The American Journal of Pathology, Vol. 184, No. 4, April 2014

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

ROUS-WHIPPLE AWARD LECTURE

How Endothelial Cells Regulate Transmigration of Leukocytes in the Inflammatory Response

William A. Muller

From the Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, Illinois

CME Accreditation Statement: This activity ("ASIP 2014 AJP CME Program in Pathogenesis") has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the American Society for Clinical Pathology (ASCP) and the American Society for Investigative Pathology (ASIP). ASCP is accredited by the ACCME to provide continuing medical education for physicians.

The ASCP designates this journal-based CME activity ("ASIP 2014 AJP CME Program in Pathogenesis") for a maximum of 48 *AMA PRA Category 1 Credit(s)*™. Physicians should only claim credit commensurate with the extent of their participation in the activity.

CME Disclosures: The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose.

Accepted for publication December 9, 2013.

Address correspondence to William A. Muller, M.D., Ph.D., Magerstadt Professor and Chairman, Department of Pathology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave., Chicago, IL 60611. E-mail: wamuller@northwestern.edu.

Leukocytes attach to vascular endothelial cells at the site of inflammation via a series of intercellular adhesive interactions. In a separate step in leukocyte extravasation, transendothelial migration is regulated by molecules that play no role in the preceding steps of tethering, rolling, adhesion, and locomotion. Transendothelial migration itself can be dissected into a series of distinct interactions regulated sequentially by molecules concentrated at the endothelial cell border; these include platelet/endothelial cell adhesion molecule, poliovirus receptor (CD155), and CD99. These molecules are components of the lateral border recycling compartment (LBRC), a perijunctional network of interconnected tubulovesicular membrane that traffics to surround the leukocyte as it passes across the endothelial cell. This targeted recycling of LBRC requires kinesin to move the membrane along microtubules, and interfering with LBRC trafficking blocks transmigration of neutrophils, monocytes, and lymphocytes. The LBRC is also recruited to mediate transcellular migration when that occurs. Movement of the LBRC is coordinated with events on the luminal surface, such as clustering of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 under the migrating leukocyte, as well as movement of vascular endothelial cadherin and its associated catenins out of the junction at the site of transendothelial migration. How these events are coordinated is not known, but their regulation shares common signaling pathways that may serve to connect these steps. *(Am J Pathol 2014, 184: 886–896; http://dx.doi.org/10.1016/j.ajpath.2013.12.033)*

Transendothelial Migration

The Point of No Return in the Inflammatory Response

The inflammatory response, the body's rather stereotyped response to tissue damage of any kind, evolved to heal wounds and fight infections. However, recent improvements in human health mean that we are living long enough to experience the other side of the double-edged sword of inflammation. There has not been time enough on an evolutionary scale to select against the baggage that comes with our ability to heal wounds and kill foreign microorganisms. Inflammation is at the root of virtually all pathology. Thus, there has been great interest in understanding how inflammation is regulated and so to design pharmaceutical and other interventions to control it.

Supported by NIH grants R01-HL046849 and R37-HL064774.

The Rous–Whipple Award is given by the American Society for Investigative Pathology to a senior pathologist with a distinguished career in experimental pathology research and continued productivity at the time of the award. William A. Muller, recipient of the 2013 ASIP Rous–Whipple Award, delivered a lecture entitled "How Endothelial Cells Regulate Transmigration of Leukocytes in the Inflammatory Response," on April 21, 2013, at the annual meeting of the American Society for Investigative Pathology in Boston, MA.

Disclosures: None declared.

http://dx.doi.org/10.1016/j.ajpath.2013.12.033

Acute inflammation involves soluble and cellular mediators that act over the first minutes to hours of the inflammatory response. At the site of inflammation, generally in postcapillary venules, inflammatory mediators such as histamine secreted by mast cells cause local endothelial permeability, allowing preformed agents such as antibody and complement to cross endothelial junctions into the tissue. It is a common misconception that the increase in vascular permeability is causally related to increased leukocyte extravasation. Although these phenomena are both part of the acute inflammatory response, peak vascular leakage occurs well before leukocyte extravasation begins.[1] Furthermore, the gaps between endothelial cells created by these agents measure only a few hundred angstroms (tens of nanometers), not nearly large enough to allow white blood cells to pass through. Other mechanisms are therefore required to allow leukocytes into the area. Recent *in vivo* studies demonstrate conclusively that vascular permeability and transendothelial migration (TEM) are separate phenomena. Mice made genetically deficient in the actin-bundling protein cortactin have vessels that are constitutively leaky and have an exaggerated vascular leak response to inflammation. However, leukocyte emigration in these mice is actually reduced, compared with wild-type littermates, because of reduced intercellular adhesion molecule 1 (ICAM-1) clustering and therefore reduced leukocyte adhesion and transmigration.[2]

The Multistep Extravasation Paradigm

The process of leukocyte extravasation has been broken down into a series of adhesive and signaling interactions between leukocytes and endothelial cells. Over the past two decades, we have learned much about the cellular and molecular interactions that take place during this process.[3–5] Histamine, thrombin, and other mediators of acute inflammation stimulate exocytosis of Weibel–Palade bodies from endothelial cells, bringing P-selectin to the luminal surface. The same increased vascular permeability that allows plasma to leave the bloodstream causes local hemoconcentration, which decreases the rate of blood flow, allowing selectin ligands expressed on the surface of the slowly moving leukocytes to contact P-selectin and thus causing the leukocytes to roll along the luminal surface. Rolling brings the leukocytes close enough for their chemokine receptors to be activated by chemokines attached to the endothelial surface via glycosaminoglycans. Under certain conditions, other cell-surface receptors (eg, platelet-activating factor receptor) bind to and are activated by nonchemokine ligands expressed on the endothelial surface. The activated receptors, through an inside-out signaling cascade, activate leukocyte integrins for tighter adhesion to endothelial surface. This leads first to slow rolling on the endothelium and then to firm arrest. Attached leukocytes then locomote over the endothelial surface, using primarily the β_2 integrin CD11b/CD18 bound to ICAM-1[6–8] to reach the junction. When viewed *in vivo* by intravital microscopy, leukocytes are sometimes seen to migrate over several endothelial cells (occasionally migrating against the direction of blood flow) before they cross the endothelial cells, usually passing through the endothelial junctions.

Rolling, activation, adhesion, and locomotion (intraluminal crawling) are all critical for leukocyte extravasation, but these are reversible. In fact, most leukocytes that enter a venule at the site of inflammation do not roll, most leukocytes that roll do not adhere, and most that adhere do not extravasate.[9] However, once leukocytes make the commitment to cross the endothelium into the tissue, with notable exceptions,[10] they never go back—at least not as the same type of cell. And all of the good, the bad, and the ugly of inflammation takes place once leukocytes cross blood vessels. Thus, diapedesis or TEM is a logical process to study if one hopes to control an ongoing inflammatory response.[5,11]

