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Immunol Rev. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Immunol Rev. 2016 September ; 273(1): 61–75. doi:10.1111/imr.12443.

Transendothelial Migration: Unifying Principles from the Endothelial Perspective

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Summary

Transendothelial migration (TEM) of PMN involves a carefully orchestrated dialogue of adhesion and signaling events between leukocyte and endothelial cell. This chapter will focus on the endothelial cells' contribution to transmigration. The initiation of TEM itself generally requires interaction of PECAM on the leukocyte with PECAM at the endothelial cell border. This is responsible for the transient elevation of cytosolic free calcium ions in endothelium that is required for TEM and for recruitment of membrane from the lateral border recycling compartment (LBRC). TEM requires LBRC to move to the site at which TEM will take place and for VE-cadherin to move away. Targeting of the LBRC to this site likely precedes movement of VE-cadherin, and may play a role in clearing VE-cadherin from the site of TEM.

The process of TEM can be dissected into steps mediated by distinct pairs of PMN/endothelial interacting molecules. CD99 regulates a step at or close to the end of TEM. CD99 signals through soluble adenylyl cyclase to activate PKA to trigger ongoing targeted recycling of the LBRC. Paracellular transmigration predominates (≥ 90% of events) in the cremaster muscle circulation, but transcellular migration may be more important at sites such as the blood brain barrier. Both processes involve many of the same molecules as well as recruitment of the LBRC.

Keywords

transmigration; endothelial cell junctions; platelet/endothelial cell adhesion molecule 1 (PECAM); lateral border recycling compartment (LBRC); CD99

Introduction

This chapter will cover the process of transendothelial migration (TEM) or diapedesis, the stage in leukocyte extravasation in which leukocytes squeeze across the endothelial lining of blood vessels *en route* to the site of inflammation. There are many comprehensive reviews of transmigration (1–6) to which the reader is referred; our knowledge of this process has increased remarkably in the past decade. In keeping with the spirit of *Immunological*

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DISCLOSURES

The author has no conflicts of interest to declare, financial or otherwise.

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Reviews, we will "review our own work within the context of the field…" and point out where controversies lie and where important information still awaits discovery. Since there are other chapters in this volume on interactions of neutrophils (PMN) with epithelial cells and even transmigration of PMN, this chapter will discuss transendothelial migration from the point of view of the endothelial cells. The focus will be on TEM of neutrophils. Many of the same molecules and mechanisms mediate TEM of monocytes and lymphocytes. However, where there are known differences, these will be pointed out.

The molecules and signaling pathways that are most important for TEM vary depending on the leukocyte type, the vascular bed, the inflammatory stimulus, and the chronicity of the inflammatory response. PMN generally participate in the acute inflammatory response, but are otherwise subjected to the same caveats. The molecules and signaling pathways described here have been found to be generally relevant and robust using both *in vitro* and *in vivo* models. However, there will undoubtedly be exceptions, since blocking any single molecule or pathway never inhibits leukocyte rolling, adhesion, or TEM completely.

Most transmigration in response to inflammation takes place at postcapillary venules. At these sites transmigration follows the steps of capture, rolling, activation, adhesion, and intraluminal crawling in the extravasation cascade (3, 7, 8). Exceptions occur in the lung in which most TEM takes place through capillaries thinner than the diameter of PMN so this elaborate sequence of adhesive events is not necessary to arrest them at the site (9–11). These steps are reversible, and most of the neutrophils (PMN) that enter the postcapillary venule feeding the site of inflammation do not roll; most of those that roll do not adhere, and even most of the adherent PMN do not extravasate (12). However, once a PMN commits to diapedesis, with rare exceptions (13), it traverses the endothelium and underlying basal lamina to enter the inflamed tissue. Most of the benefits as well as the unintended consequences of inflammation take place after leukocytes cross the blood vessels. Thus, we have felt that TEM is the "point of no return" of the inflammatory response and will focus on the molecules and mechanisms regulating it.

There are two pathways for TEM. In paracellular migration leukocytes move between apposing endothelial cells; in transcellular migration they move through the body of the endothelial cell (1, 14–16). Transcellular migration was controversial for a long time, since evidence to support it was limited to static thin section electron micrographs in which TEM at a tricellular junction (a preferred site for PMN transmigration (17)) could resemble transcellular migration close to an endothelial cell junction (18). However, studies employing extensive serial sectioning (15) as well as high resolution intravital microscopy (13) demonstrate that transcellular migration can and does occur *in vivo*. Based in intravital microscopic observations few PMN (< 10%) transmigrate transcellularly *in vivo* (13). Nevertheless, there is evidence that a greater proportion of lymphocytes transmigrate transcellularly at special sites such as the blood-brain barrier, and in some *in vitro* models (19, 20). Paracellular and transcellular migration seem at first glance to be very different pathways. However, as we learn more about them, we find more similarities than differences (1, 14, 21, 22).

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While increased vascular permeability at the site of inflammation and leukocyte transmigration occur in spatial and temporal proximity, they are distinct events (23). Plasma leakage takes place more proximally in smaller (20 – 40 μm) postcapillary venules, while PMN exit from larger (40 – 60 μm) venules (24, 25). This makes sense, since leakage of plasma provides the hemoconcentration and slower flow that allows leukocytes to make productive contacts with the endothelial wall. These processes can even be dissected genetically. Cortactin-deficient mice have constitutively elevated vascular permeability but defective TEM (26). Many of the same signaling molecules and pathways regulate vascular permeability as well as TEM. What likely contributes to the maintenance of the endothelial barrier during leukocyte diapedesis is that these signals are localized to the portion of the endothelial cell interacting with the leukocyte during TEM, while they are more diffusely activated in response to soluble mediators that trigger vascular permeability (23).

Preludes to transendothelial migration

As PMN approach the site of transmigration (whether it is paracellular or transcellular) intercellular cell adhesion molecule-1 (ICAM-1) on the endothelial cell becomes concentrated below the leukocyte (14, 27, 28) due to interactions with leukocyte function associated antigen (LFA-1, CD11a/CD11b) on the leukocyte (27). Vascular cell adhesion molecule-1 (VCAM-1) shows a similar concentration under and around lymphocytes and monocytes (29). Contemporaneously, tetraspanin molecules on the endothelial cell become co-localized with these molecules (30). Engagement of ICAM-1 activates the actin-bundling molecule cortactin, which helps bring f-actin into this site and also recruits more of the cytoplasmic tail of ICAM-1 to continue the process in what may be a positive feedback loop (31, 32).

