Filling the gaps

The endothelium in regulating vascular leakage and leukocyte extravasation

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Stiffness-induced endothelial DLC-1 expression forces leukocyte spreading through stabilization of the ICAM-1 adhesome

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Summary

Stiffening of blood vessels upon aging is a key driver of cardiovascular diseases and inflammation. Leukocytes follow the well-defined stages of rolling, spreading and crawling prior to diapedesis through the endothelial cells (ECs). Here we show the molecular mechanism of translating substrate stiffening by ECs to leukocytes via upregulation of endothelial DLC-1. DLC-1 expression was increased in stiffness associated diseases like atherosclerosis. Depletion of DLC-1 in ECs cultured on stiff substrates mimicked transmigration kinetics observed for ECs cultured on physiological representative soft substrates. Leukocytes display impaired spreading due to destabilization of the ICAM-1 adhesome. Mechanistic studies revealed that DLC-1-depleted ECs fail to recruit the actin adapter proteins Filamin B, α-actinin-4 and cortactin to clustered ICAM-1, thereby preventing the formation of the ICAM-1 adhesome. Finally, the spreading inability of leukocytes on ECs cultured on soft substrates was overcome by raising expression levels of endothelial DLC-1 and subsequently stabilizing the ICAM-1 adhesome.
Introduction

Aging is a major risk factor for cardiovascular diseases, and is associated to age-related stiffening of the vascular wall resulting in increased leukocyte influx induced by increased chronic inflammation. An increase in matrix stiffness of the vasculature strongly promotes leukocyte transendothelial migratory capacity. However, the mechanism how endothelial cells (ECs) translate the underlying matrix stiffness towards leukocyte transendothelial migration (TEM) remains unclear. Both chronic and acute inflammation are characterized by extravasation of activated leukocytes through the EC layer lining the interior vessel wall. In order to cross the endothelium, leukocytes follow a defined multi-step process also referred to as TEM. Tight-ly regulated interactions between leukocytes and the ECs orchestrate the different steps; going from the initial adhesion by rolling to firm adhesion and spreading, followed by diapedesis through the endothelial barrier either in a trans- or paracellular way. The different leukocyte-vessel wall interactions during the subsequent specific steps are clearly described; P- and E-selectins for rolling, ICAM-1 and VCAM-1 for spreading and finally CD99, PECAM-1, JAMs and VE-cadherin for diapedesis. However, the transition from one stage to the next remains a largely understudied territory. Intracellular ICAM-1 signaling, upon clustering of the adhesion molecule by binding of leukocytes, includes recruitment of actin adaptor proteins Filamins, α-actinin-4 and cortactin, connecting ICAM-1 to the actin cytoskeleton. Each of these adaptor proteins can specifically modify the actin cytoskeleton network: cortactin induces F-actin branching, Filamins promote actin fiber crosslinking and α-actinin-4 stabilizes the induction of antiparallel F-actin fibers. Interestingly, recruitment of α-actinin-4 was shown to be a regulator of endothelial stiffness and thereby influencing the spreading of adhesive polymorphonuclear cells (PMNs) to the endothelium and subsequent diapedesis efficiency.

Not only actin adaptor proteins are important to modify the endothelial cytoskeleton during the different steps of leukocyte TEM, also small GTPases, mainly the Rho-family are crucially involved. In turn Rho GTPases are regulated by Guanine-nucleotide Exchange Factors (GEFs) for activation and GTPase Activating Proteins (GAPs) for deactivation. However, no GTPases or GEF or GAP proteins have been described to be involved in the initial rolling or firm adhesion stage.

Deleted in liver cancer 1 (DLC-1) (ARHGAP7, STARD12) is a Rho-GAP and a regulator for cell proliferation, adhesion, and migration and complete loss or downregulation of DLC-1 in not only liver, but also lung, breast, stomach, brain, colon and prostate cancer establishes its strong tumor suppressor function. Besides tumor cells, DLC-1 is also expressed in ECs,
where it is mainly described to regulate angiogenesis via RhoA inactivation through its GAP domain. However, a possible role for endothelial DLC-1 in regulating leukocyte TEM is to date unknown.