Diapedesis Is a Distinct Step in TEM

Electron microscopy studies of leukocytes in the process of TEM show that the leukocytes squeeze in amoeboid fashion between tightly apposed endothelial cells.[12,13] Neutrophils have the pharmacological armamentarium to blow a hole in the vessel wall, but instead of barging in like a SWAT team, they sneak in like spies, minimizing leakage of vascular contents into the interstitial tissues. It was this tight apposition of leukocyte and endothelial cell membranes that fascinated me. Considering that it takes only 1 to 2 minutes for the leukocyte to completely traverse the endothelial border *in vitro*[6] or *in vivo*,[10] this suggests that very specialized membrane–membrane interactions are taking place. In the 1980s, when I started my training, leukocytes were assumed to direct the inflammatory response. They were known to have receptors for specific chemoattractants as well as adhesion molecules (later defined as integrins) promoting their migration. Endothelial cells were assumed to serve only as a relatively inert Teflon-like lining to blood vessels, preventing blood from clotting. However, the ability to culture endothelial cells *in vitro*, which was pioneered at the Gimbrone and Jaffe laboratories in the early 1970s,[14,15] catapulted vascular biology into the molecular era. It soon became apparent that endothelial cells play a major role in the regulation of most metabolic and physiological processes, including blood pressure, glucose metabolism, thrombosis, hemostasis, and inflammation. I wondered whether there were specific endothelial molecules at the junctions important for intercellular adhesion that regulate diapedesis, in the same way that the selectins and vascular cell adhesion molecule 1 (VCAM-1) and ICAM-1 on the apical surface are important for leukocyte rolling and adhesion, respectively.[16–19]

Adhesion Molecules at the Junction with Unique Roles in TEM

Monoclonal antibodies (mAbs) were made against endothelial cells and selected for those that bound to antigens concentrated

at endothelial cell borders. This strategy yielded mAbs that recognize platelet endothelial cell adhesion molecule (PECAM),[20] vascular endothelial cadherin (VE-cadherin),[21] and CD99.[22] Blocking antibodies against the homophilic interaction domain of PECAM, when added to either leukocytes or endothelial cells in *in vitro* transmigration assays, block TEM as completely as adding the antibodies to both cell types at the same time, demonstrating the homophilic nature of this interaction.[23] Moreover, blocking PECAM−PECAM interactions has no effect on adhesion of leukocytes to[23] or locomotion on[6] the endothelium. Removing the PECAM blockade allows leukocytes to resume transmigration.[23] These experiments demonstrate that TEM is a distinct step in leukocyte emigration that is molecularly dissectable from the other steps. This finding has been replicated in many models of acute inflammation *in vivo*, and antibodies or soluble PECAM-Fc chimeras that interfere with the homophilic interaction of mouse PECAM (or genetic deletion of PECAM) block inflammation in peritonitis,[24,25] dermatitis,[26] arthritis,[27] experimental autoimmune encephalitis,[28] and antigen-specific T-cell trafficking in the central nervous system.[29] Anti-PECAM antibodies also block inflammation in various cat and rat models (reviewed in ref. 9).

This role for PECAM in extravasation has been borne out in all mouse strains tested except for C57BL/6 mice,[30,31] which have a way to bypass the requirement for PECAM for leukocyte extravasation. In the thioglycollate peritonitis model, this trait maps to a small region on the short arm of mouse chromosome 2.[31] It is important to note that PECAM has a separate immunosuppressive role in T cells,[32,33] and knocking out PECAM in C57BL/6 mice may in some cases lead to hyperactive immune responses.[34,35] Thus, animal models using PECAM-deficient mice of the C57BL/6 strain that are measuring both the afferent and efferent limbs of the immune response may show enhanced disease.[36] Nevertheless, observations with PECAM led the way for discovery of other endothelial cell molecules that regulate TEM. Some of these, such as CD99 and poliovirus receptor (CD155), appear to function only in the process of diapedesis. Others, such as ICAM-1, ICAM-2, VCAM-1, and a number of the junctional adhesion molecule (JAM) family members, have functions in earlier steps in the pathway, as well as potential roles in diapedesis.[5,11]

Diapedesis Is a Multistep Process

CD99 is another molecule expressed on both leukocytes and endothelial cells. Similar to PECAM, homophilic interaction between leukocyte CD99 and endothelial CD99 is required for TEM, and blocking either side blocks TEM as efficiently as blocking both.[23,37] Furthermore, like PECAM, CD99 has no effect on the earlier stages of rolling, adhesion, or locomotion.

We have examined the interactions of leukocytes with endothelial cells when TEM is blocked by either PECAM or CD99. When PECAM−PECAM interactions are blocked, whether by mAb, soluble decoy PECAM-Fc, or PECAM knockout, leukocytes are arrested on the apical surface of the endothelial cell. They remain tightly adherent and crawl over the endothelium, usually along the junctions, probing with lamellipodia but unable to transmigrate.[6,23] This was observed by both scanning and transmission electron microscopy and also on living cells *in vitro*. This phenotype is also observed by intravital confocal microscopy *in vivo* (unpublished data). By contrast, when CD99 is blocked, leukocytes are arrested partway through the junction with the leading edge below the endothelial monolayer and the trailing uropod on the apical surface.[22] Observed in living cells *in vitro*, the leukocytes are seen to extend further into the junction but then retract part of the way back out as they migrate along the endothelial borders, unable to complete transmigration.[37] This unusual phenotype is also observed *in vivo* (unpublished data).

The two different morphologies suggest that PECAM and CD99 control different steps in TEM. Alan Schenkel in my research group took advantage of the fact that the blockade of TEM by both anti-PECAM and anti-CD99 mAbs is reversible. The block to TEM with anti-PECAM could be removed, and TEM could be subsequently blocked by anti-CD99. However, if TEM were first blocked with anti-CD99 and this block subsequently removed, TEM could no longer be blocked by anti-PECAM.[22] This demonstrates that PECAM functions at a step upstream of CD99 and suggests that TEM can be dissected into at least two steps, controlled by two separate molecules (but begging the question of whether there are other molecules controlling steps in TEM intermediate or distal to these).

Recently, David Sullivan and Michael Seidman[38] in my research group showed that poliovirus receptor (CD155), another molecule at the endothelial borders, regulates a step in TEM in between those regulated by PECAM and CD99. Using the same sequential blocking technique as used for CD99, they showed that monocytes released from a block in TEM due to PECAM could still be blocked by antibodies against CD155 or against its monocyte ligand, DNAM-1 (CD226), and monocytes released from a block in TEM due to anti-CD155 or anti−DNAM-1 antibodies could still be blocked by anti-CD99, although these antibodies could not block TEM of monocytes released from blockade in TEM due to anti-CD99.[38]

CD99 is important for leukocyte extravasation *in vivo*, as demonstrated by monoclonal[39] and polyclonal[40] antibody blocking experiments. Similarly, other molecules implicated in TEM through *in vitro* experiments, such as CD47 and JAM-A, have been validated *in vivo*.[41−43]

The Lateral Border Recycling Compartment

How do these molecules promote TEM? The paradigm initially was that proinflammatory cytokines such as IL-1 or tumor necrosis factor (TNF) up-regulate expression of

endothelial molecules involved in leukocyte extravasation. However, such treatments change neither the expression level nor the apparent distribution of PECAM or CD99.[22] Zahra Mamdouh and Xia Chen[44] found that approximately one third of the PECAM at the endothelial cell border is present in a reticulum of membrane within the endothelial cell close to the borders. This compartment is unique. Although PECAM is internalized into this compartment, it is distinct from the endocytic recycling pathway of transferrin. Ultrastructurally, using horseradish peroxidase (HRP)—labeled anti-PECAM mAb, they found that at 37°C anti-PECAM is brought into these parajunctional arrays of 50-nm-diameter vesicle-like structures that are connected to each other and connected to the cell border at intervals. These structures often appear to be many vesicle diameters away from the border, but biochemical experiments demonstrate that they are all connected to each other and to the junction (Figure 1). Although resembling caveolae, they are a distinct set of vesicle-like structures, as shown by numerous biochemical and immunohistologic experiments.[44] Not only is PECAM in this compartment, but also other molecules implicated in TEM, such as CD99, JAM-A, and CD155.[38,46] Interestingly, although VE-cadherin is seen along the endothelial borders, it does not enter this compartment.[46]

Membrane from the junction is internalized rapidly into this compartment, but the total levels of PECAM, CD99, and the other molecules remain constant. We hypothesized that the internalized membrane was recycled. At 4°C, antibody penetrated through the entire junction, but it did not enter this compartment. Stereologic analysis shows that there was just as much membrane in this compartment as there was at 37°C, but it was inaccessible. We developed a way to label the membrane as it returns to the surface, based on the fact that the compartment is inaccessible to the antibody at 4°C. When the temperature is increased, membrane from the compartment is detected back on the surface within 5 minutes, and it returns with a half-time of approximately 10 minutes, being distributed randomly and evenly along the cell.[46] Because of the rapid and continuous flux of membrane between the endothelial cell border and the compartment, we refer to this as the lateral border recycling compartment (LBRC).[47] Similar vesicles are also seen in mouse postcapillary venules *in situ* (unpublished data).