Cortactin-deficient mice have defective neutrophil TEM due to defective ICAM-1 clustering. They also have inherently leaky venules due to reduced levels of Ras-related protein 1 (Rap1) the absence of activation by cortactin (26). In addition to confirming a role for cortactin in both of these phenomena, this paper demonstrated genetically what many in the field had recognized from much indirect evidence: That TEM and opening of endothelial junctions are distinct and separable events.

When tracked with fluorescent molecules or stained *post hoc* with fluorescent antibodies, the co-localization of ICAM-1 and LFA-1 appears to continue during the course of transmigration, with LFA-1 redistributing to the uropod (lagging edge) of the PMN to keep in contact as the PMN migrates below the endothelium (27). As diapedesis begins ICAM-1, VCAM-1, tetraspanins, and actin become highly concentrated along the endothelial cell membrane surrounding the leukocyte as it transmigrates, giving the appearance of a ring by immunofluorescent staining (27, 29, 30). Orthogonal views of confocal immunofluorescence images of these molecules show intense staining surrounding the transmigrating leukocyte (Figure 1) whether it is passing paracellularly or transcellularly (14, 33). In addition to these rings, fingers of fluorescence stretching above the apical surface of the endothelial and partially surrounding the leukocytes have been reported by some (28–30, 33, 34) but not others (14, 27, 35–37). The differences are likely due to differences in the *in vitro* assays used and the time it took for TEM to be completed (38). These have been interpreted as

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apical membrane forming “docking structures” (29) or “transmigratory cups” (33) or artifacts due to of out-of-focus light over the rings in the X-Y plane being reconstructed in the X-Z plane (1, 38). Their presence or absence does not seem to affect the rate or extent of TEM (28, 33).

Initiating transmigration: Major membrane movements

At least two events in the endothelial cell are critical for efficient transendothelial migration: Mobilization of VE-cadherin away from the site of TEM and influx of membrane from the lateral border recycling compartment (LBRC) to the site of TEM (36, 37, 39, 40). Both occur early in the process of transmigration and continue until TEM is complete (36, 41). Blocking either process reduces TEM by up to 90%, depending on the inflammatory model.

Mobilization of vascular endothelial cell cadherin (VE-cadherin)

VE-cadherin (cadherin-5, CD144) is a type I transmembrane protein that (after birth) is exclusively expressed in endothelial cells. It is concentrated at adherens junctions where it forms calcium dependent homophilic interactions and participates in barrier function and transendothelial migration (42–44). VE-cadherin surface expression is stabilized by its association with the cytoplasmic protein p120 catenin, and it can link to the actin cytoskeleton via its interaction with β-catenin and plakoglobin, members of the armadillo gene family (39, 45–47).

Adhesion of leukocytes to the endothelial cell activates downstream signaling pathways that induce VE-cadherin to be cleared from the site of transmigration to produce what appears as a gap in VE-cadherin staining along the junction (41, 48). VE-cadherin is internalized into clathrin coated vesicles under a variety of conditions (49–51). However, in the setting of TEM, VE-cadherin may not be internalized but rather pushed aside along the plane of the membrane and diffuse back to refill the junction once transmigration is complete (39, 41).

Under resting conditions, the vascular endothelial specific protein tyrosine phosphatase (VE-PTP) is associated with VE-cadherin via plakoglobin, maintaining it in a hypophosphorylated state. Interaction of activated endothelial cells with leukocytes triggers dissociation of VE-PTP from VE-cadherin, allowing the latter to be phosphorylated (52, 53). Engagement of endothelial ICAM-1 and VCAM-1 by leukocyte integrins transduces signals that phosphorylate VE-cadherin and its associated catenins (54–57). Phosphorylation of VE-cadherin at its cytoplasmic tail results in the weakening of the junctions as the cytoplasmic tail is uncoupled from the actin cytoskeleton. Two key tyrosine residues, Y658 and Y731 on the cytoplasmic tail of VE-cadherin have been implicated in this process (40, 58). (Note that Y658 is distinct from the Y685 implicated to have a role in permeability in response to soluble mediators (58).) The Vestweber group recently showed that mice in which VE-cadherin was replaced with the Y731F mutant to mimic the dephosphorylated state of VE-cadherin had a specific defect in leukocyte transmigration (58).

Targeted recycling of membrane from the LBRC

When a non-blocking monoclonal antibody against platelet/endothelial cell adhesion molecule 1 (PECAM, CD31) is added to endothelial cells at 37°C, it is seen by

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immunoelectron microscopy not only along the endothelial border but also in a subjunctional structure of interconnected 50 nm vesicles and tubules (36). PECAM from this compartment exchanges with the surface membrane at the cell border with a half-time of approximately 10 minutes. Therefore this compartment was called the Lateral Border Recycling Compartment or LBRC (36). The LBRC is contiguous with the plasma membrane, suggesting that this compartment is a complex invagination of the junctional membrane that extends several hundred nanometers into the cell. Interestingly, the compartment is labeled well at 37°C, but incubating endothelial cells with the same antibodies at 4°C only labeled the junction (36, 59). Electron micrographs clearly show that the compartment still exists under this condition, indicating that PECAM in the LBRC is protected from large molecules at 4°C. This is not because the vesicles have pinched off, as protons and small molecules such as biotin can still enter (36, 60). Under resting conditions roughly 30% of the total PECAM is in the LBRC (36).

The LBRC is distinct from other endothelial vesicular organelles such as the vesiculo-vacuolar organelle (VVO) and caveolae. VVOs are much larger (>150 nm) in size, more heterogeneous in shape, and typically communicate between the apical and basal surfaces (61). VVOs open and close in response to VEGF whereas the LBRC is completely and continuously accessible to the exterior of the cell (62). VVOs have not been observed in vitro. Likewise, caveolae are rare in endothelial cells in vitro and are typically observed as single vesicles or single invaginations on the apical and basal membranes, seldom present at the junction. By immunofluorescence, caveolin-1 (a marker of both caveolae and VVOs (63, 64)) does not co-localize with PECAM at the junction or during TEM (14, 36). In addition, biochemical analysis of PECAM and caveolin-1 shows that the two localize to different membrane microdomains (36). This was examined using sucrose gradients to purify cholesterol-rich membrane microdomains from endothelial cells solubilized with cold non-ionic detergent (36). In this analysis, caveolin-1 was predominantly recovered in the buoyant fractions that correspond to lipid rafts whereas PECAM was recovered from the dense fractions which contained the solubilized proteins that are excluded from membrane microdomains. (This is in distinction to monocytes, where activation of PECAM led to its phosphorylation and partition into cholesterol-rich microdomains (65) and in platelets where a small fraction of palmitoylated PECAM can be recovered in such domains (66).)

