstream of ICAM such as cortactin (99, 151, 163, 164, 166) and VE-cadherin (168). Interestingly, SGEF and RhoG have also been associated with engulfment of *Salmonella* by phagocytic cup structures on the apical surface of epithelial cells (196). While apical cups have been discussed so far in the context of paracellular TEM, it is important to note that these structures have also been observed during leukocyte transcellular migration, independently of EC junctions (188, 189, 191). This alternate route of TEM is discussed in the next section.

6. TRANSCELLULAR TEM

Thus far, leukocyte TEM has mostly been described in terms of crossing the endothelial barrier by breaching the intercellular junctions that connect adjacent ECs, i.e. the “paracellular” route (Figure 7). However, as mentioned in the introduction, this is not the only route, or possibly even the most prevalent means by which leukocytes extravasate. In the early 1960s, detailed morphological studies of leukocyte TEM revealed that a significant amount of leukocytes seemed to transmigrate without disrupting the EC junctions (197).

Instead of using the paracellular course, they transmigrated directly “across” or “through” an individual EC cytoplasm, using this so-called “transcellular” route (198) (Figure 7). One reason why the notion of transcellular TEM fell out of favor is likely due to the advances made in identifying the molecular constituents of cell-cell junctions. The ability to inhibit or activate known protein components gave researchers new experimental tools that were ideally suited to study paracellular (junctional) TEM. A second reason why the idea of transcellular TEM was slow to gain widespread acceptance had to do with the technical challenges of capturing it in an experimental setting. Advances in electron microscopy techniques and computer generated three-dimensional reconstructions ultimately permitted visualization of neutrophils migrating through a transcellular pore that passed cleanly through the cytoplasm of the EC very close to, but decidedly distinct from EC junctions (189). This also raised the caveat that many studies might be underestimating the amount of transcellular TEM if migration occurs so close to the EC junctions that they are mistaken for paracellular events. Other *in vivo* studies have since confirmed the existence of a transcellular route (199, 200). Besides the inherent difficulty in visualizing transcellular TEM, the last major obstacle to the acceptance of this phenomenon was probably conceptual. It is rather more difficult to envision a leukocyte penetrating and passing straight through the cytoplasm of another cell, than it is to imagine the leukocyte squeezing in between adjacent cells in a zipper-like model of junction disassembly. The recent resurgence in the recognition of the transcellular mode of TEM may in part be due to more in-depth descriptions of membrane structures which could aid transcellular pore formation. The subsequent section will describe these structures, putting them in context with the current experimental evidence for transcellular TEM.

6.1. EC membranous structures in relation to transcellular TEM

ECs contain subcellular membranous structures such as caveolae, fenestrae, and vesiculo-vacuolar organelles (VVOs) which function in regulating microvascular permeability and inflammation. These specialized membrane microdomains have been implicated as the sites of transendothelial exchange of fluid and plasma proteins in and out of the bloodstream (201), but may also directly or indirectly impact leukocyte TEM. Fenestrae are specialized plasma membrane microdomains, where the membrane “thins out” into circular openings usually spanned by a non-membranous diaphragm. Fenestrated endothelium is normally present only in specialized vascular beds that are functionally required to have high permeability e.g. in endocrine organs where secretion of hormones into the bloodstream occurs (11). Interestingly, tumor neovasculature is also highly fenestrated (202), and VEGF application *in vivo* rapidly (<10 min) induced the formation of fenestrae in skeletal muscle and dermal endothelium which are both normally non-fenestrated (continuous) (203). In addition to inducing fenestrations, VEGF-treatment *in vitro* was also correlated with increased appearance of fused, clustered, caveolae-like vesicles separated by diaphragms (204). Such aggregated vesicles have been referred to as vesiculo-vacuolar organelles (VVOs) (205). An important unresolved question is whether caveolae fuse and give rise to VVOs and/or fenestrations. Caveolae, but not the VVO or fenestrations contain caveolin-1 (204), which suggests that caveolin-1 must be specifically excluded if there is a transition from caveolae to fused clustered vesicles (VVO's) and fenestrae. The hypothesis that membrane fusion events are required to form a transcellular channel permissive for leukocyte passage is bolstered by the observation that lymphocytes transmigrate at caveolin-1 enriched areas, and that reduction of caveolin-1 expression by siRNA specifically decreases transcellular TEM (206). Local fusion of caveolae that are concentrated around the transmigrating leukocyte might therefore be one way to form a transcellular pore linking the apical with the basal EC membrane (Figure 7). Caveolin-independent mechanisms may exist as well, since the site of leukocyte transcellular TEM is often associated with an abundance of vesicles or VVO-like structures in the adjacent EC cytoplasm (8). In these