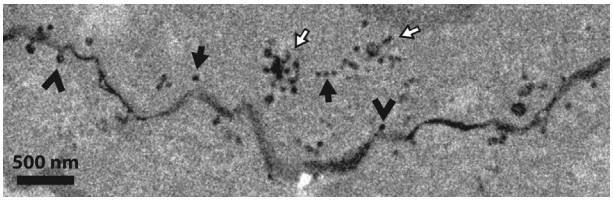


Figure 1 The LBRC as observed by immunoelectron microscopy. Horseradish peroxidase—labeled nonblocking anti-PECAM mAb was incubated with human umbilical vein endothelial cells for 1 hour at 37°C to label PECAM in the junction as well as within the LBRC. After fixation, the location of the antibody was revealed by incubation with DAB and H_2O_2.[45] The interconnected vesicle-like structures of the LBRC can be seen budding off the intercellular junction (**arrowheads**), interconnected in cisternae (**white arrows**), or as apparently individual vesicles (**black arrows**). Scale bar = 500 nm.

The LBRC Is Critical for Paracellular Transmigration

During TEM, the membrane from the LBRC is redirected. Instead of returning evenly along the cell junction, it traffics to the site where the leukocyte is transmigrating. The membrane surrounding the transmigrating leukocyte is enriched with membrane from the LBRC for the entire duration of its passage (Figure 2). We call this process targeted recycling of the LBRC. It is not just an epiphenomenon—it appears to be the sine qua non for transmigration.

Everything that we can do to block targeted recycling blocks TEM. Interfering with leukocyte and endothelial cell PECAM—PECAM interactions blocks targeted recycling and TEM.[44] The LBRC is trafficked along cortical microtubules, so depolymerizing microtubules or blocking kinesin molecular motors under proper experimental conditions selectively blocks targeted recycling of the LBRC and blocks TEM.[47] The appearance of the blocked leukocytes in all cases is the same. There is no effect on adhesion or ability to migrate to the endothelial cell border; leukocytes are arrested over the junctions and are unable to initiate TEM.[47] Mutating tyrosine 663 on the cytoplasmic tail of PECAM blocks the ability of PECAM to enter and exit the LBRC and to engage in targeted recycling. Cells in which native PECAM is replaced with PECAM Y663F are unable to support TEM, but normal levels of TEM can be rescued by re-expressing wild-type PECAM.[48]

Antibodies against endothelial cell PECAM, CD155, and CD99 block TEM, but only when added at 37°C, conditions under which they enter the LBRC.[22,23,38] If the same antibodies are added to endothelial cells at 4°C, conditions under which they saturably bind to all of their antigens present at the junction but do not enter the LBRC, there is no blockade of TEM when the unbound antibody is washed away and leukocytes are added at 37°C.[22,23,38] Thus, it is possible that only the fraction of junctional adhesion molecules within the LBRC is required for TEM.

The LBRC Is Critical for Transcellular Transmigration

Targeted recycling of the LBRC seems to be necessary for TEM of neutrophils, monocytes, and lymphocytes under all conditions tested—even conditions in which we could not block TEM by anti-PECAM antibodies. However, what highlights the importance of the LBRC in TEM is the exception that proves the rule. Greater than 90% of leukocyte TEM takes place at endothelial cell borders, but under certain conditions leukocytes can migrate through the endothelial cell cytoplasm in what is called transcellular migration. Under certain *in vitro* conditions, up to 30% of neutrophils could be made to migrate transcellularly.[49,50]

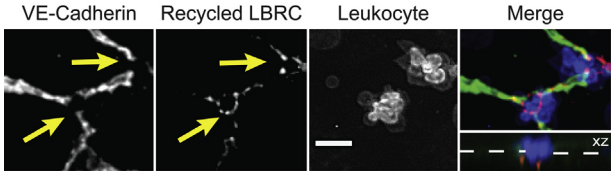


Figure 2 Targeted recycling of the LBRC during TEM. Two monocytes caught in the act of TEM by paraformaldehyde fixation. The monolayer was stained with antibodies to VE-cadherin (green), to mark the cell junctions; to recycling LBRC (red), to show targeted recycling; and to CD18 (blue), to visualize the monocytes. Note the gap in VE-cadherin staining and the enrichment of recycling LBRC where the leukocytes are passing across the endothelial cells (**arrows**). An orthogonal view of one of the transmigration events (XZ plane, shown below the merged image) shows a collar of LBRC around the migrating monocyte. The level of the endothelial monolayer is indicated by a **broken line**. Scale bar = 10 μm.

Generally, these are conditions in which the leukocyte is activated directly by a chemokine or other agent exposed to it on the apical surface of cytokine-activated endothelium. Zahara Mamdouh and Alexei Mikhailov[46] found that, during transcellular migration, the LBRC migrates from the endothelial border to surround the leukocyte for the duration of its passage. Microtubule depolymerization, which prevents movement of the LBRC, blocks both transcellular and paracellular transmigration. Furthermore, anti-PECAM or an anti-CD99 mAb can block transcellular migration once it has begun, even though these molecules are not present on the apical surface of the endothelial cells.[46] Thus, the LBRC traffics to wherever the leukocyte is transmigrating, even if it is far from the endothelial border.

Other Regulators of TEM

So far, I have highlighted the focus in my research group on the molecular mechanisms in the endothelial cell that regulate TEM. There are other equally important molecules and mechanisms at work in endothelial cells that regulate TEM. Some of these are outlined below, to put our work into context.

ICAM-1 and VCAM-1 Clustering

As a prelude to TEM, ICAM-1 and VCAM-1 cluster under the leukocyte as it approaches the junction.[51–53] Clustering of ICAM-1 requires c-Src-dependent phosphorylation of cortactin to start the remodeling of actin[54,55] and to perpetuate ICAM-1 clustering,[5] along with joining of tetraspanin molecules to these clusters.[56] Although it is not universally observed, under certain *in vitro* conditions the apical surface membrane appears to extend upward, to partially envelop the leukocyte during TEM.[50,57] These so-called transmigratory cups are enriched in ICAM-1, VCAM-1, and tetraspanins overlying a core of actin filaments.[49,58]

Clustering of ICAM-1 induces Src phosphorylation,[59] which is required for several steps in TEM[38,54,60] (in addition to the role in activating cortactin). ICAM-1 clustering also activates RhoA.[61,62] Interestingly, just like the self-enforcing interactions between cortactin phosphorylation and ICAM-1 clustering, active RhoA enhances ICAM-1 clustering.[63] RhoA in turn activates Rho kinase (ROCK), which inactivates protein phosphatase 1c and inhibits the inactivation of myosin light chain kinase (MLCK). The net result is promotion of the activity of MLCK, which tugs on actin filaments and effectively loosens the junctions.

