Segregation of VE-cadherin from the LBRC Depends on the Ectodomain Sequence Required for Homophilic Adhesion

Running Title: Protein Sorting at the Lateral Border

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ABSTRACT

The lateral border recycling compartment (LBRC) is a reticulum of perijunctional
tubulovesicular membrane continuous with the plasmalemma of endothelial cells that is essential
for efficient transendothelial migration (TEM) of leukocytes. The LBRC contains molecules
involved in TEM such as PECAM, PVR, and CD99, but not VE-cadherin. Despite its
importance, how membrane proteins are included in or excluded from the LBRC is not known.
Immunoelectron microscopy and biochemical approaches demonstrate that inclusion into the
LBRC is the default pathway for transmembrane molecules present at endothelial cell borders.
A chimeric molecule composed of the extracellular domain of VE-cadherin and cytoplasmic tail
of PECAM (VE-CAD/PECAM) did not enter the LBRC, suggesting that VE-cadherin was
excluded by a mechanism involving its extracellular domain. Deletion of the homophilic
interaction domain EC1 or the homophilic interaction motif RVDAE allowed VE-CAD/PECAM
and even native VE-cadherin to enter the LBRC. Similarly, treatment with RVDAE peptide to
block homophilic VE-cadherin interactions allowed endogenous VE-cadherin to enter the LBRC.
This suggests that homophilic interactions of VE-cadherin stabilize it at cell borders and prevent
entry into the LBRC.
INTRODUCTION

Efficient cell function depends upon segregation and compartmentalization of membrane domains (Furuse et al., 2002; Shin et al., 2006). The specialized membrane composition of the apical and basolateral surfaces of polarized epithelial cells is maintained by selective membrane delivery and retention at these surfaces. Even within a membrane, microdomains such as lipid rafts and caveolae efficiently compartmentalize specific functions locally (Mukherjee and Maxfield, 2004; Ushio-Fukai, 2009). The lateral borders of endothelial cells are specialized regions containing adhesion and signaling molecules related to maintaining barrier function (Dejana and Vestweber, 2013; Mehta and Malik, 2006), sensing shear (Tarbell, 2010; Tzima et al., 2005), and allowing immune surveillance (Ley et al., 2007; Muller, 2011). There is a subcompartment of junctional membrane, the lateral border recycling compartment (LBRC), which is critical for promoting transendothelial migration (TEM) of leukocytes.

Previous studies demonstrated that the LBRC, plays a critical role in TEM under all inflammatory conditions studied (Mamdouh et al., 2003; Mamdouh et al., 2008; Mamdouh et al., 2009). The LBRC is a reticulum of interconnected membrane below the cell borders. It is defined by its function, as there are no identified unique markers (Sullivan et al., 2014). Membrane constitutively cycles between the border and this compartment evenly along the junctions between endothelial cells. However, when leukocytes transmigrate, LBRC membrane is actively trafficked along microtubules to surround the leukocyte at the site of TEM (Mamdouh et al., 2003; Mamdouh et al., 2008; Mamdouh et al., 2009; Muller, 2011). Another unique feature of the LBRC is that major molecules regulating TEM, including platelet/endothelial cell adhesion molecule (PECAM, CD31), CD99, poliovirus receptor (PVR; CD155), and Junctional adhesion molecule A (JAM-A), all reside in the LBRC and display the same targeted recycling pattern during leukocyte TEM. Blockade of PECAM, PVR, or CD99; or inhibition of kinesin molecular motors disrupt the targeted recycling of the LBRC and thus block TEM (Dasgupta and Muller, 2008; Mamdouh et al., 2003; Mamdouh et al., 2008; Mamdouh et al., 2009; Muller, 2011; Sullivan et al., 2013).

Vascular endothelial cell specific cadherin (VE-cadherin), a major component of the endothelial cell border, is not in the LBRC (Mamdouh et al., 2009; Sullivan et al., 2014). VE-cadherin plays
a central role in the formation and maintenance of adherens junctions and barrier function in endothelium (Dejana and Giampietro, 2012; Dejana et al., 2008; Vestweber et al., 2009). It is exclusively expressed in endothelium and localized to the intercellular border. Like other cadherins, VE-cadherin contains five homologous extracellular domain repeats, (EC1-5) (Dejana et al., 2008; Vestweber, 2008; Vincent et al., 2004). The extracellular domains mediate homophilic binding of VE-cadherin between adjacent cells and stabilize the adherens junction. It has been well demonstrated that the EC1 domain is the core element required for the homophilic interaction of VE-cadherin (Dejana et al., 2008; Vestweber, 2008; Vincent et al., 2004).

Despite its critical importance in the process of TEM, the membrane composition and selection mechanism for molecules residing in the LBRC is unknown. We approached this question by studying PECAM, the best known component of the LBRC. We hoped that understanding how PECAM entered the LBRC would provide clues to how other components enter. Previous studies demonstrated that mutation of tyrosine 663 in the PECAM cytoplasmic tail to phenylalanine interfered with the efficient movement of PECAM between the endothelial cell border and the LBRC (Dasgupta et al., 2009). However, standard signaling functions of PECAM were not altered (Dasgupta et al., 2009). This suggested the possibility that, similar to some other cytoplasmic tail tyrosine motifs (Lipardi et al., 2002; Rohrer et al., 1996); there was a recognition signal on the cytoplasmic tail of PECAM for inclusion into the LBRC.

We tested this hypothesis by expressing chimeric molecules containing the cytoplasmic tail of PECAM and the extracellular domains of other transmembrane proteins. While the cytoplasmic domain of PECAM enhanced the ability of chimeric molecules to get to the lateral border, it was not necessary to bring exogenous molecules into the LBRC. Contrary to our hypothesis, we found that entrance into the LBRC was a default pathway for molecules present on the endothelial cell borders. Moreover, even exogenous molecules that enter the LBRC move with it as the LBRC traffics to surround transmigrating leukocytes. In contrast, VE-cadherin was excluded apparently via homophilic interactions with other VE-cadherin molecules at the cell surface.
RESULTS

PECAM does not use a positive sorting signal to enter the LBRC

If the cytoplasmic tail of PECAM contained a sorting motif to direct it to the LBRC, we hypothesized that the cytoplasmic tail of PECAM, when expressed on a molecule not normally in the LBRC, could direct that molecule into the LBRC. To test this hypothesis, we constructed a chimera that contained the transmembrane and cytoplasmic domains of PECAM fused to the extracellular domain of Tac (interleukin 2 receptor α chain, CD25), a protein not expressed by endothelial cells (Fig. 1).