cases, membrane fusion events leading to transcellular pore formation was dependent on the SNARE-containing membrane fusion complex. Membrane fusion events need not be restricted to transcellular TEM. Another related membrane structure, apparently distinct from VVOs, concentrates adjacent to lateral EC junctions (78). This novel “EC surface-connected compartment” is comprised of PECAM-containing membrane vesicles that continually recycle in and out of this network under steady state, but upon leukocyte adhesion, membrane recycling becomes specifically targeted to junctional leukocyte adhesion sites in a kinesin and microtubule-dependent process (136). The disgorgement of membrane would increase the surface area of PECAM-containing membrane in contact with the leukocyte, assisting in maintaining the tight seal between EC and leukocyte during paracellular TEM (207).

6.2. Factors regulating transcellular migration

Mechanistic study of transcellular TEM requires the ability to observe it in an experimentally malleable setting. Further bolstered by the *in vivo* studies, several groups began to redouble their efforts to observe transcellular TEM *in vitro*. Ultimately, high resolution confocal microscopy provided *in vitro* evidence that leukocyte TEM can occur by both a paracellular and transcellular route (191). The physiological factors that promote transcellular TEM are currently an area of intense research. Are specific vascular beds more prone to using one route over the other, and/or do certain leukocyte subtypes utilize different modes preferentially? Are there other signaling factors upstream of cup formation such as inflammatory stimulus? The remainder of this section will address these intriguing issues.

By and large, transcellular TEM has been more readily observed *in vivo*, while *in vitro* it is the less preferred route. Furthermore, microvascular ECs support transcellular TEM more readily than macrovascular ECs, although the reason for this phenomenon is not known (8, 206). In one experimental system, transcellular movement of lymphocytes accounted for ~30% of the total TEM across microvascular cells, but less than 10% across macrovascular cells such as HUVECs (8). With respect to leukocyte type, one study reported that T-lymphocytes migrated exclusively paracellularly (208), yet other studies observe a detectable level of T-cell transcellular migration across the same endothelial cell type (8, 192). Another group observed that neutrophil intraluminal crawling was an important mechanism for determining which route was taken i.e. Mac-1 deficient leukocytes, which adhere but do not crawl on the EC apical surface, mainly use the transcellular pathway (7). This group further made the observation that ECs form “domes” that encapsulate neutrophils undergoing transcellular TEM, and formation of these structures help preserve barrier integrity while TEM is occurring (200). A final hypothesis is that exposure to shear forces might affect transmigration route preference (209). Although shear flow can globally increase TEM, in one study it did not promote one route over the other (208). Thus, whether any of these factors affect transcellular TEM is still under investigation. Another factor that can influence TEM route preference has to do with endothelial ICAM-1 levels. Yang *et al.* (208) demonstrated that transcellular TEM events were positively correlated with increased expression of ICAM-1, for example after 24 hour treatment with TNF-alpha. They surmised that ICAM-1 levels influence the route of TEM due to higher occupancy of the cognate receptor on the leukocyte (LFA-1) promoting rapid, non-junctional arrest and thus transcellular TEM. This phenotype could be reconstituted by artificially raising ICAM-1 levels by overexpression of full-length ICAM-1, but not ICAM-1 lacking the cytoplasmic domain. In fact, this cytoplasmic domain deletion of ICAM-1 not only decreased overall TEM as previously described (91, 153, 154), but this was specifically due to a decrease in the transcellular fraction (208).

Another reason for differences in route choice may depend on strength of intercellular junctions within the monolayer in question, which in turn depends on locally regulated