The LBRC also contains other molecules reported to positively regulate TEM: junctional adhesion molecule A (JAM-A) (14), CD99 (14), PVR (59), and nepmucin (67), which regulates lymphocyte TEM in high-endothelial venules. The LBRC is also observed in vivo as EM examination blood vessels from mice injected intravenously with anti-mouse PECAM-HRP shows an identical sub-junctional staining pattern (unpublished results).

The same immunoelectron microscopy experiments show that VE-cadherin, a molecule that negatively regulates TEM, is not in the LBRC (14, 59). We have recently developed a way to isolate the LBRC from endothelial cells (68). A proteomic approach was taken to identify proteins unique to or enriched in the LBRC. These studies confirmed that caveolin-1 is not a component of the LBRC and revealed that in addition to VE-cadherin, its associated catenins were also excluded from the LBRC. We were unable to identify any unique proteins in the

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LBRC; however, both IQ motif GTPase-activating protein 1(IQGAP1) and vimentin were specifically enriched in the LBRC fraction (69).

We recently used chimeric molecules of CD25, PECAM, and VE-cadherin to study the signals for inclusion of membrane proteins into the LBRC (60). Inclusion of proteins from the lateral border of the endothelial cell plasma membrane into the LBRC seems to be the default pathway. VE-cadherin is actively excluded by its homophilic interaction motif (RVDAE). Removal of this domain from VE-cadherin or competition with a soluble RVDAE peptide allowed its entry into the LBRC. This explains why no unique proteins were found in the isolated LBRC and enriched proteins were associated with the cytoplasmic side (69). This study also demonstrated that the constituents of the LBRC move in concert during TEM; there is no separation of membrane enriched in PECAM or CD99 (60).

During TEM, the LBRC moves along microtubules to engage the transmigrating leukocyte in a process called ‘targeted recycling.’ This is a critical step in diapedesis. Disrupting PECAM-PECAM homophilic interactions ablates both TEM and targeted recycling (36). The enrichment of the LBRC at the interface with the leukocyte is similarly blocked when the endothelial cells are treated with reagents demecholcine or taxol, which depolymerize microtubules or cause microtubule bundling, respectively (37). Neither treatment affected the size or the constitutive recycling of the compartment suggesting instead that microtubules are essential for LBRC movement during TEM. This is supported by the finding that LBRC targeted recycling is also impaired upon microinjection of function blocking antibodies against the conserved motor domain of kinesin, a molecular motor that facilitates intracellular traffic along microtubules toward the plus end (37).

There are 45 kinesin heavy chains that comprise 15 kinesin families. We recently found that knockdown of kinesin-1 with shRNA or blockade by intraendothelial cell injection of a blocking monoclonal antibody specific for kinesin-1 arrested targeted recycling of the LBRC and TEM of monocytes to 10–15% of control levels (70). Kinesin-1 associates with several known light chains, each of which has multiple isoforms generated by alternative splicing. Light chains assist in binding of cargo to the kinesin heavy chain and the alternatively spliced carboxy terminal ends help define the cargo specificity (71–74). We found that kinesin light chain 1 (KLC1) was the light chain responsible for targeted recycling of the LBRC and surprisingly, a single splice form of KLC1, KLC1 variant 1 (also known as KLC1C) was the major—perhaps the only—isoform involved: Knockdown of KLC1C alone blocked targeted recycling of the LBRC and TEM maximally; re-expression of KLC1C restored both. Other splice variants had no effect (70). These results were true for PMN as well as for monocytes.

Available data suggest that the molecules in the LBRC, not those on the surface of the junction are the ones that are required for TEM. When endothelial cells were treated with blocking antibodies against PECAM, PVR, or CD99 at 4°C, which leaves the protected LBRC pool of these molecules untouched, TEM remained unaffected, while the same antibodies given at 37°C (conditions under which they enter the LBRC) blocked TEM (59, 75).

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Relationship of VE-cadherin movement to LBRC targeted recycling

Removal of VE-cadherin from the endothelial junction at the site of TEM and targeted recycling of the LBRC to the site of TEM are required for efficient TEM. Are these processes independent, cooperative, or sequential? Does one depend on the other? We recently investigated this question for transmigration of both PMN and monocytes (76). By reversibly depolymerizing microtubules in endothelial cells or injecting a function-blocking kinesin antibody, we were able to inhibit targeted recycling and TEM, as previously published (37). Under these conditions VE-cadherin was not cleared from the membrane underneath the adherent leukocytes (76). We then performed the converse experiment, transfecting endothelial cells to express a form of VE-cadherin with tyrosines 658 and 731 mutated to phenylalanine so they could not leave the junction (40). Under these conditions, TEM was blocked; however, targeted recycling of LBRC still took place (76). Thus, targeted recycling of the LBRC is triggered and can occur in the absence of movement of the LBRC.

This suggests that LBRC movement occurs first in response to leukocytes positioned at the endothelial border and may actually participate in moving VE-cadherin-containing membrane out of the way (68, 76). Recall that the LBRC does not contain VE-cadherin; in fact it is actively excluded from the LBRC (60). The influx of LBRC membrane to surround the leukocyte could either dilute or push aside VE-cadherin and its associated catenins. When the leukocyte has passed across the endothelium, retrieval of this membrane back into the LBRC would allow VE-cadherin to become enriched at the junction again by exclusion from the LBRC or by lateral movement along the plasma membrane. This would allow junctions to re-form rapidly without having to reassemble their complex three dimensional structure (1, 60, 68, 76). In fact, not only adherens junctions but also tight junctions reform very rapidly after transmigration (77).

The presence within the LBRC of the molecules that positively regulate TEM suggests that it could act as a reservoir of these molecules for TEM as well as a reservoir of junctional membrane. By simple geometry, about 2/3 of the membrane surface area necessary to surround the transmigrating leukocyte could come from the junctional plasma membrane at the cell borders immediately adjacent to the leukocyte (68). The additional 1/3 could come from the LBRC; this is precisely the fraction of membrane that resides in the LBRC (36). In this way, transmigration could proceed without retraction of the endothelial cells.