In order to compare endothelial cells consistently expressing the same levels of chimera, we made stable transfectants in immortalized human umbilical vein endothelial cells (iHUVEC), that faithfully recapitulate the known characteristics of primary endothelial cells (Ancuta et al., 2003; Yang et al., 2005; Yang et al., 2006). Both iHUVEC and HUVEC displayed the same level of PECAM, VE-cadherin, and CD99 (Fig. S1A) and PECAM-dependent TEM percentage (Fig S1B). More important, iHUVEC maintains the LBRC, which has the same morphology (compare EM images in Fig. S1C to Fig 2 of (Mamdouh et al., 2003)) and function in supporting leukocyte TEM as it does in primary endothelium (Fig. S1D). Therefore, we will refer to these as endothelial cells (EC) henceforth. We then established stable Tac/PECAM and Tac transfectants from EC; and the expression of each construct was confirmed using flow cytometry, western blots, and immunofluorescence (Fig. 2). As seen in Fig. 2A, EC do not express Tac. However, after transduction of Tac/PECAM or Tac, the anti-Tac Ab readily detected surface expression of these molecules. Expression of Tac/PECAM was also confirmed using western blots by Ab against the cytoplasmic tail of PECAM (Fig. 2B). Native PECAM was detected in all cell lines; and Tac/PECAM transfectants showed an additional band that corresponds to the expected molecular weight of the chimera. The expression of Tac/PECAM protein was 1.6 times native PECAM. The expression of endogenous PECAM (Fig. S2A) and TEM of monocytes (Fig. S2B) were not changed in these transfectants.

As expected, both the Tac/PECAM and Tac constructs were expressed diffusely on the membrane, indicating that neither construct has a defect in trafficking through the rough
endoplasmic reticulum-Golgi pathway (Fig. 2C). The Tac/PECAM was partially enriched at the intercellular junctions, however the degree of enrichment was highly variable and significantly less than the clear junctional localization of PECAM. This is to be expected, as Tac has no ligand on the apposing cell to trap it at the cell border.

Immuno-EM studies demonstrated that the LBRC remained intact in both transfectants. For both lines PECAM was detected in the subjunctional interconnected vesicles that are typical of the LBRC (Fig. 2D), as previously reported (Mamdouh et al., 2003; Sullivan et al., 2013). We then examined the exogenous proteins and found that Tac/PECAM molecules not only localize partially to junctions, but also enter the LBRC (Fig. 2D), consistent with the hypothesis that the cytoplasmic tail of PECAM contains a sorting signal for entry. Surprisingly, even though Tac was expressed diffusely over the cell (Fig. 2C), it was still found in the LBRC (Fig. 2D).

While immuno-EM is the gold standard for identifying LBRC components, it only examines a small fraction of the cell borders. To determine whether our results were representative of the entire cell population, we exploited the fact that Abs are unable to enter the LBRC at 4°C. We previously used an immunoprecipitation assay to examine the distribution of phosphorylated PECAM between the surface and LBRC (Dasgupta and Muller, 2008). In the current study, we applied a similar rationale to develop a Sequential Immunoprecipitation Assay (See Methods) to determine whether a membrane protein was in the LBRC. Using this assay we can readily distinguish between the surface and LBRC pools of PECAM (Fig. 3A). Consistent with our previous observation, about 1/3 of total PECAM is in the LBRC (Dasgupta and Muller, 2008; Mamdouh et al., 2003). On the other hand, VE-cadherin, which has previously been shown to absent from the LBRC (Mamdouh et al., 2009; Sullivan et al., 2013), was almost undetectable in the LBRC (Fig. 3A).

This assay cannot distinguish between pools protected in the LBRC and internal pools (i.e. proteins found in endosome, or late stage biosynthetic precursors) that may be more abundant for exogenously expressed proteins. To control for this, we treated monolayers with NHS-sulfo-LC-biotin, a membrane impermeant biotinylation reagent. Our previous work has shown that the
LBRC membrane is continuous with the plasma membrane and accessible to small compounds, but not Fab fragments of antibody, at 4°C (Mamdouh et al., 2003). Therefore, the surface and LBRC pools but not the internal pools of the target protein should be biotinylated and detected (Fig. 3B). This modification ensures that we detect the protein that is only on the surface and LBRC but not in the internal pools. This method measures the distribution at steady state, not at a time after incubation with antibody. Furthermore, since the labeling is performed in the absence of antibodies, this verifies that the compartmentalization of membrane is not influenced by interaction with the antibodies. Modification with biotin does not affect the outcome; the distribution of VE-cadherin and PECAM are similar in both assays (compare 3A and 3B). The trace amount of VE-cadherin sometimes detectable in the LBRC fraction may represent residual surface molecules that escaped binding by the antibody in the first round, since no more protected VE-cadherin was seen when we used biotin to restrict labeling to surface and LBRC (Fig. 3B).

Using this assay, we examined the ability of the Tac/PECAM and Tac to enter into the LBRC. As shown in the immuno-EM, both Tac/PECAM (Fig. 3C) and Tac (Fig. 3D) are clearly detectable in the LBRC. The presence of PECAM in the LBRC was used as a control to ensure that the LBRC was properly formed in these two (Fig.3, C and D) and all other transfectants used in this assay. Apparently, molecules of Tac that were present at the cell borders were capable of entering. This was not consistent with our original hypothesis, and instead suggests that any protein that can get to the endothelial cell border can potentially enter the LBRC.