We found an stiffness-dependent increase in endothelial DLC-1 expression both in vitro and in vivo upon disease-induced vascular stiffening in human atherosclerotic plaques and in pulmonary arterial hypertension (PAH) patients. Increased DLC-1 expression is correlated with increased leukocyte diapedesis upon increased substrate stiffness, and therefore we hypothesized that DLC-1 expression level is linked to the defect in PMN spreading and subsequently decrease TEM, seen on soft substrate stiffness. Through mechanistic studies, we found that leukocyte-induced recruitment of actin adaptor proteins upon ICAM-1 clustering required presence of DLC-1 to stabilize the ICAM-1 complex (i.e. the ICAM-1 adhesome) in a GAP-independent way.

In conclusion, we discovered the signaling pathway that translates substrate stiffness towards physiological processes during cardiovascular diseases and inflammation like leukocyte extravasation. Our results reveal an essential role for substrate stiffness-regulated endothelial DLC-1 in stabilizing the ICAM-1 adhesome in order to promote leukocytes to proceed from rolling to spreading in stiff microenvironments.
Results

DLC-1 expression is substrate stiffness-dependent and determines PMN spreading

In order to study the consequences of increased vascular stiffness in vivo, we studied DLC-1 expression in two cardiovascular-related diseases, both associated with increased vascular stiffness. First, the left iliac artery obtained from an organ donor presented with non-centrical plaque formation on the left side of the vessel was examined on DLC-1 expression by immunohistochemistry (Figure 1A). Due to atherosclerotic plaque formation, a stiff substrate is presented to ECs at the side of the plaque and shoulder region, where a softer substrate is present at the non-plaque region. Zooms of the plaque shoulder region and non-plaque region showed significant more DLC-1 expression in the ECs that cover the plaque shoulder region compared to the ECs that cover the non-plaque region (Figure 1B-D). Second, isolated lung microvascular ECs from patients suffering from pulmonary arterial hypertension (PAH) are known to be subjected to increased stiffness due to intimal fibrosis, increased media and adventitia thickness and accumulation of extracellular matrix proteins like collagen. Compared to ECs from healthy controls, ECs from PAH patients express higher levels of DLC1 (Figure 1E) that are significantly increased upon quantification of 3 patients (Figure 1F). Moreover, aged mice were used as an in vivo model for increased vascular stiffness and the effect on EC protein expression. Additional analysis of isolated lung ECs (mLECs) from 13 weeks young, and 20 months old mice showed a trend towards increased DLC-1 expression upon aging (Figure S1B).

Stiffness-dependent expression of DLC-1 was confirmed in vitro by culturing human umbilical vein endothelial cells (HUVECs) on hydrogels with varying stiffness. When lowering the substrate stiffness, the expression levels of DLC-1 decreased drastically (Figure 1G-H). But we did not observe a change in TNFα-induced ICAM-1 expression, indicating that the inflammatory response of HUVECs is not affected by substrate stiffness.

Both atherosclerosis and PAH are associated with increased leukocyte extravasation. To address if substrate stiffening is translated by the ECs to affect leukocyte TEM behavior in vitro, we compared transmigration of PMNs through a monolayer of HUVECs cultured on stiff or soft substrates. The results revealed major differences on PMN spreading and crawling prior to diapedesis. Specifically, PMNs on the apical surface of ECs that were cultured on the soft substrate showed limited spreading prior to diapedesis when compared to ECs that were cultured on stiff substrate (Figure 1I-J).