Initiating transmigration: Major signaling events

When leukocytes arrive at what will become the site of transmigration on the apical surface of the endothelial cell, at least two critical signaling events occur: 1) PECAM on the leukocyte engages PECAM at the endothelial cell border and 2) there is a transient increase in cytosolic free calcium ion concentration ($\uparrow[Ca^{+2}]_i$) in the endothelial cells (1, 3, 78, 79). Interfering with either process results in a similar phenotype: Neutrophils (80) or monocytes (81) arrested over the endothelial cell borders tightly adherent, but unable to transmigrate (75). Live imaging (82) confirms what was suspected from transmission and scanning electron micrographs (75, 83): that the leukocytes are actively moving along the endothelial borders probing with lamellapodia, as if looking for a spot to transmigrate. There is no

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change in the ability of neutrophils, monocytes, or lymphocytes to adhere to the endothelial cells or move to the junctions; only TEM is inhibited (37, 75, 84).

The initial reports of these phenomena were published the same year (75, 80). Despite the similarity of these phenotypes, *in vitro* studies did not show a connection between PECAM ligation and ↑ $[Ca^{+2}]_i$ in endothelial cells (85)(and unpublished results from our lab) that could be detected by calcium sensitive fluorescent dyes *in vitro*. However, recently a link has been established.

PECAM-PECAM engagement

PECAM is comprised of six extracellular immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tail containing two immunotyrosine inhibitory motifs (ITIM) (86, 87). Interaction of the homophilic interaction domain (the amino-terminal immunoglobulin family domain 1) (83, 88) of PECAM on the leukocyte with PECAM domain 1 on the endothelial cell is required for TEM (75, 83). Monoclonal antibodies recognizing this domain (75) or PECAM domain 1-IgG fusion proteins (83) block TEM of PMN and monocytes *in vitro* and *in vivo* (89–91). Incubating either leukocytes or endothelial cells with anti-PECAM antibody blocks equally efficiently, and as well as blocking both together, consistent with a homophilic mechanism (75).

Activating PECAM in platelets, lymphocytes, and endothelial cells results in phosphorylation of the ITIM domain tyrosines 663 and 686 (87, 92–94). PECAM has no endogenous tyrosine kinase activity, but is phosphorylated by src and fer family kinases (95–97) to recruit src homology region 2 domain-containing tyrosine phosphatase 2 (SHP-2) (98–100). In T cells and platelets, it has an inhibitory role (92, 101, 102). In most of these cases, tyrosine 686 is the critical phosphorylation site with 663 playing a secondary role.

It therefore came as a surprise when we found that when interacting with monocytes, phosphorylation of tyrosine 663 on endothelial cell PECAM was critical for TEM; tyrosine 686 phosphorylation was not necessary (103). Gain of function experiments showed that expression of PECAM containing tyrosine 663 in ECV-304 cells could impart the ability to support TEM even if tyrosine 686 was mutated to phenylalanine (Y686F). On the other hand, expression of PECAM with Y663F could not support TEM. In this case, TEM was rescued by transfection of PECAM in which Y663 was not mutated (103). Mechanistically, wild-type PECAM and PECAM Y686F could enter the LBRC and undergo targeted recycling. On the other hand, PECAM Y663F had difficulty entering and leaving the LBRC and was not targeted efficiently to the site of monocyte interaction with the endothelial junction (103).

The mechanism by which phosphorylation of tyrosine 663 on the cytoplasmic tail of PECAM affects targeted recycling of the LBRC is not known. To be honest, we do not even know that phosphorylation is necessary. We know that mutation of tyrosine 663 to phenylalanine inhibits the process (103). It is possible that an unmodified tyrosine residue at that position is required for efficient targeted recycling. In a somewhat related manner, de-phosphorylation of mouse VE-cadherin tyrosine 731 is thought to be critical for VE-cadherin movement during TEM (58).

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Transient increase in endothelial cell cytosolic free calcium ion concentration (↑ $[Ca^{2+}]_i$)

As mentioned above, pharmacological chelation of endothelial Ca^{2+} during TEM results in a phenotype in which leukocytes adhere normally to the apical surface of endothelial cells, but are unable to transmigrate across (33, 80, 81, 104, 105) similar to blocking PECAM homophilic interactions with primary antibody (75), suggesting that these two processes might be related.

Further evidence came when we found that chelation of endothelial Ca^{2+} inhibited targeted recycling of the LBRC to the same extent that it inhibited TEM (84). Transient receptor potential canonical family member six (TRPC6) is a non-selective cation channel that is five-fold more permeable to Ca^{2+} than Na^{+} (106–108). It is the most abundant TRPC channel in endothelial cells (109, 110) and is concentrated along endothelial cell borders, where it seems to be further enriched in the endothelial membrane surrounding transmigrating PMN (84).

Expression of a dominant negative TRPC6 or knockdown of endogenous TRPC6 in human umbilical vein endothelial cells dramatically reduced targeted recycling of the LBRC and TEM of PMN and monocytes (84). Both processes were restored in endothelial cells by expression of a rescue construct expressing TRPC6 resistant to the shRNA (84). In TRPC6-deficient mice reconstituted with wild-type bone marrow, PMN were still recruited to a site of inflammation in the skin, but there was a selective defect in transmigration (84). PMN were arrested on the luminal surface of postcapillary venules, similar to the phenotype seen when PECAM was blocked (89, 91, 111, 112). The block was qualitatively and quantitatively similar to that achieved in the global TRPC6 knockout, demonstrating that absence of TRPC6 in endothelial cells was primarily responsible for the effect (84). TRPC6-deficient mice have previously been shown to have defects in the inflammatory response (106, 113–122). This work provides a mechanism to explain the failure to recruit leukocytes in these models.

Transendothelial migration as a multistep process

After the LBRC has been recruited to the site of TEM via PECAM-PECAM interactions, and VE-cadherin has left the site of TEM, migration still requires additional molecular interactions. This is demonstrable because one can selectively and reversibly block the transmigration step using antibodies against specific molecules. In a sequential transmigration assay (Fig. 2), one can block TEM using monoclonal antibody (mAb) against molecule A for an appropriate time, then wash out the block, add a mAb against molecule B, and resume the TEM assay. If molecule B is involved in a process downstream of A, mAb B will block any further TEM. If molecule B is involved in a step of TEM before molecule A, it will not (59, 68, 123). This was first shown for the relationship of PECAM and CD99 (123). CD99 is expressed at the borders of EC and is in the LBRC (14). If TEM is blocked by anti-CD99 monoclonal antibody, the initial round of PECAM-mediated targeted recycling takes place (124), and PMN (124, 125) or monocytes (123, 124) begin TEM, but cannot complete it. They are stuck partway through, with their leading edge below the endothelial monolayer, but their uropod remaining on the apical surface (123–125). Recent

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studies showed that after the initial round of targeted recycling, further targeted recycling does not occur under these conditions (124).