**Exogenous LBRC molecules traffic along with endogenous molecules**

Tac/PECAM and Tac were found in compartments resembling the LBRC, but do these compartments function like part of the LBRC? Furthermore, while it is assumed that all proteins localized to the LBRC traffic together, there has never been any direct demonstration of this, since we do not have Abs against any of the known LBRC constituents, except PECAM, that do not block LBRC function and transmigration. The presence of exogenous Tac in the LBRC gave us the opportunity to test this. The targeted recycling assay (Mamdouh et al., 2003) tracks membrane coming from the LBRC to surround a leukocyte during TEM. When we performed
this assay on Tac/PECAM and Tac, neither construct was enriched around the migrating leukocyte (Fig. 4E). We speculated that the constructs were targeted with the LBRC, but did not remain enriched around the leukocyte because they had no cognate binding partner on the leukocyte and get outcompeted by molecules that do. To provide a functional interaction with a transmigrating leukocyte, we modified the Tac/PECAM and Tac constructs to each contain IgG Domain 1 and 2 (D1,2) from extracellular part of PECAM, which is known to facilitate homophilic interactions, to make chimeras D1,2-Tac/PECAM and D1,2-Tac and establish transfectants (Fig. 4A). As expected, both constructs were largely localized to the junction since they now bore the homophilic interaction domain of PECAM (Fig. 4B). By utilizing the Sequential Immunoprecipitation Assay, we also observed that both constructs entered the LBRC and the distribution of PECAM and VE-cadherin did not change (Fig. 4, C and D). Neither construct interfered with PECAM expression (Fig. S2C) and TEM (Fig. S2D). Labeling the LBRC pools of these constructs, we found that they both were readily enriched around the migrating leukocyte (Fig. 4E). Quantitation of fluorescence intensity demonstrated that D1,2-Tac and D1,2-Tac/PECAM were 3-4 fold enriched around the migrating leukocyte, similar to levels of enrichment of PECAM (3.7 fold) (Fig. 4E). This indicates that the D1,2- Tac/PECAM and D1,2-Tac move with the LBRC.

It is possible that the homophilic interaction domains of PECAM on the D1,2-Tac/PECAM and D1,2-Tac artifactually allowed them to participate in targeted recycling through cis interaction with native PECAM. We thought this unlikely, since D1,2 on the chimeras would be much closer to the membrane (alongside domains 5 and 6, with which they do not interact) than D1,2 on native PECAM. Nonetheless, to test this possibility, we determined whether these chimeras co-migrated with native PECAM when PECAM was cross-linked in a patching assay. In this assay, in the absence of intercellular junctions, PECAM, D1,2-Tac/PECAM and D1,2-Tac were diffusely distributed on the cell surface (Fig. S3). Upon crosslinking of PECAM using a mAb that binds to domain 5 but not D1,2, the native PECAM on the cell surface formed many concentrated foci (Fig. S3, B and D). However, D1,2-Tac (Fig. S3B) and D1,2-Tac/PECAM (Fig. S3D) did not move into these foci, but remained diffusely localized. Therefore, we
conclude that targeted recycling of Tac from the LBRC to the site of transmigration represents its intrinsic tendency to move with the LBRC.

VE-cadherin exclusion from the LBRC does not require its cytoplasmic tail

Our observation that even exogenous proteins gain access to the LBRC suggests that entrance into the LBRC is a default pathway. Since VE-cadherin is the only junctional transmembrane molecule known to be excluded from the LBRC, we investigated the mechanism behind this exclusion. VE-cadherin is connected to the actin cytoskeleton through the p120 and β-catenins that bind to specific phosphorylated tyrosine residues (Y658 and Y731, respectively) on its cytoplasmic tail (Potter et al., 2005).

To examine the role of the cytoplasmic tail in exclusion of VE-cadherin from the LBRC, we established cell lines expressing N-terminal-FLAG tagged full length VE-cadherin (FLAG-VE-CAD) and VE-cadherin with the cytoplasmic tail of PECAM (FLAG-VE-CAD/PECAM). The presence of the FLAG epitope enables us to distinguish them from endogenous VE-cadherin. Flow cytometry (Fig. 5A) and western blots (Fig. 5B) showed that both constructs were expressed on the surface of EC at approximately the same level. Immunofluorescence staining showed that both proteins localize to the junction (Fig. 5C). Expression of FLAG-VE-CAD or FLAG-VE-CAD/PECAM did not influence PECAM expression (Fig. S2E) and TEM (Fig. S2F). Our use of only a single FLAG sequence in these constructs may have helped prevent changes in VE-cadherin adhesion.

We then examined both constructs by anti-FLAG Ab in immuno-EM studies (Fig. 5D). As expected, FLAG-VE-CAD was only observed at the junction and not found in typical LBRC vesicular structures. FLAG-VE-CAD/PECAM had the same distribution despite the PECAM cytoplasmic tail it contains. We further utilized the Sequential Immunoprecipitation Assay to verify the EM findings. Biotinylation interfered with the ability of the anti-FLAG antibody to recognize the epitope, presumably due to the two lysine residues in the FLAG sequence. Our attempts to immunoprecipitate FLAG proteins and detect them in western blots with the commercial anti-FLAG Abs were unsuccessful. Therefore, we used anti-VE-cadherin Ab for
both native VE-cadherin and its corresponding FLAG constructs (Fig 5 and 7). Notice, anti-VE-cadherin Ab cannot distinguish native VE-cadherin from the corresponding full-length or truncated FLAG-VE-CAD constructs due to closeness of molecular weight (Fig 5E, 7A, 7C, and 7E). The anti-VE-cadherin Ab can distinguish native VE-cadherin from all FLAG-VE-CAD/PECAM constructs (Fig 5F, 7B, 7D, and 7F). Both FLAG-VE-CAD (Fig. 5E) and FLAG-VE-CAD/PECAM were not detected in the LBRC (Fig. 5, E and F). This observation is consistent with our immuno-EM findings. Together, these findings suggest that the cytoplasmic domain of VE-cadherin is not necessary for its exclusion from the LBRC.

**Homophilic interactions of VE-cadherin extracellular domain 1 prevent its entry into the LBRC**

Having ruled out the cytoplasmic tail of VE-cadherin as critical to its exclusion from the LBRC, we next investigate the role of the extracellular domain. Because the amino terminal cadherin domain, EC1, and, in particular, the pentapeptide motif RVDAE within EC1, of VE-cadherin has been reported to facilitate intercellular homophilic interactions (Dejana et al., 2008; Heupel et al., 2009; Komarova et al., 2012; Vestweber, 2008; Vincent et al., 2004), we first focused on this region. We prepared a series of constructs in which we sequentially deleted portions of the VE-cadherin extracellular domain 1 in the FLAG-VE-CAD and FLAG-VE-CAD/PECAM chimeras. More specifically, we made constructs with deletion of the entire EC1 (FLAG-ΔEC1 and FLAG-ΔEC1/PECAM lacking residues 1-104 of VE-cadherin), deletion of up to and including the RVDAE motif (FLAG-ΔRVDAE and FLAG-ΔRVDAE/PECAM, lacking residues 1-51 of VE-cadherin) and deletion up to the RVDAE, leaving these amino acids intact (FLAG-ΔN and FLAG-ΔN/PECAM, lacking residues 1-46 of VE-cadherin).