cytokine exposure. This idea was tested using IL-1 β treatment which increases the amount of stress fibers, and disrupts EC junctions (210). Most importantly, this treatment caused a 40% decrease in the number of monocytes migrating transcellularly. A similar explanation was proposed for the observation that TEM of neutrophils was exclusively paracellular on HUVECs stimulated for only 4 hours with TNF-alpha, but after 24 hours of stimulation a significant (10%) number of TEM events now occurred transcellularly (208). Such short-term acute application of cytokines such as TNF-alpha has been shown to cause junctional disruption (114, 211, 212). Therefore, in this scenario, neutrophils could sense and utilize the path of least resistance; if junctions were weakened, paracellular migration was facilitated at the expense of transcellular TEM. It remains to be seen whether inhibition of paracellular migration by strengthening junctions results in a compensatory increase in transcellular TEM. This may be a relevant factor *in vivo* because historically it was transcellular, not paracellular TEM that was invariably observed across vessels of the brain microvasculature which have very elaborate and impermeable cell junctions (198). Lastly, in other studies where transcellular migration was inhibited through caveolin-1 knockdown, overall TEM was partially compensated for by enhanced paracellular migration (206). In summary, it is now acknowledged that leukocytes can in fact use the transcellular route of TEM, and the differential signaling involved in route selection will be an exciting area of intense research.

6.3. Integrating EC membrane fusion events with leukocyte invasive podosomes

Much attention has been given to EC-derived structures that are actively involved in the progression of leukocyte transcellular TEM. However, the leukocyte also plays an active role in this process. Lymphocytes have been observed extending podosome-like protrusions which probe the EC apical surface, and then initiate transcellular invasion at a permissive location of the cytoplasm (8) (Figure 7). In response to palpation by leukocyte podosomes, EC surface indentations termed “podoprints” form. Recent work by Carman *et al.* (8) demonstrated that transcellular TEM, but not paracellular TEM was highly dependent on these “invasive” podosomes, and that their formation required lymphocyte Src and N-WASP activity. The authors propose that this dynamic probing is a mechanism for leukocytes to sense areas of low endothelial resistance, and once a permissive area is identified, leukocyte protrusions can progress to become true invasive podosomes which ultimately breach the endothelium. Leukocyte invasive podosomes may act in concert with endothelial membrane protrusions (cups), which might provide a vertical traction substrate for the podosomes against the EC surface (8). Intriguingly, there was often an abundance of vesicles or VVO-like structures in the EC cytoplasm adjacent to these invasive podosomes, which also lends credence to the idea that podosomes elicit an EC response to trigger membrane fusion events that would facilitate formation of a transcellular pore.

7. SUMMARY AND PERSPECTIVE

Exciting progress is being made in elucidating the complex mechanisms that orchestrate leukocyte TEM, and a current theme is that ECs are active participants in this process. Leukocyte engagement of EC adhesion receptors triggers signaling events impacting actin cytoskeleton and EC junction remodeling, both of which contribute to the TEM response. The renewed recognition of the transcellular mode of TEM is an exciting example of how the field is constantly progressing. Perhaps the most important unresolved issue in this area of research is the classification of stimuli and signaling that contribute to the relative utilization of the paracellular versus transcellular route of TEM. Interestingly, endothelial apical cup structures may participate in both modes of TEM by synergizing with leukocyte podosome formation to help stabilize and guide TEM. Continued research into the process of leukocyte TEM will be vital to the understanding and rational design of potential

therapies for clinical conditions where an inappropriate inflammatory response leads to disease.

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Abbreviations

EC	endothelial cell
ESAM	endothelial-specific adhesion molecule
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
HUVEC(s)	human umbilical vein endothelial cell(s)
ICAM	intercellular adhesion molecule
IL-1-beta	interleukin-1-beta
JAM	junctional adhesion molecule
MAGI-1	membrane-associated guanylate kinase-inverted
MLC	myosin light chain
MLCK	myosin light chain kinase
MMPs	matrix metalloproteinases
PECAM	platelet endothelial cell adhesion molecule
ROS	reactive oxygen species
SGEF	SH3-containing GEF
TEM	transendothelial migration
TNF-alpha	tumor necrosis factor alpha
VCAM	vascular cell adhesion molecule
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VVO	vesiculo-vacuolar organelle
ZO	zonula occludens