The fact that two distinct molecules regulate different steps in TEM begs the question of whether there are other molecules that mediate additional steps in the process. In fact, poliovirus receptor (CD155, PVR), expressed at EC borders, is involved in TEM of monocytes (126). Using the sequential TEM assay, we found that PVR, interacting with the leukocyte ligand DNAX accessory molecule-1, controls a step in TEM that is between those regulated by PECAM and CD99 (59). PVR is also in the LBRC and, as expected, PVR mAb delivered to the LBRC blocked TEM (59).

Completing transmigration: The role of CD99

CD99 is a type 1 membrane protein concentrated at the cell borders of endothelial cells and expressed diffusely on the surfaces of circulating blood cells to varying degrees. It is highly glycosylated by O-linked carbohydrates, which account for 44% of its apparent molecular weight of 32 kD. It is a fairly unique molecule with only one paralog in the genome (127) and no signaling motifs on its cytoplasmic tail. As mentioned above, inhibition of homophilic interaction between CD99 on the leukocyte and CD99 on the endothelial cell arrests TEM with the leukocyte partway across the endothelial junction with its leading lamellipodium under the EC and a trailing uropod on the apical surface (123, 125), in contrast to the appearance of leukocytes blocked at the PECAM-dependent step, which appear entirely on the apical surface hovering over the junctions (75).

CD99 is constitutively in a complex at the endothelial cell border with three intracellular molecules: the A-kinase anchoring protein ezrin, the unique soluble adenylate cyclase (sAC), and protein kinase A (PKA). Engagement of CD99 activates sAC to produce cAMP locally to activate PKA and orchestrate the next wave of LBRC recruitment (124). An evolutionarily conserved basic region in the juxtamembrane region of the cytoplasmic tail is critical for maintaining CD99 in this complex. Mutation of these lysine residues to glutamate abolished the ability of CD99 to form a complex with ezrin and abolished the ability of the endothelial cells to support TEM (124).

In vivo as well as *in vitro* a small molecule inhibitor of sAC (KH7) and endothelial cell specific knockout or knockdown of sAC inhibited PMN and monocyte TEM in a model of acute inflammatory dermatitis (124). The phenotype of the block appeared identical to the blockade imparted by anti-CD99 mAb (123–125).

Completing transmigration: Membrane and cytoskeletal movements

Following TEM, the junctions must reseal and tight apposition of adjacent endothelial cells must be re-established. Unfortunately, relatively little is known about how adherens and tight junctions rapidly reform following TEM (77). Nothing is known about retrieval of the LBRC; is it continuously recycled during the leukocyte passage or is it retrieved in one large membrane movement at the end?

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How do the endothelial transmigration pores close? The increase in isometric tension in endothelial cells that has been reported to follow ↑ $[Ca^{2+}]_i$ due to phosphorylation of myosin light chain kinase was thought to help open the endothelial borders in preparation for TEM (128). Recently, this tension was shown to come during the second half of TEM when the transmigration pore was decreasing in size (129). This corresponded to contraction of f-actin fibers surrounding the transmigration pore and an increase in RhoA activity as registered by RhoA activity sensors (129). The authors hypothesize that during the initial phase of TEM, vascular leakage across the transmigration pore is minimized because the leukocyte is actively pushing against the endothelial cells to open a passage for TEM (129). In addition, continuous interaction of adhesion molecules from the LBRC with ligands on the leukocyte would help maintain tight apposition (68).

What about movement of membrane after pore closure? As a result of the loss of isometric tension in the endothelial monolayer, lamellipodia form on the ventral surface of the endothelial cells and move across the substratum to contact the neighboring endothelial cell and seal the “wound.” This process was effective for both paracellular and transcellular migration and required several Rac1 effectors as well as localized production of hydrogen peroxide (130).

Transcellular Migration

Although it is well accepted that leukocytes cross the endothelium at the cell borders (paracellular route) there is increasing evidence that leukocytes can also pass directly through endothelial cells (transcellular route). Much of the original evidence was indirect, based on single transmission electron micrographs that appeared to show leukocytes deeply indenting endothelial cells and/or passing across endothelial cells through a membrane-lined channel next to an intact junction (131–133). However, endothelial junctions are serpentine, and it was possible that the leukocyte was passing through a less structured junction (18). For a nice historical review of transcellular migration the reader is referred to reference (19). Transcellular migration was viewed with skepticism despite (or perhaps because of) the large amount of circumstantial evidence that supported it. The skeptics were to a great extent appeased by a paper published by the Dvorak lab in 1998 (15). This provided arguably the first indisputable evidence of neutrophil transcellular migration *in vivo.* In these studies neutrophil emigration was stimulated by direct injection of fMLF, a neutrophil chemoattractant and β2 integrin activator, into the skin of guinea pigs (15). The authors presented a collection of electron microscope serial sections in which neutrophils were shown to pass entirely across an endothelial cell without ever contacting a recognizable junction (15). Although the absolute frequency of transcellular migration was not addressed in this study, it demonstrated that transcellular migration was possible *in vivo*. High resolution intravital microscopy studies in which endothelial cell borders are labeled with non-blocking anti-PECAM antibody and PMN express eGFP have since demonstrated that transcellular migration does indeed occur under physiological conditions, but accounts for ≤ 10% of TEM events (13).

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Molecules implicated in transcellular migration

Recently, several *in vitro* models were established that produced reliable transcellular migration (14, 33, 35, 134). These provided data on the molecules and mechanisms involved in this process. Many of the same molecules important for paracellular migration turn out to have a role in transcellular migration.

During transcellular TEM, ICAM-1 that is uniformly expressed on the luminal surface of HUVEC redistributes and is concentrated at the site of diapedesis. Furthermore, it is enriched in the membrane channel that surrounded the crossing leukocyte as it went through the EC body (14, 22, 33, 35, 134). However, for transcellular migration, as for paracellular migration, some investigators (27, 35, 37) do not observe the “docking structures” or “transmigratory cups” formed by projections of ICAM-1 above the plane of the endothelial cell membrane reported by others (29, 33).

In addition to ICAM-1, other molecules normally thought of to be restricted to the cell borders, including PECAM, JAM-A, and CD99 are seen around the leukocyte migrating transcellularly (14, 22, 33, 35, 134). In all cases, VE-cadherin is absent (See Fig. 1). These molecules are not only present, but appear to be functional. Somewhat surprisingly, transcellular migration was found to be dependent on PECAM and CD99. Blocking antibodies arrest transcellular migration (14). The reason for this may be explained by the mechanism, discussed below.

What determines the site of transmigration?