We first tested the ability of FLAG-VE-CAD (full length and the construct without RVDAE motif) to engage in homophilic adhesion. L cells were transduced with FLAG-VE-CAD, FLAG-ΔRVDAE, or vacant pBABE-puro (as control). Flow cytometry showed that the expression FLAG-VE-CAD and FLAG-ΔRVDAE were comparable among the transfectants (Fig. S4A). In the classic aggregation assays, which first defined cadherin function (Takeichi, 1990), the ability of a molecule to support adhesion is assessed by expressing it in cells and determining whether its expression imparts on these cells the ability to aggregate (Albelda et al., 1991; Bronner-
As seen in Fig. S4B, expression of FLAG-VE-Cadherin in L cells imparts on them the ability to aggregate. However, deletion of the RVDAE motif of VE-cadherin significantly impaired this ability and only background levels of aggregation were seen (Fig. S4B). Experiments using mixtures of fluorescently labeled and unlabeled transfected and non-transfected cells confirmed that FLAG-VE-cadherin aggregation was homophilic, while the small numbers of aggregates seen with FLAG-ΔRVDAE transfectants were mixtures of transfected and non-transfected cells (data not shown).

Next, EC transfectants expressing each of these truncated constructs were created and validated using flow cytometry (Fig. 6A) and immunofluorescence (Fig. 6B). All chimeras were expressed on the cell surface, all at roughly double the level of endogenous VE-cadherin, and all were mostly localized to intercellular junctions. Our anti-VE-cadherin mAb, hec1, recognizes the EC4 domain (Corada et al., 2001) and thus, its ability to bind the truncated constructs is not affected. Similar to other constructs used, truncated FLAG-VE-CAD and FLAG-VE-CAD/PECAM constructs did not interfere with PECAM expression (Fig. S2G) and leukocyte TEM (Fig. S2H).

To examine the ability of these constructs to enter the LBRC, we performed the immuno-EM studies by labeling cells with HRP-conjugated anti-FLAG Abs (Fig. 6C). All constructs that lacked the RVDAE motif were readily detected in LRBC-like structures (black arrows), including truncated native VE-cadherin, whereas the constructs with the RVDAE motif (FLAG-ΔN and FLAG-ΔN/PECAM) were only detected at the junction whether they bore the cytoplasmic tail of VE-cadherin or PECAM, even though unstained LBRC-like structures were observed in those samples (white arrows).

To confirm these findings, we performed the Sequential Immunoprecipitation Assay as described above. As seen in Fig. 7, all the constructs that lack EC1 domain or RVDAE motif had significant proportions detected in the LBRC fractions, consistent with LBRC localization (Fig. 7, C-F). Densitometry analysis from FLAG-ΔEC1/PECAM and FLAG-ΔRVDAE/PECAM shows that they have the same distribution pattern as PECAM: ~1/3 of chimera is in the LBRC (Fig. 7 D and F). For FLAG-ΔEC1 and FLAG-ΔRVDAE, since the close Mr does not allow us to
distinguish endogenous VE-cadherin from exogenous FLAG constructs, the densitometry analysis actually compares the portion of FLAG proteins in the LBRC to the combination of FLAG proteins and native VE-cadherin on the surface. Because native VE-cadherin is only on the surface, the percentage of FLAG proteins in the LBRC fraction is therefore an underestimate. Densitometry analysis is consistent with this idea in that their percentage in the LBRC is lower compared to PECAM (Fig 7 C and E) but clearly demonstrate the presence of VE-cadherin lacking the RVDAE motif in the LBRC. The two truncation constructs that still contain the RVDAE motif were excluded from the LBRC (Fig. 7, A and B), a finding consistent with our Immuno-EM results (Fig. 6C).

To further verify these findings, we tested whether interrupting homophilic interactions of native VE-cadherin would allow it to enter the LBRC. It has been reported that the blocking peptide (RVDAE) but not the scrambled peptide (ADVRE) can interrupt the homophilic interaction of VE-cadherin in other in vitro models (Heupel et al., 2009; Komarova et al., 2012). We measured the distribution of VE-cadherin in wild-type EC treated with blocking peptide or scrambled peptide. Since we had to maintain monolayer integrity, we had to use submaximal blocking conditions. Nevertheless, blocking the RVDAE motif did cause significant amounts of VE-cadherin to enter the LBRC (Fig. 7, G-I). We also observed that under these conditions the blocking peptide but not scrambled peptide increased monolayer permeability (Fig. S4C). Together these findings suggest that VE-cadherin is excluded from the LBRC by means of the RVDAE motif in EC1.

Discussion
We hypothesized the cytoplasmic tail of PECAM carried a recognition motif for selectively sorting into the LBRC. Our findings indicated that the transmembrane and cytoplasmic tail of PECAM can at least partially concentrate a protein at the endothelial cell border. However, ultrastructural studies showed that Tac, a molecule not expressed on endothelium, when exogenously expressed, could enter into the LBRC, even though it did not show any junctional enrichment (Fig. 2). This finding suggested that even though the transmembrane and cytoplasmic tail of PECAM may have some role in directing molecules to the EC border, it is not the required
to bring the molecule into the LBRC. All of the known membrane constituents of the LBRC are molecules concentrated at the endothelial cell borders. Therefore, these molecules are quantitatively more likely to enter the LBRC. However, our data suggest that any molecule that finds itself at the endothelial border might enter the LBRC unless it was actively excluded. These results may explain why we did not find any conserved sequence motifs on the cytoplasmic tails of other known LBRC proteins (PVR, CD99, and JAM-A).