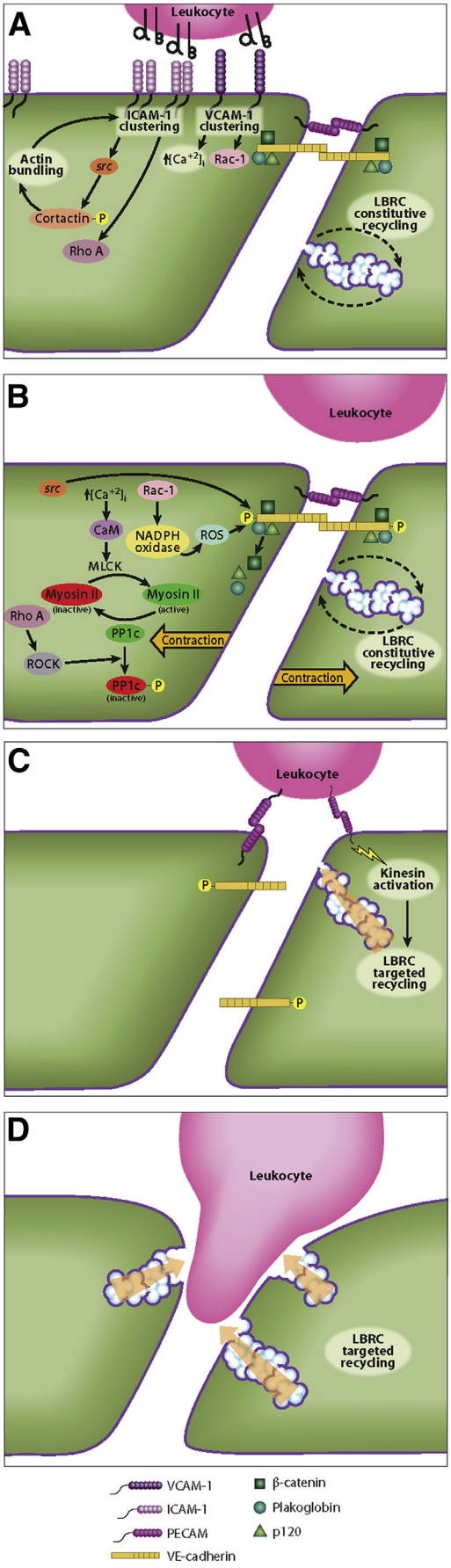
Calcium Signaling

Elevation of endothelial cytosolic free calcium ion concentration ($[Ca^{+2}]_i$) is known to be required for transmigration to proceed. Blocking the ability of endothelial cells to flux calcium using a cell-permeable calcium chelator leads to the same phenotype as blocking PECAM, in which leukocytes arrest on the apical surface of the endothelium, tightly adherent but unable to transmigrate.[64] It has been demonstrated that increased $[Ca^{+2}]_i$ leads to activation of MLCK through calmodulin (CaM).[65] The source of the increased $[Ca^{+2}]_i$ is not clear, although VCAM-1 clustering and cross-linking of P- or E-selectin[66] have been implicated. MLCK activates nonmuscle myosin in endothelial cells for interaction with actin filaments, leading to tension development within the cell, which likely promotes loosening of the junctions.

Movement of VE-Cadherin

Although we have been focusing on the recruitment of the LBRC to the site of transmigration, there is extensive evidence that certain membrane proteins, in particular VE-cadherin and its associated catenins, transiently leave the site of TEM.[67,68] Under resting conditions, vascular endothelial cell protein tyrosine phosphatase (VE-PTP) associates with VE-cadherin, the main adhesion molecule of endothelial cell adherens junctions, and maintains it in a state of reduced phosphorylation at the endothelial junctions.[69] Leukocyte adhesion to activated endothelial cells triggers dissociation of VE-PTP from VE-cadherin, allowing phosphorylation of the latter.[69,70] A recent study[71] suggests that this occurs through a signaling pathway in which VCAM-1 activates Rac1, which activates endothelial NADPH oxidase, the reactive oxygen metabolites of which activate Pyk2. Pyk2 is hypothesized to phosphorylate an alternative substrate of VE-PTP, which then displaces binding to VE-cadherin.

Displacement of VE-PTP from VE-cadherin is the first step in its dissociation from the membrane. This allows access to VE-cadherin by protein kinases. VE-cadherin is tethered to the cortical actin cytoskeleton by α-catenin, to which it binds via p120 and β-catenin.[72] Phosphorylation of VE-cadherin on tyrosine 658 by Src and on tyrosine 731 by Pyk2 inhibits binding of p120 and β-catenin, respectively. Dissociation of VE-cadherin from p120 and β-catenin



facilitate its removal from the adherens junction. These interactions not only regulate endothelial cell permeability, but also regulate TEM. Mutation of tyrosine residues 658 or 731 to phenylalanine inhibits the release of p120- and β-catenin, maintains more VE-cadherin at the cell border, and reduces TEM.[72] This demonstrates that removal of VE-cadherin from the endothelial cell border at the site of TEM is not just an epiphenomenon—it is a requirement for transmigration. The relationship between the influx of membrane from the LBRC and the apparent removal of VE-cadherin from the site of TEM is the subject of ongoing study. In Figure 3, I summarize and try to integrate some of the major adhesion and signaling events that occur during the transmigration process.

Other Molecules Involved in TEM

Many molecules are reported to play a role in TEM, because antagonizing these molecules blocks leukocyte migration in transmigration assays. Many of these studies do not distinguish between a true block in diapedesis and a block in leukocyte adhesion, chemotaxis, ability to migrate per se, detachment from the underside of the endothelial cell, migration across the extracellular matrix, or (in some assays) migration through the pores of a filter support.[73] However, even discounting such findings, several other endothelial cell molecules have been reported to play a role in mediating TEM, in that blockade by antibodies or knockdown of these molecules reduces TEM. In addition to PECAM, CD155, CD99, and the recently reported CD99L2,[74–76] these other molecules include JAM-A,[77,78] JAM-C,[10,79] ICAM-2,[43,80,81] CD47,[82] MUC18 (CD146),[83] activated leukocyte cell adhesion molecule (ALCAM),[84] nepmucin,[85] and even the purinergic receptor $P2Y_2$.[86] Restoration of TEM by re-expressing a candidate molecule after it has been knocked down is a more reliable way to establish that the molecule plays a role in transmigration than by simply knocking it down or blocking its function. This has now been demonstrated with a number of molecules.[5] Nevertheless, to our knowledge, PECAM is the only molecule demonstrated to play a role in TEM by a true gain of function. Expression of PECAM in

Figure 3 A unified schematic view of TEM. **A:** Clustering of ICAM-1 and VCAM-1 through engagement of their leukocyte integrin counter-receptors (shown as black αβ) initiates activation of Src, RhoA, and Rac-1, as well as increased cytosolic free calcium ion. Phosphorylation of cortactin by Src stimulates F-actin rearrangements in the cortical cytoplasm, which facilitates more ICAM-1 clustering. **B:** These signals lead to activation of MLCK, inactivation of PP1c, and phosphorylation of VE-cadherin, inducing release of the associated catenins. **C:** Leukocyte PECAM engagement of endothelial cell PECAM and/or other leukocyte—endothelial cell interactions at the apical surface of the endothelial border activate kinesin molecular motors in the endothelial cell and stimulate targeted trafficking of LBRC membrane to the vicinity of the leukocyte. **D:** Targeted trafficking of LBRC membrane continues as the leukocyte moves into the border between endothelial cells, now enlarged by the contribution of membrane from the LBRC. This process continues until transmigration is complete. Circled P, phosphorylated state; ROS, reactive oxygen species. Reproduced from Muller[5] with permission of Annual Reviews.