PMN spreading on ECs cultured on soft substrates was significantly reduced compared to those that spread on ECs cultured on stiff substrates.
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Figure 1

DLC1 protein expression

E

PAH-1 CTRL PAH-2
DLC1 actin

F Quantification of expression level

G

DLC1 ICAM1

H Quantification of expression level

K

Spreading

L

Circularity

154
Figure 1. DLC-1 expression is substrate stiffness-dependent and determines PMN spreading

Overview of left iliac artery obtained from an organ donor presented with non-centri- cal atherosclerotic plaque formation on the left side immunohistochemically stained for DLC-1 (A). Zoomed images of shoulder region (B) and non-plaque region (C) with open arrowheads indicating EC layer and filled arrowheads indicate internal elastic membrane which is not visible in the shoulder region due to increased intima thickness (C). Relative DLC-1 expression in ECs in the shoulder and non-plaque regions based on staining intensity, each data point represents one field of view (D). Representative western blot of total cell lysates of lung microvascular ECs from 2 PAH patients showing increased DLC-1 expression compared to 1 healthy control (E) and quantification of 3 patients and 3 healthy controls showing significant increase in DLC-1 expression in PAH patients (F). Representative western blot of total cell lysates of HUVECs cultured on stiff (plastic) or soft (2 kPa) substrates showing reduced DLC-1 expression of soft substrates in both control and TNFα treated conditions (G) and quantification showing significant reduced DLC-1 expression on soft substrates (H). Stills from PMN spreading (back dashed line) and diapedesis (red dashed line) on HUVECs cultured on stiff (glass) (I) or soft (1.5 kPa) (J) substrates show reduced spreading which is reflected by quantification of surface area (K) and circularity index (L) of PMN after spreading on the apical surface of ECs.

as was quantified by PMN surface area and circularity index, where round cells have a circularity index of 1 and more spread cells have an index <1 (Figure 1K-L). Thus, substrate stiffness alters DLC-1 expression, which is functionally translated to PMN spreading on ECs.

DLC-1 contains a GTPase activating protein (GAP) domain through which it can regulate small GTPases activity, mainly RhoA, and thereby affect actin cytoskeletal rearrangements. Therefore we took a closer look at the effects of substrate stiffness on the EC actin cytoskeleton. Immunofluorescent examination of HUVECs grown on stiff and soft substrates revealed a decrease in cortical actin, F-actin stress fibers and focal adhesions after TNFα stimulation when substrate stiffness is lowered (Figure S1A). To understand these substrate stiffness-induced changes in the actin cytoskeletal are cause by a general effect of protein expression levels on EC morphology, we decided to study potential involvement of other Rho-GEFs and Rho-GAPs. For this, GEF and GAP protein levels were screened in ECs upon culturing on varying stiffness substrates (Figure S1C-E). Our data reveal that DLC-1 expression strongly depends on high substrate stiffness in contrast to the expression of PDZ-GEF1, P190RhoGEF, P115RhoGEF, Ect2, ELMO1, and ASEF. Taken together, increased substrate stiffness, either due to aging or disease, is related to increased DLC-1 expression and promotes PMN spreading on ECs.
Figure 2. PMN spreading is impaired upon endothelial DLC-1 depletion
PMN transmigration characteristics through DLC-1 depleted ECs and control cells under physiological flow showing number of adhesive cells (A) the number of TEM cells (B) and percentage of cell that undergo TEM (C). Lateral migration of PMNs on top of ECS from adhesion until start of diapedesis represented as migration distance (D) and migration velocity (E). The duration of lateral migration on top of the ECs from adhesion until start of diapedesis (F) and duration of actual diapedesis, the time that PMNs need to breach through the EC layer (G). Quantification of adhesive PMNs based on spreading or round phenotype on control ECs and on DLC-1 silenced ECs (H). Lateral migration tracks of adhesive PMNs before diapedesis on top of control ECs (I) and on top of DLC-1 silenced ECs (J) were the direction of flow is from right to left. Data are obtained from 3 independent experiments.
Endothelial DLC-1 depletion prolongs the rolling stage of PMNs before diapedesis