There are clearly additional factors that influence the efficiency by which proteins may get into the LBRC. One is obvious: Proteins concentrated at the cell borders are more likely to get in because of their proximity. PECAM is more concentrated at the endothelial borders than Tac/PECAM because homophilic interactions on the apposing cell keep it there by diffusion trapping (Sun et al., 2000). Tac/PECAM may be more concentrated at the endothelial borders than Tac by virtue of its interaction with native PECAM through the cytoplasmic tail. Differences in the rate at which membrane proteins transit through the LBRC could also potentially affect their distribution on the surface vs. LBRC. In a previous study we showed that substitution of phenylalanine for tyrosine at position 663 in the cytoplasmic tail of PECAM decreases the efficiency of trafficking of PECAM into and out of the LBRC (and hence targeted recycling required for leukocyte transmigration), but does not totally eliminate it (Dasgupta et al., 2009). The constitutive recycling assays performed in that paper exposed the endothelial cell to antibody for one hour, which was sufficient time to saturate native PECAM, but may not have been enough time for a slowly recycling mutant. In the present study we show the distribution of membrane proteins at steady state, so potential differences in recycling rate do not affect our conclusions.

Since our data failed to demonstrate a positive sorting mechanism to bring membrane proteins into the LBRC, we next directed our attention to understanding how they may be excluded from the LBRC. VE-cadherin is the only known transmembrane protein at the endothelial border that is not in the LBRC. A variety of previous studies suggest the cytoplasmic tail of VE-cadherin is involved in regulation of endocytosis and downstream signaling by interacting with catenins and the cytoskeleton; but no study provides direct evidence of its role on subcellular localization.
(Dejana and Giampietro, 2012; Nanes et al., 2012; Vestweber, 2008). Replacing the cytoplasmic tail of VE-cadherin with that of PECAM did not allow the chimera to enter the LBRC (Fig. 5). Thus, the cytoplasmic tail of VE-cadherin is not required for the exclusion of VE-cadherin from the LBRC. Furthermore, all of the VE-cadherin constructs in this study contained the transmembrane domain of VE-cadherin, whether or not they were able to enter the LBRC. Therefore, the transmembrane domain is not sufficient for sorting into the LBRC. However, we cannot formally rule out that the cytoplasmic tail of VE-cadherin may play an additional role in exclusion from the LBRC.

We next turned our attention to the EC1 domain of VE-cadherin, which mediates homophilic interactions (Ahrens et al., 2003; Corada et al., 2001; Hewat et al., 2007; Legrand et al., 2001). The RVDAE motif of the EC1 domain, a pentapeptide relatively specific for VE-cadherin, has recently been identified as the core element for the homophilic interaction of VE-cadherin. Eliminating the RVDAE motif or blocking it with the inhibitory peptide significantly inhibits VE-cadherin trans-interaction, as shown in current study (Fig. S4) and previous reports (Heupel et al., 2009; Komarova et al., 2012). Our findings suggest that the EC1 domain and its RVDAE motif regulate subcellular localization of VE-cadherin and block it from entering the LBRC (Fig. 6 and 7). It was somewhat surprising that VE-cadherin without its cytoplasmic domain bound tightly enough by its homophilic interaction domain to exclude it from the LBRC. However, native VE-cadherin was able to enter the LBRC when homophilic VE-cadherin interactions were blocked by incubation with RVDAE peptide (Fig. 7, G-I). These data demonstrate that exclusion of VE-cadherin from the LBRC depend on the integrity of its homophilic interaction domain and strongly suggest that hemophilic adhesions (in trans) keep VE-cadherin out of the LBRC.

This begs the question of how the VE-cadherin mutants lacking RVDAE are able to be concentrated at the cell junctions. We do not know, but suspect that interactions of the VE-cadherin cytoplasmic tail (or the PECAM cytoplasmic tail for the chimeric molecules) with elements of the cytoskeleton favor its association at the junctional plasmalemma, much as Tac/PECAM chimeras are concentrated at the cell borders. Furthermore, homophilic interactions in cis (Dejana and Giampietro, 2012; Hewat et al., 2007) may stabilize them at the
plasmalemma. In our studies native VE-cadherin was present for these mutated molecules to interact with. However, the interactions in \textit{cis} in the absence of the \textit{trans} homophilic interactions are apparently not enough to prevent internalization into the LBRC. This also raises the question of how PECAM, CD99, and JAM-A, all of which normally engage in homophilic interactions with apposing cells are not excluded from the LBRC? Again, we do not know. However, these molecules do not engage in extensive \textit{cis} interactions and their homophilic \textit{trans} interactions are relatively weak. Therefore, disengaging them would be much easier than for VE-cadherin.

In addition, we were able to demonstrate, using our Tac and Tac/PECAM chimeras, that all LBRC molecules participate in targeted recycling and move in concert to surround the transmigrating leukocyte. Due to the technical constraints, this could not be demonstrated previously. Tac and Tac/PECAM carrying the PECAM homophilic interaction domains (D1,2) at the N-terminal displayed the same targeted recycling pattern as PECAM. While the D1,2 Tac/PECAM chimera could have been drawn there due to association with the cytoplasmic tail of endogenous PECAM, the D1,2 Tac chimera has no such association and the PECAM extracellular domains attached to it would not be expected to be in a position to physically associate with endogenous PECAM, since they would be too close to the membrane, adjacent to endogenous PECAM domains 5 and 6. Furthermore, control experiments show no physical interaction of the chimeras with native PECAM (Fig. S3). It is important to reiterate that in these experiments, chimeric or FLAG-tagged molecules were introduced into EC containing normal levels of VE-cadherin, PECAM, etc. so that the integrity of the junctions and of the LBRC would be maintained and we would observe only an effect on the partitioning of exogenous molecules into the LBRC (and be able to compare with the localization of endogenous molecules as internal controls) rather than a substitution of these molecules for the normal components. Indeed, TEM was not affected by these manipulations.

While the membrane of the LBRC moves to surround the leukocyte as it transmigrates, VE-cadherin, which maintains barrier function at the adherens junction, leaves the site of transmigration (Allport et al., 1997; Allport et al., 2000; Shaw et al., 2001). VE-cadherin is excluded from the LBRC. This difference on protein distribution may have direct impact on EC
function during TEM. VE-cadherin may be internalized into clathrin-coated vesicles (Xiao et al., 2005) and removed from the site of TEM as recycling LBRC membrane moves in, or it may be pushed aside in the plane of the membrane (Allport et al., 1997; Allport et al., 2000; Shaw et al., 2001). Both the influx of LBRC and efflux of VE-cadherin are required for TEM to occur. How their movements are coordinated is the subject of ongoing investigation.