PECAM-deficient ECV-304 cells imparts the ability to support transmigration in a manner that could be blocked by anti-PECAM.[48] Interestingly, at baseline these cells express ICAM-1 and CD99; however, in the absence of PECAM, this is not sufficient to support transmigration.[48]

Where these molecules exert their control over TEM and how they relate to PECAM, CD99, and the LBRC are questions for future research. In fact, how all of the mechanisms discussed in this section relate to each other is not known. Do they occur sequentially? Is there a hierarchy? One consideration is that inflammation, although a stereotyped response, is not identical at all times and in all places. Depending on the stimulus, the vascular bed, and the timing, different leukocyte types and mechanisms may predominate. However, considering that during diapedesis the leading edge of the leukocyte is under the endothelial cell interacting with the basement membrane while the trailing uropod is still on the apical surface, it is likely that many of these molecular interactions and mechanisms occur simultaneously in different parts of the endothelial cell and leukocyte.

Frontiers and Future Directions

Isolating the LBRC

Significant progress has been achieved in our understanding of the TEM process. However, as with all important scientific problems, the answers to one question lead to even more questions. How is the LBRC formed, and what is it composed of? We are taking two approaches to answering these questions. Homogenization and subcellular fractionation of endothelial cells on sucrose density gradients is yielding some important information. David Sullivan in my research group (unpublished data) took advantage of the fact that the LBRC is accessible by antibodies at 37°C but not at 4°C to bind HRP—conjugated antibody to PECAM on the surface of the cell border but not in the LBRC. After homogenization and flotation away from contaminating organelles, membrane vesicles were briefly exposed to 3,3′-diaminobenzidine (DAB) and H_2O_2. In vesicles containing membrane from the endothelial cell surface, the HRP oxidizes DAB to insoluble multimers that precipitate on the membrane and increase the density of the vesicles containing them. On the other hand, vesicles of LBRC do not contain HRP—labeled antibody and consequently are considerably less dense than the vesicles containing surface membrane. The spectrum of proteins seen is compared to that seen when both surface and LBRC are labeled. This variation of the DAB density shift[87,88] is being used to purify LBRC membrane and analyze it for novel LBRC-associated proteins. Dr. Sullivan has found an unsuspected cytosolic protein associated with the LBRC that appears to play a major role in its targeted recycling (unpublished data).

What Directs Molecules Into and Out of the LBRC?

We have previously demonstrated that mutation of tyrosine 663 on the cytoplasmic tail of PECAM blocks TEM without affecting classic PECAM signaling.[60] We found that this mutation interfered with the ability of PECAM to enter and leave the LBRC. This suggests that Y663 might be a part of a localization signal that allows PECAM to traffic into the LBRC. Dr. Gong Feng in my research group has been making chimeric molecules in which the extracellular and cytoplasmic domains of molecules known to be included in the LBRC (eg, PECAM) and those known to be excluded from the LBRC (eg, VE-cadherin) are swapped to determine the rules for sorting of molecules into the LBRC (unpublished data).

The Minimal Machinery Necessary for TEM

Interest in transcellular migration of leukocytes has been far greater than its physiological presence might suggest. Direct *in vivo* measurements show that polymorphonuclear leukocytes migrate transcellularly less than 10% of the time.[10] Nonetheless, we are interested in this phenomenon because it could allow us to understand the mechanisms necessary for transmigration itself, without the potential confounding factors related to junctional integrity. Drs. Annette Gonzalez and Alexei Mikhailov have been studying this process using

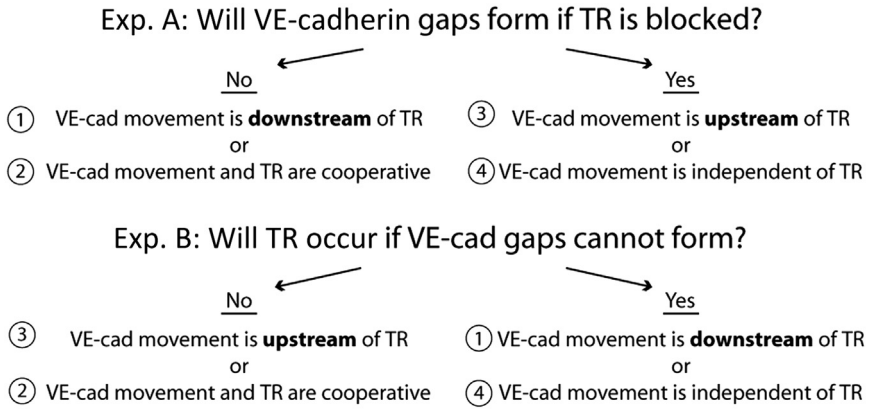


Figure 4 The experimental logic for determining the relationship of VE-cadherin gap formation and targeted recycling (TR) of the LBRC. The two independent experiments yield four possible results. However, only one of the four proposed relationships is consistent with successful completion of both experiments **A** and **B**.

human umbilical vein endothelial cells (HUVECs) in which the endogenous PECAM has been replaced by PECAM-eGFP. Studying monocyte–endothelial cell interactions by live-cell imaging, they have found that leukocytes tightly adherent to the apical surface of the endothelial cells attract a cloud of fluorescent PECAM-bearing membrane (presumably the LBRC, as in ref. 45) that follows them as they locomote along the apical surface. When a monocyte transmigrates, the cloud coalesces into a ring that tightly surrounds the leukocyte as it migrates through the endothelial cell, contracts as the uropod vanishes beneath the endothelial cell, and dissipates after transcellular migration. Presumably, the membrane reverts to interconnected tubulovesicular structures and relocalizes to the perijunctional zone, but optical limitations preclude direct observation.

Which molecular motors and adaptor proteins are responsible for moving the LBRC toward the leukocyte and recovering it after TEM? Bita Cyrus in my research group is studying these questions using a combination of biochemical, microinjection, and knockdown strategies.

The Plasticity of Endothelial Junctions

One of the difficulties limiting study of the mechanisms of transcellular migration is that even under the most conducive circumstances only approximately 30% of polymorphonuclear leukocytes and approximately 10% of monocytes migrate transcellularly. It has been postulated that transcellular migration occurs more frequently at the blood–brain barrier, where true tight junctions exist and paracellular migration would be expected to be particularly difficult.[58,89] To study the mechanisms of transcellular TEM in a robust system, Ryan Winger in my research group has developed ways to make the endothelial cell borders tighter. Using a combination of methods reported in the literature to decrease junctional permeability, he has been able to routinely grow endothelial cell monolayers with transendothelial electrical resistances of 250 $\Omega \cdot cm^2$, which is as high as has been reported *in vitro* with human cells.[34,35,90] In preliminary experiments, however, transmigration rates of monocytes are not diminished, transmigration is still PECAM and CD99 dependent, and, surprisingly, 99% of the transmigration events are paracellular. Data show the remarkable plasticity of the endothelial junctions, because during TEM the electrical resistance does not decrease.