To unravel the signaling pathway of substrate stiffness-dependent PMN spreading and DLC-1 expression, we investigated the link between DLC-1 expression levels and PMN spreading deficiency. Therefore, PMN TEM through DLC-1-silenced HUVEC was performed under physiological flow conditions. Analysis of these experiments showed no differences in the absolute number of both adhesive and transmigrated PMNs, as well as the percentage of the adherent PMNs that transmigrated (Figure 2A-C). Prior to diapedesis however, the lateral migration distance and velocity of PMNs on the apical surface of ECs is significantly increased upon silencing of DLC-1 (Figure 2D-E). The total time from PMN interaction with ECs until the start of diapedesis is not affected by DLC-1 depletion (Figure 2F). Also the actual diapedesis duration, from start of PMN breaching until complete transmigration underneath the EC monolayer is not affected (Figure 2G). This suggests a decreased crawling phase of PMNs on the apical surface of DLC-1 silenced ECs.

Indeed, PMNs showed an extended rolling phase prior to the diapedesis on DLC-1-depleted HUVECs compared to shCTRL-transduced HUVECs (Movie S1). Quantification of the PMN phenotype during the rolling phase showed reduced spread PMNs upon silencing of endothelial DLC-1 (Figure 2H). Migration plots were created by tracking PMNs on the apical EC surface from first PMN-EC contact until start of diapedesis. The rolling phenotype was demonstrated in the actual PMN migration path, where PMNs that migrated on DLC-1-silenced HUVECs showed a path in the direction of flow, typical for rolling, in contrast to the multidirectional crawling path of more spread PMNs on control HUVECs (Figure 2I-J). In summary, silencing endothelial DLC-1 resulted in increased rolling and reduced crawling duration, where the absolute numbers of adhered and transmigrated PMNs are not affected. Thus, endothelial DLC-1 regulates the transition from the rolling to the firm adhesion and crawling stage.

Adhesion molecule expression and ICAM-1 mobility are not hampered upon DLC-1 depletion

To understand how DLC-1 regulates the transition from rolling to adhesion, we first analyzed the composition of the actin and microtubule network in ECs. HUVECs depleted from DLC-1 still form a continuous monolayer, and the cytoskeletal actin and microtubule structures show no major differences in both basal and TNFα-treated conditions (Figure S2A-D). To explain the observed rolling phenotype of PMNs on DLC-1-depleted ECs, we next measured the adhesion receptor expression levels. Where selectins are described to be involved in tethering and rolling 29, ICAM-1 and VCAM-1 are essential for the firm adhesion and spreading 5. Total protein expression analyzed
Figure 3. Actin adaptor proteins recruitment upon ICAM-1 clustering depend on DLC-1 and substrate stiffness

Representative Western blot of ICAM-1 IP after clustering with anti-ICAM-1 dynabeads on control- or DLC-1-shRNA transduced HUVECs showing recruitment
of actin adaptor proteins Filamin B, α-actinin-4 and cortactin (A). Quantification of 3 independent experiments of actin adaptor recruitment upon ICAM-1 IP (B) and of protein expression levels in TCL (C). Recruitment of actin adaptor proteins Filamin A, Filamin B, α-actinin-4 and cortactin towards ICAM-1 upon clustering with anti-ICAM-1 dynabeads on EC cultured on high, medium or low substrate stiffness (D). Quantification of 3 independent experiments of actin adaptor recruitment upon ICAM-1 IP (E) and quantification of protein expression levels in TCL (F). Immunoprecipitation with anti-DLC-1 or control IgG (Ctrl) on TNFα treated HUVECs show interaction between DLC-1 and Filamin B and α-actinin-4 (G). Immunofluorescent images of HUVECs transduced with indicated expression vectors showing recruitment of ICAM-1-mCherry and control-GFP or ICAM-1-mCherry and DLC-1-FL-GFP towards anti-ICAM-1 coated beads after 60 minutes (H). Line plot along the white dashed line shows gray values for DLC-1 (green line) above background GFP levels co-localizing with ICAM-1-mCherry (red line) at the edges of the bead (gray dashed line) (I). Mean Gray Value within 0.8 µm from gray dashed line in Figure 3I (J). Data are obtained from 3 independent experiments.