Whether the leukocyte migrates paracellularly or transcellularly may depend on the relative tightness of the endothelial junctions and the ability of the leukocyte to breach them. Carman and Springer (16) speculated that the leukocytes take the “path of least resistance” across the endothelium. If junctions are very tight, as for example, in the blood brain barrier, migrating across the cell at a thin point may be easier, as seen in cerebral inflammation (20, 135). However, that cannot be the only explanation since postcapillary venules, which are the sites of most inflammatory diapedesis (including transcellular migration (15)) have relatively leaky junctions. In fact, they are specialized for permeability, since this is the site of fluid re-uptake from the interstitium (136). Endothelial cells in culture, where transcellular migration has been best demonstrated, form a monolayer of low electrical resistance. When grown under conditions that result in ten-fold higher resistance, the frequency of transcellular TEM was no higher than under standard conditions (137).

Transcellular migration may also occur if the leukocyte has difficulty reaching the junction, such as cells in which CD11b is non-functional (82) or deficient (138). Phillipson, *et al.* (138) reported that neutrophils deficient in CD11b/CD18 adhered to the endothelium at a site of inflammation, but transmigrated poorly due to their inability to locomote along the endothelial surface. However, those that transmigrated tended to go transcellularly, perhaps because they achieved sufficient activation to transmigrate before they reached the cell border. Similarly, T cells deficient in the Rac activator Tiam1 are deficient in polarization and locomotion on endothelium, and tend to migrate transcellularly (139).

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The role of leukocyte activation

We hypothesize that for low-resistance endothelia at least, transcellular migration may occur when leukocytes are highly and/or directly activated. The first indisputable evidence of neutrophil transcellular migration *in vivo* came from studies in which emigration was stimulated by direct injection of fMLF, a neutrophil chemoattractant and β2 integrin activator, into the skin of guinea pigs (15). The published in vitro studies of transcellular migration used MCP-1, platelet activating factor, or SDF-1 to stimulate migration of monocytes, neutrophils, and T cells, respectively (33) or employed mitogen-activated T lymphoblasts. (16, 134) Furthermore, these agents were added to the apical side of the endothelium where they would activate leukocytes, but not provide a chemotactic gradient. In the standard TEM assay system in our lab (140), paracellular migration predominates and transcellular migration is almost never seen across cytokine activated HUVEC. However, when we applied chemokine or chemoattractant to the apical side of endothelial monolayers, 10 to 30 % of leukocytes migrated transcellularly (14).

Leukocyte activation would promote polymerization of actin in their lamellipodia, which has been associated with transcellular migration in vivo (15) and shown to be necessary for transcellular migration in vitro (22). Leukocytes probe the apical surface of endothelial cells with “invasive podosomes” that contain polymerized actin. These deeply invaginated the surface in the areas of eventual transcellular migration (22).

The role of the Lateral Border Recycling Compartment

Membrane vesicles were seen accumulating in the region of transcellular migration (22). Millan *et al.* reported that caveolin-1 accumulated in the regions of transcellular migration implying that these might be caveolae (134). However, other groups did not see any enrichment in caveolin-1 at the site of diapedesis (14, 22, 33).

Mamdouh, *et al.* reported that the Lateral Border Recycling Compartment (LBRC) was critical for transcellular migration of leukocytes (14), similar to its involvement in paracellular migration (Fig. 3). The LBRC membrane components PECAM, CD99, and JAM-A moved in concert to surround neutrophil and monocytes migrating transcellularly. The source of the membrane was demonstrated to be the LBRC, and similar to paracellular migration (37), was dependent on functioning microtubules (14). Microtubule depolymerizing agents blocked targeted recycling and blocked transcellular as well as paracellular migration (14).

Using transfected cell lines, Yang, *et al.* showed that overexpression of ICAM-1 promoted transcellular migration (35), implicating a role for ICAM-1 in this process. However, when LBRC recycling was inhibited, there was no effect on ICAM-1 enrichment around adherent leukocytes, even though both paracellular and transcellular migration were blocked (14). Therefore, while enrichment of ICAM-1 may help promote transcellular migration, and may even be a necessary prerequisite, it is not sufficient to promote transmigration in the absence of a functional LBRC.

The role of the LBRC in transcellular migration explains a number of observations made by several groups of investigators. The appearance in electron micrographs of membrane

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vesicles clustered around the leukocyte as it transmigrates (22) is consistent with recruitment of the LBRC. Many of the studies that provide evidence for transcellular migration *in vivo* often show electron micrographs of leukocytes passing through the cell within a micron or two of an intact endothelial junction (20, 135). This is exactly where the LBRC is situated and would put it in a prime location for its role in promoting transcellular as well as paracellular migration. Transcellular migration is also dependent on PECAM and CD99 (14). Since expression of PECAM and CD99 on the cell surface is essentially restricted to the cell borders, this was unexpected. However, once the LBRC is recruited and becomes part of the membrane forming the transmigration pore, interaction of leukocyte and endothelial cell PECAM and CD99 is required for transcellular passage.

Carman *et al.* (22) showed leukocytes appearing to probe the endothelial surface with lamellipodia (podosomes), often deeply invaginating the surface of the endothelial cell. They were highly enriched for actin filaments (15, 22), which were required for their function (22). Actin polymerization and lamellapodia formation are events triggered in leukocytes by integrin activation, so prominent podosomes in cells migrating transcellularly could be manifestations of high levels of leukocyte activation. The podosomes were hypothesized to initiate the formation of the transcellular channel. In previous reports such podosomes were observed invaginating the endothelial surface away from the junctions even under conditions where leukocytes crossed at the cell borders (141, 142) as well as under conditions where leukocytes are blocked in their attempts to cross at the junctions (Figure 4a in ref (83)). These lamellapodia may be a general mechanism for leukocytes to crawl across the endothelial surface. However, if they have a role in promoting transmigration, it is conceivable that they signal the recruitment of LBRC membrane to the leukocytes when they are at the cell borders as well as when they are not. In this case, the mechanisms of paracellular and transcellular migration would have even more in common.

At least one potential mechanistic difference between paracellular and transcellular migration remains. Since the LBRC is connected to the lateral endothelial cell surface at the cell borders, fusion of the LBRC membrane with the plasma membrane is not necessarily required to bring the LBRC in contact with the leukocyte for paracellular TEM. On the other hand, to bring this membrane compartment in contact with the leukocyte on the apical surface for transcellular migration would require membrane fusion. In fact, Carman *et al.* (22) provide evidence that membrane fusion is required for transcellular migration. However, since the LBRC vesicles are connected to each other (36), rather than multiple fusion events involving dozens or hundreds of vesicles, the entire surface area of the LBRC could be brought to surround the leukocyte with perhaps only two fusion events necessary: One at the apical surface and one at the basal surface of the endothelial cell.