The Relationship of LBRC and VE-Cadherin Movements

How are the movements of the LBRC and VE-cadherin coordinated? During TEM, VE-cadherin is removed from the cell border at the site of transmigration, whereas the LBRC moves in.[67,68] VE-cadherin gap formation can be prevented by mutations in the cytoplasmic tail of VE-cadherin that prevent it from interacting with β-catenin and p120.[72] Just like targeted recycling of the LBRC, VE-cadherin gap formation is required for TEM, and manipulations that inhibit gap formation also reduce TEM. Does VE-cadherin leave first and then the LBRC moves in to fill this vacuum? Does the LBRC move in and push VE-cadherin out of the way? Are these two movements of membrane somehow coordinated or cooperative? Or are they totally independent? Dr. Annette Gonzalez is approaching this problem using a series of experiments in which she selectively blocks either targeted recycling or VE-cadherin gap formation and determines the effect on the other membrane compartment. The results of these experiments should unambiguously determine the relationship of these events, both of which are essential for TEM (Figure 4).

Other Challenges

We have known for more than 20 years that a transient increase in cytosolic free calcium ion concentration[64] is required for TEM. However, we still do not know which step or steps in transmigration are regulated by the calcium flux. Evan Weber, a student in my research group, is working on this problem using an *in vitro* TEM assay that can dissect the contributions of adhesion, locomotion, diapedesis, and migration across the basement membrane[91] to the leukocyte extravasation process. The role of CD99 in TEM was first demonstrated more than a decade ago, but CD99 is a unique molecule with no homologs in the genome (other than the somewhat distantly related paralog CD99L2[92]) and no known signaling motifs on its cytoplasmic tail.[22] Understanding its mechanism of action has therefore been a challenge. Richard Watson, another student in my research group, has been studying signaling events that can overcome a CD99 blockade to transmigration and is tracing these pathways back to CD99.

Several other unanswered questions remain. How is it that the molecules that regulate TEM are all present together in the LBRC, yet PECAM, CD155, and CD99 act sequentially? Why are there so many molecules on leukocytes and endothelial cells that, when blocked with antibodies or knocked down or out, have such a large effect on TEM? If interfering with the function of any one of them blocks TEM by more than 50%, then the others should act in a common step (otherwise, it would easily be possible to block 100% of TEM, which is never seen). What is that common step? Why don't the other molecules compensate for the one molecule that has been deleted or inhibited in these studies? When we fully understand the mechanisms of TEM, we will have the answers to such questions and will, we hope, have a much better handle on controlling the inflammatory response.

Acknowledgments

I thank those who nominated me for this award: Drs. Yashpal Kanwar, Michael Gimbrone, Carl Nathan, Sussan Nourshargh, and the late Ralph Steinman. I also thank my family for their support and patience over my career. I have

benefitted from the advice and friendship of innumerable colleagues and mentors; thank you all. Finally and most importantly, I thank the students and postdoctoral fellows with whom I have been fortunate to work over the years. You have taught me far more than I have taught you.

References

1. Majno G, Palade GE: Studies on inflammation. 1. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. J Biophys Biochem Cytol 1961, 11:571–605
2. Schnoor M, Lai FP, Zarbock A, Kläver R, Polaschegg C, Schulte D, Weich HA, Oelkers JM, Rottner K, Vestweber D: Cortactin deficiency is associated with reduced neutrophil recruitment but increased vascular permeability in vivo. J Exp Med 2011, 208:1721–1735
3. Butcher EC: Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 1991, 67:1033–1036
4. Ley K, Laudanna C, Cybulsky MI, Nourshargh S: Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007, 7:678–689
5. Muller WA: Mechanisms of leukocyte transendothelial migration. Annu Rev Pathol 2011, 6:323–344
6. Schenkel AR, Mamdouh Z, Muller WA: Locomotion of monocytes on endothelium is a critical step during extravasation. Nat Immunol 2004, 5:393–400
7. Phillipson M, Heit B, Colarusso P, Liu L, Ballantyne CM, Kubes P: Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. J Exp Med 2006, 203:2569–2575
8. Sumagin R, Prizant H, Lomakina E, Waugh RE, Sarelius IH: LFA-1 and Mac-1 define characteristically different intralumenal crawling and emigration patterns for monocytes and neutrophils in situ. J Immunol 2010, 185:7057–7066
9. Muller WA. PECAM: regulating the start of diapedesis. Adhesion Molecules: Function and Inhibition. Edited by Ley K. Basel, Birkhäuser Verlag, 2007, pp 201–220
10. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, Nash GB, Chavakis T, Albelda SM, Rainger GE, Meda P, Imhof BA, Nourshargh S: The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. Nat Immunol 2011, 12:761–769
11. Muller WA: Mechanisms of transendothelial migration of leukocytes. Circ Res 2009, 105:223–230
12. Marchesi VT, Florey HW: Electron micrographic observations on the emigration of leukocytes. Q J Exp Physiol Cogn Med Sci 1960, 45:343–347
13. Marchesi VT: The site of leukocyte emigration during inflammation. Q J Exp Physiol Cogn Med Sci 1961, 46:115–118
14. Gimbrone MA Jr, Cotran RS, Folkman J: Endothelial regeneration: studies with human endothelial cells in culture. Ser Haematol 1973, 6:453–455
15. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973, 52:2745–2756
16. Bevilacqua MP, Stengalin S, Gimbrone MA Jr, Seed B: Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 1989, 243:1160–1165
17. Marlin SD, Springer TA: Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell 1987, 51:813–819
18. Dustin ML, Springer TA: Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J Cell Biol 1988, 107:321–331
19. Osborn L, Vassallo C, Benjamin CD: Activated endothelium binds lymphocytes through a novel binding site in the alternately spliced domain of vascular cell adhesion molecule-1. J Exp Med 1992, 176:99–107
20. Muller WA, Ratti CM, McDonnell SL, Cohn ZA: A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. J Exp Med 1989, 170:399–414
21. Ali J, Liao F, Martens E, Muller WA: Vascular endothelial cadherin (VE-cadherin): cloning and role in endothelial cell-cell adhesion. Microcirculation 1997, 4:267–277
22. Schenkel AR, Mamdouh Z, Chen X, Liebman RM, Muller WA: CD99 plays a major role in the migration of monocytes through endothelial junctions. Nat Immunol 2002, 3:143–150
23. Muller WA, Weigl SA, Deng X, Phillips DM: PECAM-1 is required for transendothelial migration of leukocytes. J Exp Med 1993, 178:449–460
24. Bogen S, Pak J, Garifallou M, Deng X, Muller WA: Monoclonal antibody to murine PECAM-1 (CD31) blocks acute inflammation in vivo. J Exp Med 1994, 179:1059–1064
25. Liao F, Ali J, Greene T, Muller WA: Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. J Exp Med 1997, 185:1349–1357
26. Schenkel AR, Chew TW, Muller WA: Platelet endothelial cell adhesion molecule deficiency or blockade significantly reduces leukocyte emigration in a majority of mouse strains. J Immunol 2004, 173:6403–6408
27. Dasgupta B, Chew T, deRoche A, Muller WA: Blocking platelet/endothelial cell adhesion molecule 1 (PECAM) inhibits disease progression and prevents joint erosion in established collagen antibody-induced arthritis. Exp Mol Pathol 2010, 88:210–215
28. Reinke EK, Lee J, Zozulya A, Karman J, Muller WA, Sandor M, Fabry Z: Short-term sPECAM-Fc treatment ameliorates EAE while chronic use hastens onset of symptoms. J Neuroimmunol 2007, 186:86–93
29. Qing Z, Sandor M, Radvany Z, Sewell D, Falus A, Potthoff D, Muller WA, Fabry Z: Inhibition of antigen-specific T cell trafficking into the central nervous system via blocking PECAM1/CD31 molecule. J Neuropathol Exp Neurol 2001, 60:798–807
30. Schenkel AR, Chew TW, Chlipala E, Harbord MW, Muller WA: Different susceptibilities of PECAM-deficient mouse strains to spontaneous idiopathic pneumonitis. Exp Mol Pathol 2006, 81:23–30
31. Seidman MA, Chew TW, Schenkel AR, Muller WA: PECAM-independent thioglycollate peritonitis is associated with a locus on murine chromosome 2. PLoS One 2009, 4:e4316
32. Newton-Nash DK, Newman PJ: A new role for platelet-endothelial cell adhesion molecule-1 (CD31): inhibition of TCR-mediated signal transduction. J Immunol 1999, 163:682–688
33. Newman DK, Hamilton C, Newman PJ: Inhibition of antigen-receptor signaling by Platelet Endothelial Cell Adhesion Molecule-1 (CD31) requires functional ITIMs, SHP-2, and p56(lck). Blood 2001, 97:2351–2357
34. Wong MX, Roberts D, Bartley PA, Jackson DE: Absence of platelet endothelial cell adhesion molecule-1 (CD31) leads to increased severity of local and systemic IgE-mediated anaphylaxis and modulation of mast cell activation. J Immunol 2002, 168:6455–6462
35. Wilkinson R, Lyons AB, Roberts D, Wong MX, Bartley PA, Jackson DE: Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) acts as a regulator of B-cell development, B-cell antigen receptor (BCR)-mediated activation, and autoimmune disease. Blood 2002, 100:184–193
36. Tada Y, Koarada S, Morito F, Ushiyama O, Haruta Y, Kanegae F, Ohta A, Ho A, Mak TW, Nagasawa K: Acceleration of the onset of collagen-induced arthritis by a deficiency of platelet endothelial cell adhesion molecule 1. Arthritis Rheum 2003, 48:3280–3290