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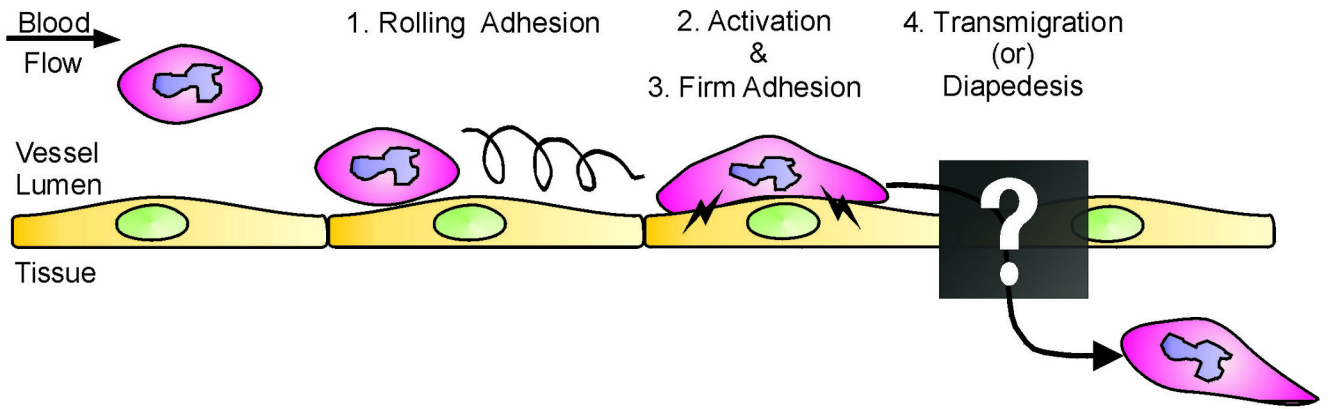


Figure 1.

Schematic diagram of the leukocyte adhesion and transmigration cascade. Given an inflammatory stimulus, leukocytes initially loosely adhere on the vascular ECs, rolling along the blood vessel wall via transient selectin-mediated interactions (1). During the activation stage, both the EC and leukocyte begin to upregulate expression and/or activity of adhesion receptors on the cell surfaces (2), and this is required for initiating the firm adhesion stage (3). Finally, leukocytes exit the bloodstream, crossing the endothelium by the process known as transmigration or diapedesis (4). The mechanisms and pathways by which transmigration occurs are poorly understood and thus will be the focus of this review.

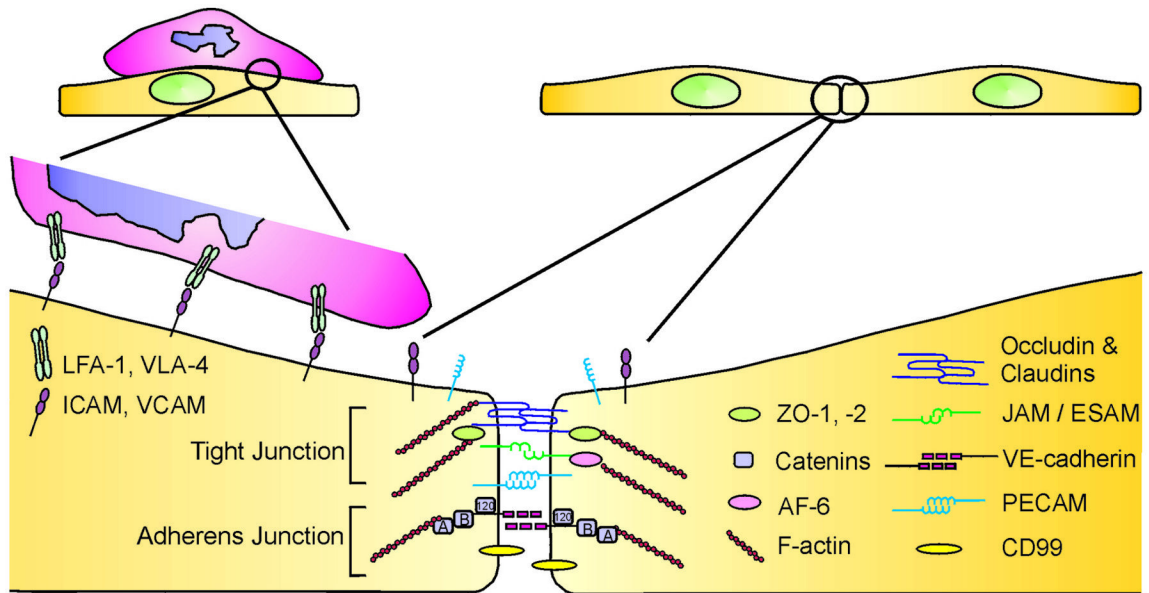


Figure 2. Molecular components of EC and EC-leukocyte interactions. ECs contain both adherens junctions and tight junctions. Transmembrane proteins located along the paracellular cleft of two adjoining ECs interact, and thus provide the physical barrier to the transmigrating leukocyte. Cytoplasmic junctional proteins provide a link between the transmembrane proteins and the cell cytoskeleton. Leukocytes interact via integrins to EC adhesion molecules present on the apical surface. In endothelial cells, adherens junctions and tight junctions can be interspersed along the entire length of the lateral membrane.