The final frontier

Once the leukocyte has passed across the endothelial barrier, it still must cross the subendothelial basal lamina. This process generally requires far more time (13). Norshargh and colleagues have demonstrated that PMN (143) and monocytes (144) move between the abluminal surface of the endothelial cell and the basal lamina searching for areas where collagen IV and laminin are deposited at low density. These correspond to areas where there

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is a gap in pericyte coverage. Leukocytes exit to the interstitium in these zones. Migration to these sites is aided by interactions between LFA-1 on the leukocyte and ICAM-1 on pericytes (145). When modeled *in vitro*, there is a role for heterophilic interaction of domain 6 of leukocyte PECAM with some component of the basement membrane in this process, inasmuch as blockade of domain 6 selectively blocks migration across the basement membrane, but not across the endothelial cell (83). In vivo, PECAM-deficient leukocytes in the C57BL/6 strain show a delay in migration across the basal lamina (146), which is also associated with a failure to exteriorize α6β1 integrin, the receptor for laminin (147).

Unanswered Questions

As in other areas of science, the more we learn about neutrophil TEM, the more we realize there is to learn. Pursuing these questions will provide opportunities for future research and will ultimately lead to a more complete understanding of the inflammatory response.

Neutrophil Activation

Anyone who has ever isolated PMN from peripheral blood knows how easy it is to activate them. It seems that just looking at them the wrong way will cause them to clump and adhere to the walls of the centrifuge tube. How, then, do they get activated enough to adhere to the endothelial surface, locomote to the junctions and transmigrate (which involves squeezing the entire cell through a pore that is maximally 4–6 μm wide (129)) but remain quiescent enough not to degranulate until they enter the inflamed tissue? One presumes that there are bidirectional activating and calming signals exchanged between the PMN and endothelial cell, but the nature of these is not known.

The LBRC

Much remains to be learned about the LBRC: What are the directional signals that recruit it to the site of TEM? Are these the same for PECAM, PVR/DNAM-1, and CD99 interactions? What happens to the membrane after targeted recycling; how is membrane retrieved for another round of targeted recycling? What is the purpose of constitutive recycling? We hypothesize that it may be a mechanism for endothelial cells to adapt to rapid and/or cyclical changes in junction surface area as vessels expand and contract during the cardiac cycle (arterial circulation) or in response to changes in tonicity or fluid volume (venous circulation) (60).

The presence in the LBRC of all the molecules that positively regulate TEM and the fact that they move together during TEM (60) begs the question of how these molecules can function sequentially in TEM? This is a focus of active research in our lab.

Mouse strain differences

It is well known that biological responses vary among mouse strains, which are variably resistant to many pathogens and disease models. Transendothelial migration is no exception. The vast majority of mouse strains respond to PECAM blockade or deficiency with a phenotype that resembles what is found with human cells—leukocytes arrested on the apical surface of the junctions, tightly adherent, but unable to transmigrate efficiently (111, 112,

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148). However, the most frequently used mouse strain in immunology, C57Bl/6, has a different phenotype: Depending on the inflammatory model, PMN and monocytes are either not inhibited from transmigrating or are transiently arrested between the abluminal surface of the endothelium and the underlying basement membrane (112, 146, 148–151). A similar phenomenon is seen with CD99, although it has been less-well studied (123, 151–155).

The reason for the strain differences are not known, and the only reason to pursue understanding this discrepancy would be to gain insight into the regulation of TEM. In this regard, while the phenotype of PECAM or CD99 block is different among strains when comparing the effect of antibody blockade or genetic deletion of these molecules themselves, when interrupting the signaling pathways downstream of PECAM (TRPC6) and CD99 (sAC), the effect is consistent: The TRPC6-deficient mice in the C57Bl/6 strain showed a phenotype similar to other strains in which TEM was blocked by anti-PECAM antibody (84). Similarly, sAC knockout mice in the C57Bl/6 background showed a phenotype similar to other strains in which TEM was blocked by anti-CD99 antibody (124).

Great progress has been made in uncovering the molecules and mechanisms that govern TEM. It is likely that the pace of discovery will increase with better and faster systems for genetically manipulating mice and cells, better imaging techniques, and better reporter molecules for detecting signal transduction in living cells. Many of the unanswered questions above will be answered, although new ones will arise as we get closer to fully understanding the acute inflammatory response and the role of the neutrophil.

Acknowledgments

This work was supported by NIH Grants R01 HL046489 and R37 HL064774. I thank Dr. David Sullivan for drafting Figure 2.

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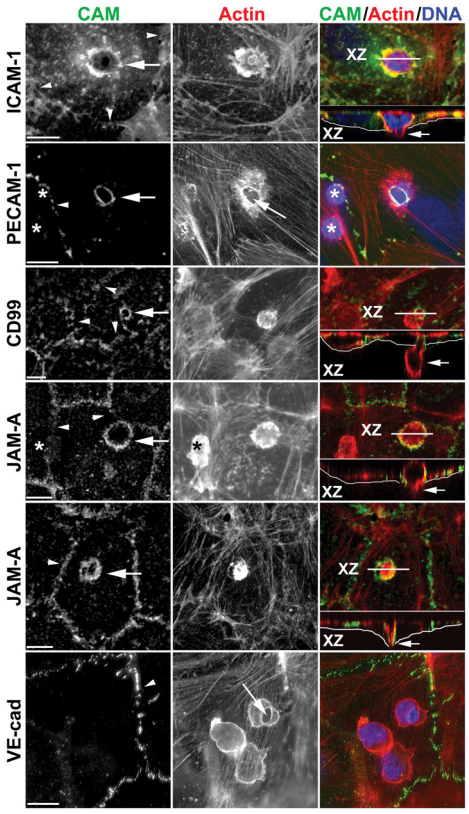

Figure 1. Enrichment of endothelial cell adhesion molecules around leukocytes migrating transcellularly

Representative confocal or deconvolved images of monocytes or PMN caught in the act of transcellular migration are shown stained with mAb for the indicated cell adhesion molecule (**CAM**, left panel, green in merge), phalloidin to mark **actin** (middle panel, red in merge), and DAPI to mark nuclei (blue in merge). Rings of enrichment of ICAM-1, PECAM, CD99, and JAM-A were consistently seen surrounding the site at which leukocytes migrated through the endothelial cell (arrows in left panel). Each event was verified by scanning through the monolayer. Orthogonal (X-Z) projections along the plane indicated by the white line (XZ) shown below the merged images demonstrate that the leukocyte was indeed crossing the endothelial cell (arrows in right panel). Thin white line underlies the basal surface of the endothlelial cell. The JAM-A panels show a monocyte (upper panel) and a PMN (lower panel). Note in the PECAM panel and upper JAM-A panel that several leukocytes are present on the endothelial cell, but only the site of transcellular migration stains for PECAM or JAM-A, respectively, demonstrating that the PECAM and JAM-A stained around the leukocyte belongs to the endothelial cell. (*) indicates leukocytes not in the act of transmigration and not stained for PECAM or JAM-A. Long arrow in PECAM and VE-cadherin panels point to the site of transcellular migration where endothelial cell actin is pushed aside. Arrowheads mark cell borders. Scale bar = 10 μm. Reproduced from (14) with permission.