MATERIALS AND METHODS
Antibodies (Abs), peptides, and endothelial cells
Monoclonal Abs (mAbs), hec1 (anti-VE-cadherin), hec2 (anti-CD99), hec7 (anti-PECAM), and IB4 (anti-CD18), were generated as described (Ali et al., 1997; Muller et al., 1989; Schenkel et al., 2002). mAb 7G7B6 (anti-Tac) (hybridoma provided by Dr. Andrew Kowalczyk, Emory University) and polyclonal Ab 301 (anti-cytoplasmic tail of PECAM) were generated in house. Non-blocking anti-PECAM mAb P1.1 was provided by Dr. Peter Newman, Medical College of Wisconsin. Anti-FLAG Ab M2 was from Sigma-Aldrich and anti-Tac Ab 2R12 was from Thermo Scientific. Fab were cut from P1.1 or 7G7B6 by using immobilized papain (Thermo Scientific). Abs for immunofluorescence were coupled to Alexa-488 and Alexa-546 (Invitrogen). Blocking peptide RVDAE and scrambled peptide ADVRE were obtained from Dr. Yulia Komarova, University of Illinois at Chicago.

iHUVEC were made by transduction of HUVEC with LSNX-16E6E7, an amphotrophic retrovirus encoding the oncoproteins E6 and E7 of human papillomavirus type 16 (provided by Dr. David Klumpp, Northwestern University).

Generation of DNA constructs and establishment of stable cell lines
Retroviral plasmid pBABE-puro was provided by Dr. Kathleen Rundell, Northwestern University. cDNA encoding human Tac was obtained from ATCC. cDNAs encoding human PECAM or VE-cadherin were generated by our lab. Cloning reagents were purchased from New England Biolabs. DNA fragments for constructs were generated by PCR using primers in Table S1. Constructs used in this project are listed in Fig. 1 and corresponding nomenclature in the legend.
All constructs were inserted into pBABE-puro with EcoRI at the 5’-end and SalI at the 3’-end, except D1,2 Tac/PECAM and D1,2 TAC, which is with SnaBI at 5’-end and SalI at 3’-end. In FLAG-VE-CAD/PECAM and its corresponding truncated constructs, the extracellular and transmembrane domain of VE-cadherin was linked with cytoplasmic tail of PECAM through BamHI. For FLAG proteins, an expression signal, containing signal peptide and propeptide of VE-cadherin, plus a FLAG peptide, was generated by PCR and linked with the N-terminal of extracellular domain through XbaI. For D1,2 Tac/PECAM and D1,2 Tac, the signal peptide and IgG-like domain 1 and 2 of PECAM was linked to extracellular domain of Tac/PECAM or Tac through KpnI. Retrovirus carrying these constructs was transduced into iHUVEC or L cells. Stable cell lines were established under puromycin selection.

Flow Cytometry
Cells were removed from the plate with Hanks’ Balanced Salt Solution (HBSS) containing 10mM EDTA. Cells suspension was incubated with the appropriate primary Ab and secondary Ab (FITC goat anti-mouse, DAKO) at 10 μg/mL. Cells Fluorescence was assayed on BD FACSCanto II flow cytometer (BD Biosciences) and data were analyzed with FlowJo (Tree Star, Inc).

Immunofluorescence Microscopy
Cells were fixed in 2% paraformaldehyde for 10 minutes, blocked with 5% bovine serum albumin (Sigma-Aldrich) for 30 minutes, and incubated with the appropriate direct-conjugated Ab at 10 μg/mL for 1 hour at room temperature. Images were acquired by using a restoration workstation (Delta Vision 3D; Applied Precision) equipped with inverted 40× objective lens; and analyzed using ImageJ software.

Immuno-Electron Microscopy (immuno-EM)
hec7, 7G7B6, and M2 Abs were conjugated to horseradish peroxidase (HRP). Monolayers of cells on Thermanox coverslips (Thermo Fisher Scientific) were incubated with the appropriate HRP-conjugated Ab for 1 h at 37°C and fixed with 4% glutaraldehyde in 0.1 M sodium
cacodylate buffer (Electron Microscopy Sciences). Cells were washed and exposed to a mixture of 10 mg/mL diaminobenzidine (Sigma-Aldrich) and 0.03% hydrogen peroxide in PBS for 20 minutes. Samples were embedded. En face sections (70 nm thick) were examined on a Tecnai Spirit electron microscope (FEI Company).

Human Leukocyte Isolation, TEM assay and Targeted Recycling Assay

Peripheral blood mononucleated cell (PBMC) were harvested as previously described (Muller and Luscinskas, 2008) and resuspended in iHUVEC medium (Invitrogen) containing 0.1% human serum albumin (Grifols Biologicals Inc). TEM assays were performed as previously described (Muller and Luscinskas, 2008). Recycling of the LBRC protein during TEM was performed as previously described (Mamdouh et al., 2003).

Western Blots

Cells were lysed with RIPA buffer (0.4% NP-40, 100mM NaCl, 10mM NaPhosphate, 50 mM NaF, 1mM EDTA, 1mM EGTA) with 1mM orthovanadate and protease inhibitor cocktail (Sigma). The lysate was run on SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The blots were then blocked, probed with primary and HRP-conjugated secondary Abs, and developed.

Sequential Immunoprecipitation Assay (Isolation of molecules from surface and LBRC)

To avoid interference from proteins in the Golgi or post-Golgi compartments, proteins on the cell surface and LBRC were selectively biotinylated. EC were incubated with 2 mM NHS-sulfo-LC-biotin (Pierce) for 30 min on ice. At this temperature there is no movement of LBRC and large molecules, like Fab fragments of Abs, cannot enter the LBRC; but protons and biotin can (Mamdouh et al., 2003). The reaction was quenched using 100mM glycine. To selectively label specific surface molecules, Abs were added at 4°C for 1 hour to saturate the binding. Abs were washed out and cells was lysed. The lysate was incubated with protein-G sepharose (Invitrogen) for several rounds to exhaustively immunoprecipitate the Ab-labeled surface protein. The samples were then incubated with Ab and protein-G sepharose to immunoprecipitate the remaining specific protein in the lysate (fraction from LBRC, which had been inaccessible to Ab
in the intact cells due to its location). Samples of the surface and LBRC fractions were analyzed by Western blots using HRP-conjugated streptavidin. Density of protein bands was analyzed with ImageJ. For experiment with blocking or scrambled peptide, EC was pre-treated with peptide at 100 μM for 1 hour at 37 °C.