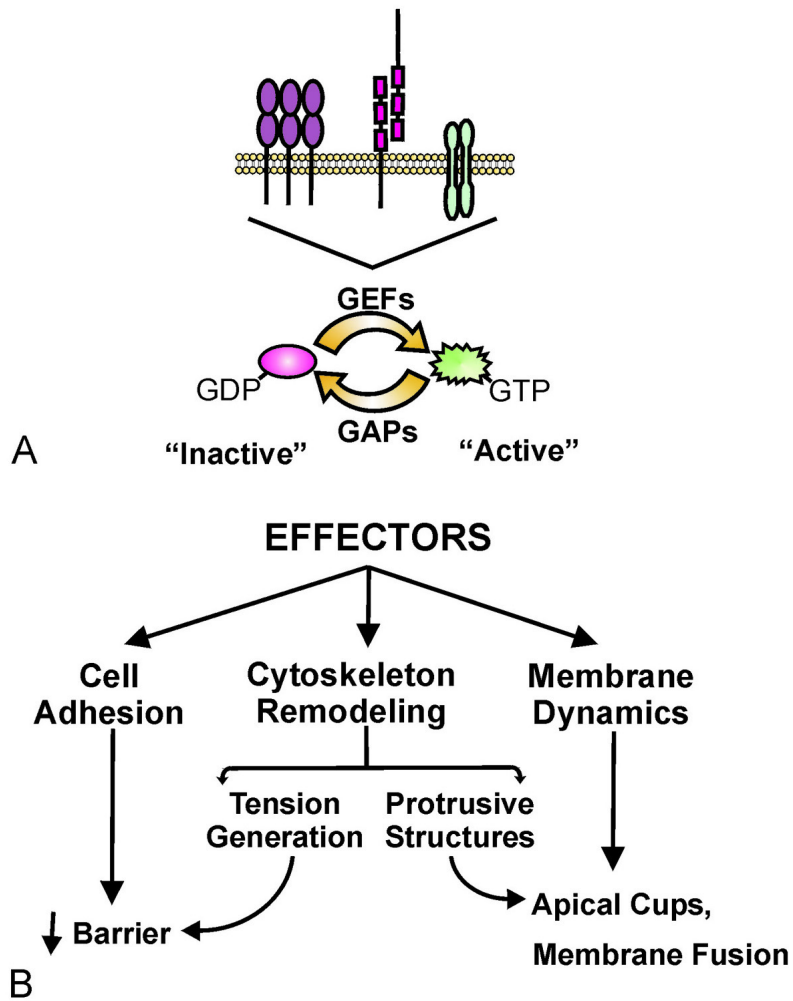


Figure 3. Low molecular weight GTPases, via downstream effectors, are key participants of EC signaling pathways involved in leukocyte TEM. (A) Signals transduced by EC adhesion molecules downstream of leukocyte engagement regulate activity of low molecular weight GTPases. GEFs activate the GTPase, enabling it to interact with downstream effectors. In turn, GAPs aid the hydrolysis of GTP to GDP, inactivating the GTPase and inhibiting downstream signaling events. (B) Effector signaling downstream from GTPase activation can control cell adhesion, cytoskeleton remodeling, and membrane dynamics. In turn, this influences barrier function, membrane fusion events, and the formation of cytoskeleton-enriched structures such as apical cups that are involved in leukocyte TEM.