by Western blot showed no difference in E-selectin, ICAM-1 and VCAM-1 when comparing DLC-1-depleted and control HUVECs (Figure S3A). Moreover, cell surface expression measured by flow cytometry, showed no significant differences upon DLC-1 silencing induced by two different shRNAs (Figure S3C-F). Apart from cell surface availability, also surface distribution of E-selectin and the formation of ICAM-1-rich filopodia is not hampered in DLC-1-deficient ECs shown by immunofluorescence (Figure S3B).

Mobility of ICAM-1 filopodia is essential to mediate clustering by leukocytes and to induce downstream ICAM-1 signaling 30,31. ICAM-1-GFP mobility in DLC-1-silenced HUVECs, assessed by Fluorescence Recovery After Photobleaching (FRAP), showed no differences in junctional or apical surface mobility, and ICAM-1 clustering by anti-ICAM-1 antibody-coated beads (Figure S3G-K).

Taken together, depletion of endothelial DLC-1 did not alter surface expression of adhesion molecules, or ICAM-1 filopodia formation and mobility.

Silencing of DLC-1 decreases actin adaptor protein recruitment upon ICAM-1 clustering in a substrate stiffness-dependent manner.

Next, involvement of DLC-1 in downstream signaling of ICAM-1 upon clustering was assessed. ICAM-1 clustering results in recruitment of actin adaptor proteins Filamin A, Filamin B, α-actinin-4 and cortactin 9,15,30,31. Immunoprecipitation (IP) of clustered ICAM-1 from either control or DLC-1-depleted HUVECs showed no significant difference in the amount of clustered ICAM-1. However, recruitment of Filamin B, α-actinin-4 and cortactin to ICAM-1 were all strongly reduced when DLC-1 is depleted from ECs, while total expres-
Figure 4. PMN spreading is independent of the GAP function of DLC-1
Physiological flow with control, knockdown and full-length (FL) or GAP-dead (GD) rescue (see Figure S4) showing number of adhesive cells (A) the number of TEM cells (B) and percentage of cells that undergo TEM (C). Lateral migration of PMNs on top of ECs from adhesion until start of diapedesis represented as migration distance (D) and migration velocity (E). Quantification of adhesive PMNs based on

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spreading or round phenotype on control, DLC-1 silenced, FL rescue or GD rescue ECs (F). Lateral migration tracks of adhesive PMNs before diapedesis on top of control ECs (G), DLC-1 knockdown ECs (H), DLC-1-FL rescue ECs (I) or DLC-1-GD rescue ECs (J). Data are obtained from 3 independent experiments.

DLC-1 and the ICAM-1 adhesome

sion levels were not affected (Figure 3A-C). Interestingly, DLC-1 itself is not able to bind directly to clustered ICAM-1 (Figure 2A bottom left panel). In line with data from Figure 3A, we show that upon lowering of substrate stiffness, DCL-1 expression is reduced and this correlated with decreased recruitment of all actin adaptor proteins to ICAM-1 (Figure 3D-F). This shows that next to α-actinin-4 also recruitment of Filamin A, Filamin B and cortactin is substrate stiffness-dependent, like DLC-1 expression. While total expression levels of Filamin A, Filamin B and α-actinin-4 are not dependent on substrate stiffness, cortactin expression is regulated by substrate stiffness (Figure 3F), leading to inconclusive cortactin recruitment upon ICAM-1 clustering.