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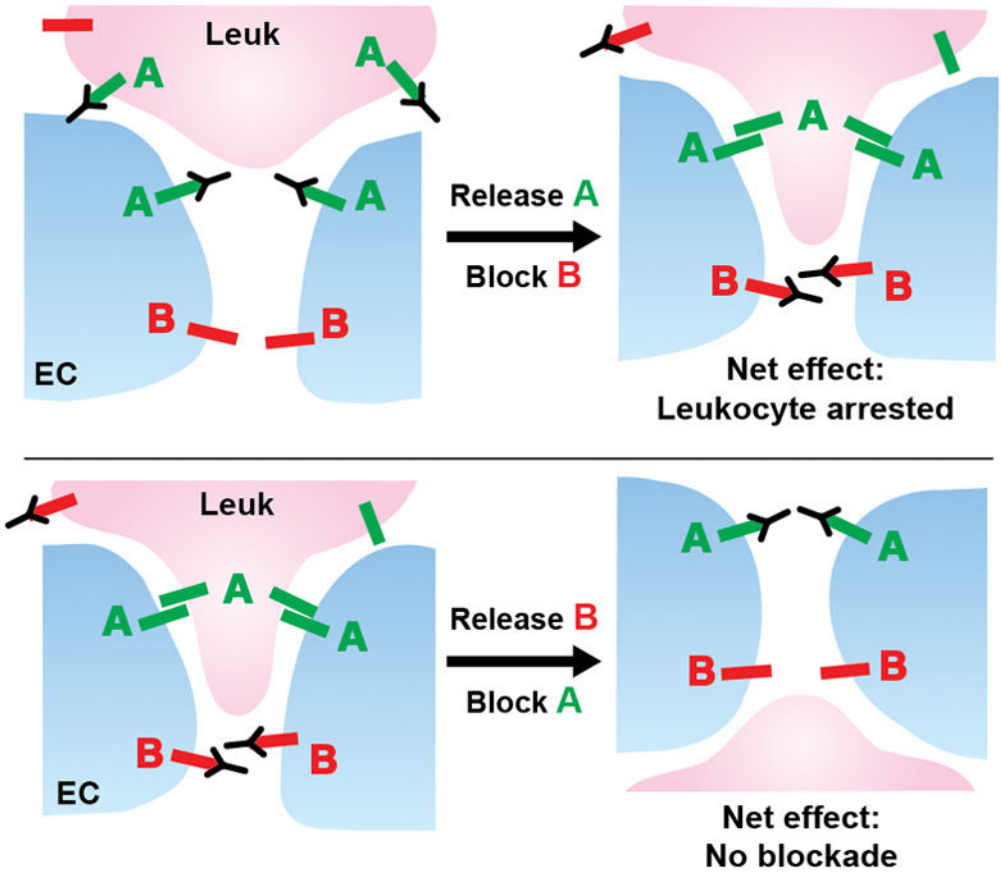

Figure 2. Schematic diagram of the sequential block assay

Molecules A and B on the EC regulate TEM in that order. (**Top**) If TEM is blocked by antibody against molecule A, and that block is released, antibody against B can still arrest TEM. (**Bottom**) If TEM is blocked by antibody against molecule B, and that block is released, TEM can no longer be blocked by antibody against A.

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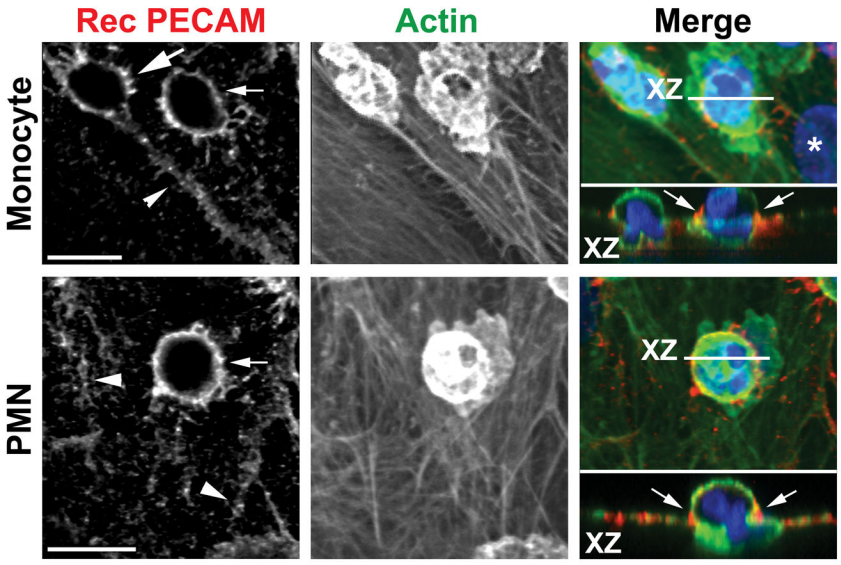

Figure 3. Targeted trafficking of membrane from the LBRC to surround leukocytes undergoing transcellular migration

Staining of recycled PECAM as a surrogate for recycling LBRC (left panel, red in overlay) surrounding monocytes (upper panel) and a PMN (lower panel) demonstrates movement of membrane from the LBRC to the endothelial surface. Recycled LBRC is seen around the leukocytes migrating transcellularly (thin arrows) far from the endothelial border (arrowheads). Recycling LBRC enrichment is as great around the monocyte migrating transcellularly as it is around the monocyte migrating paracellularly (thick arrow). Actin staining (middle panel, green in merge) shows relative positions of the cells. Orthogonal (X-Z) projection demonstrates leukocytes in the act of transcellular migration. Arrows indicate recycled PECAM around leukocytes. Bar = 10 μm. Reproduced from (14) with permission.