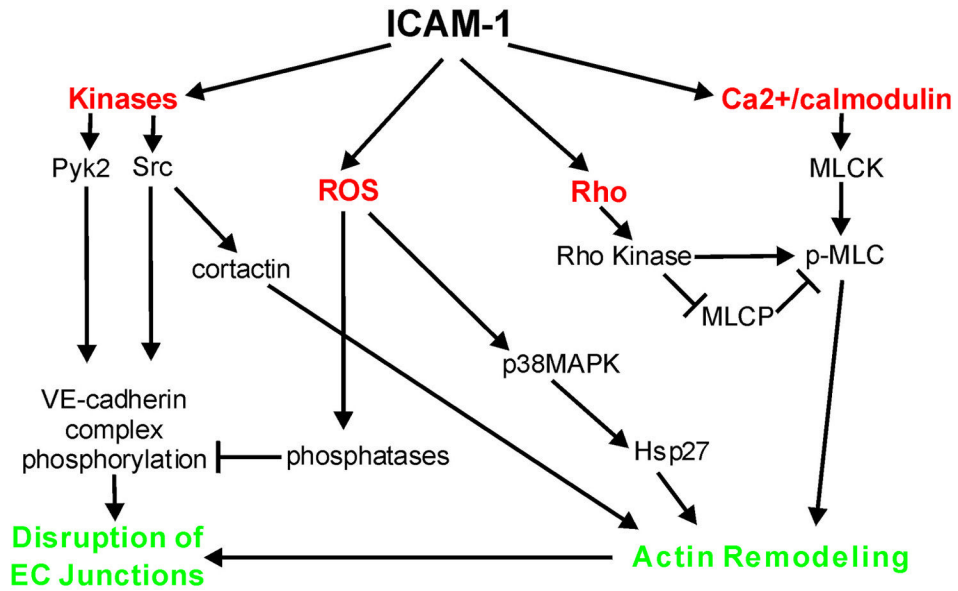


Figure 4. Schematic diagram of signaling events initiated downstream of ICAM-1 engagement. Leukocyte binding to ICAM-1 triggers diverse signaling pathways within the EC (highlighted in red). Phosphorylation of target proteins (Section 4.1.3.), particularly the VE-cadherin complex (Section 4.1.4.), production of ROS (Section 4.1.5.), activation of Rho family GTPases (Section 4.1.1.), and calcium signaling (Section 4.1.2.) are centrally involved. These pathways all contribute to the junctional disruption and/or actin remodeling that is permissive for leukocyte TEM to occur. (see text for details)

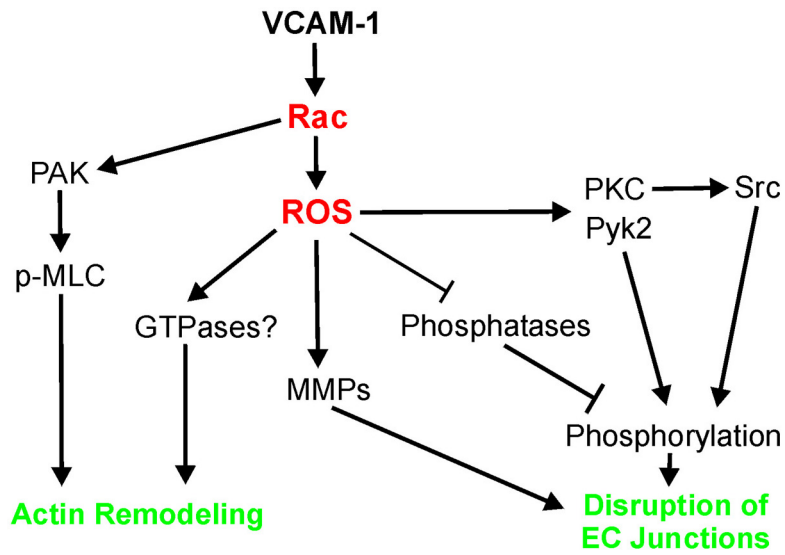


Figure 5. Schematic diagram of signaling pathways initiated downstream of VCAM-1 engagement. Leukocyte adhesion to VCAM-1 mainly signals via Rac1-mediated ROS generation. ROS inhibition of phosphatases and activation of redox-sensitive kinases serve to increase phosphorylation of junctional proteins, and together with production of MMPs, leads to junctional disruption. The Rac effector PAK has also been implicated in actin remodeling via MLC-generated tension and contractility. (see text for details)