37. Lou O, Alcaide P, Luscinskas FW, Muller WA: CD99 is a key mediator of the transendothelial migration of neutrophils. J Immunol 2007, 178:1136–1143
38. Sullivan DP, Seidman MA, Muller WA: Poliovirus receptor (CD155) regulates a step in transendothelial migration between PECAM and CD99. Am J Pathol 2013, 182:1031–1042
39. Dufour EM, Deroche A, Bae Y, Muller WA: CD99 is essential for leukocyte diapedesis in vivo. Cell Commun Adhes 2008, 15:351–363
40. Bixel G, Kloep S, Butz S, Petri B, Engelhardt B, Vestweber D: Mouse CD99 participates in T-cell recruitment into inflamed skin. Blood 2004, 104:3205–3213
41. Azcutia V, Stefanidakis M, Tsuboi N, Mayadas T, Croce KJ, Fukuda D, Aikawa M, Newton G, Luscinskas FW: Endothelial CD47 promotes vascular endothelial-cadherin tyrosine phosphorylation and participates in T cell recruitment at sites of inflammation in vivo. J Immunol 2012, 189:2553–2562
42. Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E: Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 1998, 142:117–127
43. Woodfin A, Voisin MB, Imhof BA, Dejana E, Engelhardt B, Nourshargh S: Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A and PECAM-1. Blood 2009, 113:6246–6257
44. Mamdouh Z, Chen X, Pierini LM, Maxfield FR, Muller WA: Targeted recycling of PECAM from endothelial cell surface-connected compartments during diapedesis. Nature 2003, 421:748–753
45. Graham RC Jr, Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 1966, 14:291–302
46. Mamdouh Z, Mikhailov A, Muller WA: Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. J Exp Med 2009, 206:2795–2808
47. Mamdouh Z, Kreitzer GE, Muller WA: Leukocyte transmigration requires kinesin-mediated microtubule-dependent membrane trafficking from the lateral border recycling compartment. J Exp Med 2008, 205: 951–966
48. Dasgupta B, Dufour E, Mamdouh Z, Muller W: A novel and critical role for tyrosine 663 in PECAM trafficking and transendothelial migration. J Immunol 2009, 182:5041–5051
49. Carman CV, Sage PT, Sciuto TE, de la Fuente MA, Geha RS, Ochs HD, Dvorak HF, Dvorak AM, Springer TA: Transcellular diapedesis is initiated by invasive podosomes. Immunity 2007, 26:784–797
50. Millán J, Hewlett L, Glyn M, Toomre D, Clark P, Ridley AJ: Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. Nat Cell Biol 2006, 8:113–123
51. Barreiro O, Yanez-Mo M, Serrador JM, Montoya MC, Vicente-Manzanares M, Tejedor R, Furthmayr H, Sanchez-Madrid F: Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. J Cell Biol 2002, 157:1233–1245
52. Carman CV, Jun CD, Salas A, Springer TA: Endothelial cells proactively form microvilli-like membrane projections upon intercellular adhesion molecule 1 engagement of leukocyte LFA-1. J Immunol 2003, 171:6135–6144
53. Shaw SK, Ma S, Kim MB, Rao RM, Hartman CU, Froio RM, Yang L, Jones T, Liu Y, Nusrat A, Parkos CA, Luscinskas FW: Coordinated redistribution of leukocyte LFA-1 and endothelial cell ICAM-1 accompany neutrophil transmigration. J Exp Med 2004, 200:1571–1580
54. Yang L, Kowalski JR, Zhan X, Thomas SM, Luscinskas FW: Endothelial cell cortactin phosphorylation by Src contributes to polymorphonuclear leukocyte transmigration in vitro. Circ Res 2006, 98:394–402
55. Yang L, Kowalski JR, Yacono P, Bajmoczi M, Shaw SK, Froio RM, Golan DE, Thomas SM, Luscinskas FW: Endothelial cell cortactin coordinates intercellular adhesion molecule-1 clustering and actin cytoskeleton remodeling during polymorphonuclear leukocyte adhesion and transmigration. J Immunol 2006, 177:6440–6449
56. Barreiro O, Yáñez-Mó M, Sala-Valdés M, Gutiérrez-López MD, Ovalle S, Higginbottom A, Monk PN, Cabañas C, Sánchez-Madrid F: Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. Blood 2005, 105:2852–2861
57. Carman CV, Springer TA: A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. J Cell Biol 2004, 167:377–388
58. Carman CV, Springer TA: Trans-cellular migration: cell-cell contacts get intimate. Curr Opin Cell Biol 2008, 20:533–540
59. Liu G, Place AT, Chen Z, Brovkovych VM, Vogel SM, Muller WA, Skidgel RA, Malik AB, Minshall RD: ICAM-1-activated Src and eNOS signaling increase endothelial cell surface PECAM-1 adhesivity and neutrophil transmigration. Blood 2012, 120:1942–1952
60. Dasgupta B, Muller WA: Endothelial Src kinase regulates membrane recycling from the lateral border recycling compartment during leukocyte transendothelial migration. Eur J Immunol 2008, 38:3499–3507
61. Wittchen ES, van Buul JD, Burridge K, Worthylake RA: Trading spaces: Rap, Rac, and Rho as architects of transendothelial migration. Curr Opin Hematol 2005, 12:14–21
62. Cernuda-Morollon E, Ridley AJ: Rho GTPases and leukocyte adhesion receptor expression and function in endothelial cells. Circ Res 2006, 98:757–767
63. Wójciak-Stothard B, Williams L, Ridley AJ: Monocyte adhesion and spreading on human endothelial cells is dependent on rho-regulated receptor clustering. J Cell Biol 1999, 145:1293–1307
64. Huang AJ, Manning JE, Bandak TM, Ratau MC, Hanser KR, Silverstein SC: Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. J Cell Biol 1993, 120:1371–1380
65. Hixenbaugh EA, Goeckeler ZM, Papaiya NN, Wysolmerski RB, Silverstein SC, Huang AJ: Stimulated neutrophils induce myosin light chain phosphorylation and isometric tension in endothelial cells. Am J Physiol 1997, 273:H981–H988
66. Lorenzon P, Vecile E, Nardon E, Ferrero E, Harlan JM, Tedesco F, Dobrina A: Endothelial cell E-and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. J Cell Biol 1998, 142:1381–1391
67. Allport JR, Muller WA, Luscinskas FW: Monocytes induce reversible focal changes in vascular endothelial cadherin complex during transendothelial migration under flow. J Cell Biol 2000, 148:203–216
68. Shaw SK, Bamba PS, Perkins BN, Luscinskas FW: Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. J Immunol 2001, 167:2323–2330
69. Nottebaum AF, Cagna G, Winderlich M, Gamp AC, Linnepe R, Polaschegg C, Filippova K, Lyck R, Engelhardt B, Kamenyeva O, Bixel MG, Butz S, Vestweber D: VE-PTP maintains the endothelial barrier via plakoglobin and becomes dissociated from VE-cadherin by leukocytes and by VEGF. J Exp Med 2008, 205:2929–2945
70. Broermann A, Winderlich M, Block H, Frye M, Rossaint J, Zarbock A, Cagna G, Linnepe R, Schulte D, Nottebaum AF, Vestweber D: Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. J Exp Med 2011, 208:2393–2401
71. Vockel M, Vestweber D: How T cells trigger the dissociation of the endothelial receptor phosphatase VE-PTP from VE-cadherin. Blood 2013, 122:2512–2522
72. Allingham MJ, van Buul JD, Burridge K: ICAM-1-mediated, Src- and Pyk2-dependent vascular endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration. J Immunol 2007, 179:4053–4064
73. Muller WA, Luscinskas FW: Assays of transendothelial migration in vitro. Methods Enzymol 2008, 443:155–176