**Aggregation Assay**

The aggregation assay was performed as previously described (Albelda et al., 1991; Bronner-Fraser, 1985; Muller et al., 1992). L cells carrying vacant pBABE-puro, FLAG-VE-CAD, or FLAG-ΔRVDAE were used for the assay. In brief, confluent cells were detached with 10mM EDTA and then re-suspended in HBSS containing 2 mM CaCl$_2$ and 1 mM MgCl$_2$ to final cell density of 1 X 10$^6$ cells/ml. Cell suspension was shaken at 37 °C, 90 rpm.

**Patching Assay**

Patching assay was used to determine whether D1,2 Tac or D1,2-Tac/PECAM would be directly affected by the movement of native PECAM. EC transfectants, D1,2 Tac/PECAM and D1,2 Tac, were seeded at density of 2000 cells/cm$^2$. PECAM on the surface was labeled with P1.1 (which binds to the 5$^{th}$ Ig-like domain on PECAM and thus will not interact with D1,2 Tac/PECAM and D1,2 Tac) for 30 min at 4 °C. To crosslink PECAM, cells were incubated with rhodamine-conjugated goat anti–mouse IgG (Jackson ImmunoResearch) for 10 min at 37 °C, washed, and fixed. For non-crosslinked control, after the labeling with P1.1, cells were fixed first and then incubated with rhodamine-conjugated goat anti–mouse IgG for 10 min at room temperature. Both crosslink and non-crosslink groups were then incubated with Alexa 488-conjugated 7G7B6 to label D1,2 Tac/PECAM or D1,2 Tac. Images of samples were acquired and analyzed as described above.

**Permeability Assay**

Experiments were performed as previously described (Winger et al., 2014) with some modifications. iHUVEC was pre-treated with either blocking or scrambled peptide at 37 °C for 1 hour. Peptide was washed away and cells were incubated with FITC-dextran in culture medium.
for 1 hour. The relative fluorescence intensity (RFU) was measured by a FilterMax F5 microplate reader (Molecular Devices).

Statistics
Experiments with quantitation were performed at least three times. The Student's t-test (one-way ANOVA in Aggregation Assay) was used to evaluate statistical significance and data in figures were presented as Mean ± SD.
We are grateful to Drs. Kathleen Green (Northwestern University), Kathleen Rundell, David Klumpp, Peter Newman, Andrew Kowalczyk, and Yulia Komarova for valuable suggestions, reagents and help. We thank Northwestern University Center for Advanced Microscopy for EM support.

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References


Figure Legends

Figure 1  **Schematic diagram of constructs.**

Tac/PECAM: chimera contains extracellular and transmembrane domain of Tac and cytoplasmic tail of PECAM. FLAG-VE-CAD: full length wild-type VE-cadherin with integrated FLAG peptide at N-terminal. FLAG-ΔN: truncated FLAG-VE-CAD with deletion of residue 1-46 on extracellular domain of VE-cadherin. FLAG-ΔRVDAE: truncated FLAG-VE-CAD with deletion of residue 1-51 (including RVDAE motif, residue 47-51) on extracellular domain. FLAG-ΔEC1: truncated FLAG-VE-CAD with deletion of residue 1-104 (the full-length of EC1 domain) on extracellular domain. FLAG-VE-CAD/PECAM, FLAG-ΔN/PECAM, FLAG-ΔRVDAE/PECAM, and FLAG-ΔEC1/PECAM are the corresponding chimera with the replacement of C-tail from PECAM. D1,2-Tac or D1,2-Tac/PECAM: Full length of Tac or Tac/PECAM with IgG Domain 1 and 2 (D1,2) from extracellular part of PECAM on N-terminal.

Figure 2  **Both Tac/PECAM and Tac can enter the LBRC.**

Expression and distribution of Tac/PECAM and Tac were analyzed by (A) flow cytometry, (B) Western blots, and (C) immunofluorescence staining. (A) Red curve: isotype control (CT). Filled blue curve: labeled cells. (B) Lysate was probed with anti-PECAM cytoplasmic tail Ab. (C) Tac/PECAM and Tac cells were stained with anti-Tac (red in merge) and anti-PECAM (green in merge) Abs. Scale bar: 10 μm. (D) Both Tac/PECAM and Tac are in the LBRC. Tac/PECAM and Tac monolayers were treated with HRP-conjugated anti-PECAM or anti-Tac Ab at 37°C and processed for immuno-EM. Black arrows: LBRC-interconnected vesicles visualized by diaminobenzidene staining. Scale bars: 1 μm. Data are representative of at least 20 images of each sample from 3 independent experiments. 381.5 and 370.3 μm of membrane at junctions in Tac/PECAM and Tac cells, respectively, was examined.

Figure 3  **Sequential Immunoprecipitation Assay shows both Tac/PECAM and Tac are in the LBRC.**
(A) PECAM but not VE-cadherin is in the LBRC. VE-cadherin or PECAM without biotinylation on the surface or LBRC was labeled, separated by sequential immunoprecipitation assay and detected by specific Abs. Left part: Western blots; right part: densitometry analysis. Abundance of protein in the surface / LBRC fraction was presented as percentage of total amount of protein. (B) Biotinylation does not change the distribution of PECAM and VE-cadherin between the surface and LBRC fraction. VE-cadherin or PECAM were biotinylated, labeled, separated and detected by HRP-streptavidin. (C) Tac/PECAM is in the LBRC fraction. VE-cadherin, Tac/PECAM, and PECAM in the transfectant were analyzed by the method described in (B). (D) Tac is in the LBRC fraction. VE-cadherin, Tac, and PECAM in the transfectant were analyzed by the method described in (B). **: P < 0.01, compared to PECAM in the LBRC fraction.