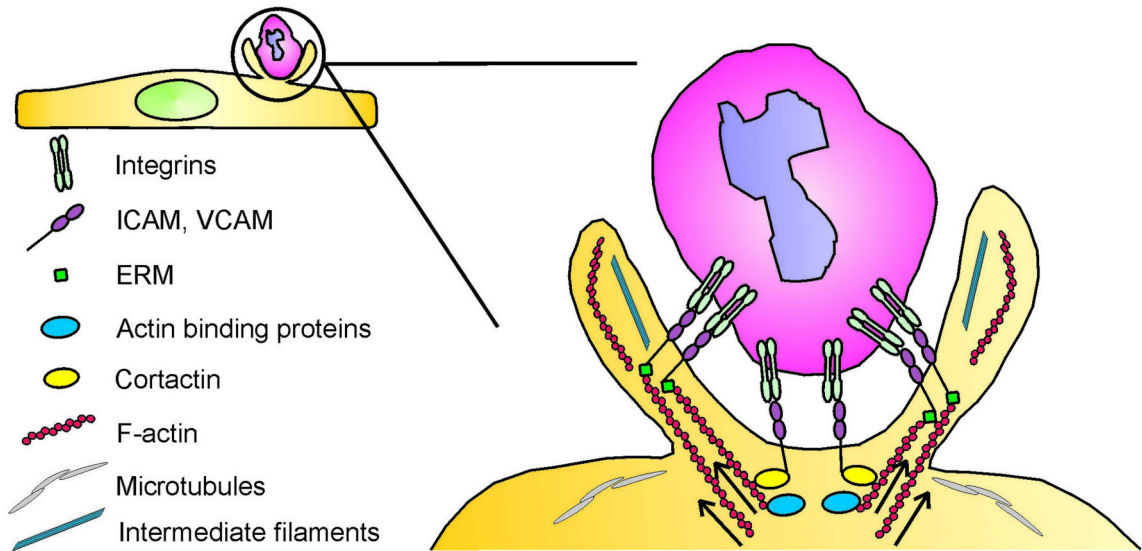


Figure 6.

Molecular architecture of EC transmigratory cup structures. EC participation in TEM is dramatically illustrated by the formation of apical cup structures. These EC-derived actin and intermediate filament-rich membrane protrusions partially surround and “embrace” the leukocyte. Assembly of apical cup structures requires clustering of EC adhesion proteins like ICAM-1 and VCAM-1, coupled to cortactin and/or ERM protein-mediated local and directed actin polymerization.

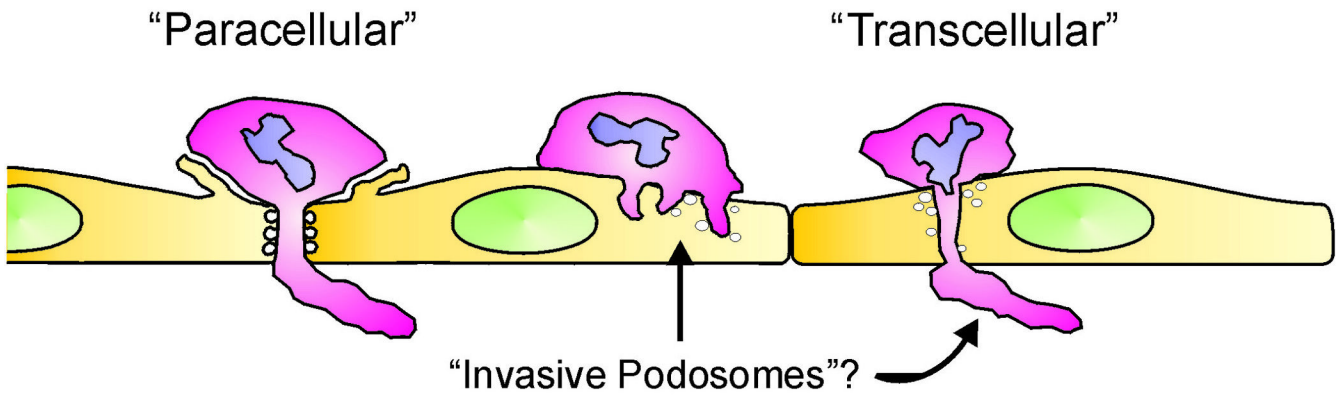


Figure 7.

Modes of leukocyte TEM: paracellular versus transcellular. Leukocytes can transmigrate across the endothelium via two independent routes. Use of the “paracellular” route requires transient junctional disruption as leukocytes migrate between adjacent ECs. Paracellular TEM may involve a series of PECAM-enriched EC surface-connected membrane compartments that are located adjacent to the junctional region. Conversely, “transcellular” TEM involves leukocyte passage directly through an individual EC, bypassing the need to disassemble EC junctions. Local fusion of caveolae or vesiculo-vacuolar organelles may be a potential mechanism of transcellular pore formation. Recent data suggest that transcellular TEM is strongly dependent on the formation of “invasive podosomes” that are extended by the leukocyte, while EC apical cup structures may aid in both types of TEM.