74. Schenkel AR, Dufour EM, Chew TW, Sorg E, Muller WA: The murine CD99-related molecule CD99-like 2 (CD99L2) is an adhesion molecule involved in the inflammatory response. Cell Commun Adhes 2007, 14:227–237
75. Bixel MG, Li H, Petri B, Khandoga AG, Khandoga A, Zarbock A, Wolburg-Buchholz K, Wolburg H, Sorokin L, Zeuschner D, Maerz S, Butz S, Krombach F, Vestweber D: CD99 and CD99L2 act at the same site as, but independently of, PECAM-1 during leukocyte diapedesis. Blood 2010, 116:1172–1184
76. Bixel MG, Petri B, Khandoga AG, Khandoga A, Wolburg-Buchholz K, Wolburg H, März S, Krombach F, Vestweber D: A CD99-related antigen on endothelial cells mediates neutrophil, but not lymphocyte extravasation in vivo. Blood 2007, 109:5327–5336
77. Del Maschio A, De Luigi A, Martin-Padura I, Brockhaus M, Bartafai T, Fruscella P, Adorini L, Martino G, Furlan R, De Simoni MG, Dejana E: Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is inhibited by an antibody to junctional adhesion molecule (JAM). J Exp Med 1999, 190: 1351–1356
78. Woodfin A, Reichel CA, Khandoga A, Corada M, Voisin MB, Scheiermann C, Haskard DO, Dejana E, Krombach F, Nourshargh S: JAM-A mediates neutrophil transmigration in a stimulus-specific manner in vivo: evidence for sequential roles for JAM-A and PECAM-1 in neutrophil transmigration. Blood 2007, 110:1848–1856
79. Bradfield PF, Scheiermann C, Nourshargh S, Ody C, Luscinskas FW, Rainger GE, Nash GB, Miljkovic-Licina M, Aurrand-Lions M, Imhof BA: JAM-C regulates unidirectional monocyte transendothelial migration in inflammation. Blood 2007, 110:2545–2555
80. Issekutz AC, Rowter D, Springer TA: Role of ICAM-1 and ICAM-2 and alternate CD11/CD18 ligands in neutrophil transendothelial migration. J Leukoc Biol 1999, 65:117–126
81. Huang MT, Larbi KY, Scheiermann C, Woodfin A, Gerwin N, Haskard DO, Nourshargh S: ICAM-2 mediates neutrophil transmigration in vivo: evidence for stimulus specificity and a role in PECAM-1-independent transmigration. Blood 2006, 107:4721–4727
82. Stefanidakis M, Newton G, Lee WY, Parkos CA, Luscinskas FW: Endothelial CD47 interaction with SIRPgamma is required for human T-cell transendothelial migration under shear flow conditions in vitro. Blood 2008, 112:1280–1289
83. Bardin N, Blot-Chabaud M, Despoix N, Kebir A, Harhouri K, Arsanto JP, Espinosa L, Perrin P, Robert S, Vely F, Sabatier F, Le Bivic A, Kaplanski G, Sampol J, Dignat-George F: CD146 and its soluble form regulate monocyte transendothelial migration. Arterioscler Thromb Vasc Biol 2009, 29:746–753
84. Masedunskas A, King JA, Tan F, Cochran R, Stevens T, Sviridov D, Ofori-Acquah SF: Activated leukocyte cell adhesion molecule is a component of the endothelial junction involved in transendothelial monocyte migration. FEBS Lett 2006, 580:2637–2645
85. Jin S, Umemoto E, Tanaka T, Shimomura Y, Tohya K, Kunizawa K, Yang BG, Jang MH, Hirata T, Miyasaka M: Nepmucin/CLM-9, an Ig domain-containing sialomucin in vascular endothelial cells, promotes lymphocyte transendothelial migration in vitro. FEBS Lett 2008, 582: 3018–3024
86. Kukulski F, Ben Yebdri F, Bahrami F, Fausther M, Tremblay A, Sévigny J: Endothelial P2Y2 receptor regulates LPS-induced neutrophil transendothelial migration in vitro. Mol Immunol 2010, 47:991–999
87. Courtoy PJ, Quintart J, Baudhuin P: Shift of equilibrium density induced by 3,3′-diaminobenzidine cytochemistry: a new procedure for the analysis and purification of peroxidase-containing organelles. J Cell Biol 1984, 98:870–876
88. Courtoy PJ, Quintart J, Draye JP, Baudhuin P: The DAB-induced density shift: principle, validity and applications to endosomes. Prog Clin Biol Res 1988, 270:169–183
89. Wolburg H, Wolburg-Buchholz K, Engelhardt B: Diapedesis of mononuclear cells across cerebral venules during experimental autoimmune encephalomyelitis leaves tight junctions intact. Acta Neuropathol 2005, 109:181–190
90. Kuchler-Bopp S, Delaunoy JP, Artault JC, Zaepfel M, Dietrich JB: Astrocytes induce several blood-brain barrier properties in non-neural endothelial cells. Neuroreport 1999, 10:1347–1353
91. Muller WA, Weigl S: Monocyte-selective transendothelial migration: Dissection of the binding and transmigration phases by an in vitro assay. J Exp Med 1992, 176:819–828
92. Suh YH, Shin YK, Kook MC, Oh KI, Park WS, Kim SH, Lee IS, Park HJ, Huh TL, Park SH: Cloning, genomic organization, alternative transcripts and expression analysis of CD99L2, a novel paralog of human CD99, and identification of evolutionary conserved motifs. Gene 2003, 307:63–76