Figure 4  **Molecules in the LBRC travel together to the site of TEM**
Expression of D1,2-Tac/PECAM and D1,2-Tac were detected by anti-Tac Ab and analyzed by (A) flow cytometry and (B) immunofluorescence staining. (A) Red curve: isotype CT. Filled blue curve: labeled cells. (B) Scale bar: 10 μm. Distribution of D1,2-Tac/PECAM (C) and D1,2-Tac (D) on the surface and LBRC was analyzed by Sequential Immunoprecipitation Assay. The left part is the Western blots and right part is densitometry analysis. **: P < 0.01, compared to PECAM in the LBRC fraction. (E) Targeted recycling of D1,2-Tac/PECAM and D1,2-Tac but not Tac/PECAM or Tac was detected during leukocyte transmigration. Recycled Protein (PECAM in EC; and D1,2-Tac/PECAM, D1,2-Tac, Tac/PECAM, and Tac in transfectants) was stained and visualized by fluorescence as described (left column). Monocytes were stained with anti-CD18 Ab (IB4, middle column). Merged images (right column) show that D1,2-Tac/PECAM and D1,2-Tac recycle and enrich at the site of TEM (white arrows), which displayed the same pattern as that of PECAM in wild-type EC (white arrow). Scale bar: 10 μm. The plot on the right bottom panel is the quantification of the enrichment of recycling proteins at the site of TEM. **: P < 0.01, compared to the enrichment of PECAM.

Figure 5  **The cytoplasmic tail of VE-cadherin is not necessary for exclusion from the LBRC.**
Expression and distribution of FLAG-VE-CAD and FLAG-VE-CAD/PECAM were analyzed by (A) flow cytometry, (B) Western blots, and (C) immunofluorescence staining. (A) EC, FLAG-VE-CAD, and FLAG-VE-CAD/PECAM cells were stained with anti-FLAG or anti-VE-cadherin Ab. Red curve: isotype control. Filled blue curve: labeled cells. (B) Lysate was probed with anti-FLAG, anti-VE-cadherin and β-actin Abs. (C) EC, FLAG-VE-CAD, and FLAG-VE-CAD/PECAM cells were stained with anti-FLAG (red in merge) and anti-VE-cadherin (green in merge). Scale bar: 10 μm. (D) Neither FLAG VE-CAD nor FLAG VE-CAD/PECAM are in the LBRC. Cells were treated with HRP-conjugated anti-FLAG Ab at 37°C and processed for immuno-EM. White arrows: likely unstained LBRC vesicles. Scale bars: 1 μm. 173.6 and 156.3 μm of membrane at junctions in FLAG-VE-CAD and FLAG-VE-CAD/PECAM cells, respectively, was examined. (E) FLAG-VE-CAD and (F) FLAG-VE-CAD/PECAM are not in the LBRC fraction. Sequential immunoprecipitation assay were performed with the method described in Figure 2B. The left part is Western blots and right part is densitometry analysis. **: P < 0.01, compared to PECAM in the LBRC fraction.

Figure 6 Extracellular domain 1 of VE-cadherin and its RVDAE motif prevent the molecule entry into the LBRC.

Expression and distribution of truncated FLAG-VE-CAD (FLAG-ΔN, FLAG-ΔRVDAE, and FLAG-ΔEC1) and FLAG-VE-CAD/PECAM (FLAG-ΔN/PECAM, FLAG-ΔRVDAE/PECAM, and FLAG-ΔEC1/PECAM) were analyzed by (A) flow cytometry and (B) immunofluorescence. (A) Cells were stained with anti-FLAG or anti-VE-cadherin Abs. Non-filled red curve: isotype control. Filled blue curve: labeled cells. (B) Cells were stained with anti-FLAG (red in merge) and anti-VE-cadherin (green in merge). (C) FLAG-VE-CAD and FLAG-VE-CAD/PECAM without RVDAE motif or EC1 domain can enter into the LBRC. Cells were treated with HRP-conjugated anti-FLAG Ab at 37°C and processed for immuno-EM. Black arrows: LBRC-interconnected vesicles visualized by diaminobenzidene staining. White arrows: likely unstained LBRC vesicles. Scale bars: 1 μm. Data are representative at least 9 images of each sample. For FLAG-ΔN, FLAG-ΔRVDAE, and FLAG-ΔEC1 cells, 180.9, 150.1, and 158.5 μm of the membrane at junction was examined, respectively. For FLAG-ΔN/PECAM, FLAG-Δ
ΔRVDAE/PECAM, and FLAG-ΔEC1/PECAM cells, 192.2, 168.1, and 172.4 μm of membrane at junctions was examined, respectively.

Figure 7  Sequential Immunoprecipitation Assay shows FLAG-VE-CAD and FLAG-VE-CAD/PECAM without EC1 domain or RVDAE motif are in the LBRC.

PECAM, VE-cadherin, or exogenous FLAG proteins on the surface of LBRC were detected by the assay described in Methods. For (A-F), the left part is the Western blots and right part is densitometry analysis. Abundance of protein in the surface / LBRC fraction was presented as percentage of total amount of protein. (A) FLAG-ΔN. (B) FLAG-ΔN/PECAM. (C) FLAG-ΔRVDAE. (D) FLAG-ΔRVDAE/PECAM. (E) FLAG-ΔEC1. (F) FLAG-ΔEC1/PECAM. (G) EC pre-treated with ADVRE scrambled peptide (S.P.). (H) EC pre-treated with RVDAE blocking peptide (B.P.). (I) Desitometry analysis of (G-H). (A) and (B) show deletion of N-terminate residue 1-46 does not facilitate the protein enter into the LBRC. However, deletion RVDAE motif (C-D) or the whole EC1 domain (E-F) in both VE-cadherin and CAD/PECAM chimera allow the proteins enter into the LBRC. (G-I) show VE-cadherin is detected in the LBRC in EC treated with B.P. but not S.P.. *: P<0.05, **: P < 0.01, compared to PECAM in the LBRC fraction.
A Wild type

Extracellular TM Cytoplasmic

D1 D2 D3 D4 D5 D6

EC1 EC2 EC3 EC4 EC5

..RVDAE..

Tac
PECAM
VE-cadherin

B Constructs

Construct Name

Tac/PECAM
FLAG-VE-CAD
FLAG-ΔN
FLAG-ΔRVDAE
FLAG-ΔEC1
FLAG-VE-CAD/PECAM
FLAG-ΔN/PECAM
FLAG-ΔRVDAE/PECAM
FLAG-ΔEC1/PECAM
D1,2-Tac
D1,2-Tac/PECAM