As mentioned earlier, DLC-1 itself did not directly bind to clustered ICAM-1, but additional IP experiments showed that DLC-1 interacted with the actin adaptor proteins Filamin B and α-actinin-4 (Figure 3G). Confocal microscopy showed recruitment of DLC-1-GFP towards anti-ICAM-1 antibody-coated beads (Figure 3H-I). Quantification of GFP signal within 0.8 µm around the edge of the bead (grey dashed line) showed a significant increase of DLC-1-FL-GFP compared to control GFP transduced HUVECs (Figure 3J). This showed that DLC-1 is present at the site of clustered ICAM-1 and although no direct interaction was observed, we suggest that DLC-1 localizes to the intracellular tail of ICAM-1 via binding of the actin adaptor proteins.

In conclusion, DLC-1 is necessary for recruitment of actin adaptor proteins upon ICAM-1 clustering, and both DLC-1 expression and actin adaptor protein recruitment to ICAM-1 are substrate stiffness-dependent. In addition, DLC-1 is present at the intracellular protein complex upon ICAM-1 clustering via binding to Filamin B and α-actinin-4, but does not directly bind to ICAM-1.

Rolling phenotype of PMNs is independent of the GAP function of DLC-1

One of the key protein domains of DLC-1 is its GAP domain which inactivates Rho-GTPases. In order to address if PMN spreading is dependent on DLC-1 GAP activity, rescue experiments were performed in ECs transduced with either GFP-tagged full length (FL) DLC-1 or a GAP-dead mutant (GD) DLC-1, while silencing endogenous DLC1 by shDLC-1-tagRFP. Next, cells were sorted by flow cytometry, taking the RFP-/GFP- population as control cells, RFP+/GFP- as DLC-1 knockdown and RFP+/GFP+ cells as either FL-
Figure 5. Actin adaptor protein recruitment and PMN spreading upon stiffness substrates are restored upon DLC-1 overexpression

Representative Western blot of ICAM-1 IP after clustering with anti-ICAM-1 dynabeads on control, knockdown and full-length (FL) or GAP-dead (GD) rescue HUVECs (see Figure S4) showing recruitment of actin adaptor proteins Filamin B, 

Filamin A, α-actinin, cortactin, ICAM-1 < DLC1-GD-GFP < DLC1. 

PMN TEM upon DLC-1-GD-GFP rescue
PMN TEM under physiological flow assays with the aforementioned sorted HUVECs were performed to assess the PMN phenotype. Either FL- or GD-DLC-1 rescue had no effect on PMN adhesion and transmigration number and percentages (Figure 4A-C). Interestingly, the increased lateral migration distance and velocity of PMNs on the apical surface of ECs with DLC-1 knockdown is fully rescued by either FL- or GD-DLC-1 (Figure 4D-E). Importantly, the round PMN phenotype on DLC-1-depleted ECs is rescued to a spread PMN phenotype on both FL- and GD-DLC-1 expressing ECs, comparable to control ECs (Figure 4F) and is also demonstrated in the random multidirectional PMN migration paths on the apical surface of FL- and GD- rescue ECs (Figure 4G-J). Taken together, the rolling phenotype of PMNs on DLC-1-deficient ECs can be completely rescued by re-expressing either full length DLC-1 or a GAP-dead mutant, indicating that the transition from rolling to spreading is controlled by DLC-1, independent of its GAP function.

Recruitment of actin adaptor proteins is independent of the GAP function of DLC-1

To validate if the DLC-1 GAP-independent rescue of PMN phenotype coincides with actin adaptor recruitment upon ICAM-1 IP and protein expression levels in TCL for DLC-1-FL rescue (B) and DLC-1-GD rescue (C). Recruitment of actin adaptor proteins Filamin A, Filamin B, α-actinin-4 and cortactin towards ICAM-1 upon clustering with anti-ICAM-1 dynabeads on control or DLC-1-GD-GFP overexpressing ECs cultured on high, medium or low substrate stiffness (D). Quantification of 4 independent experiments of ICAM-1 IP (E) and TCL (F) shown as fold increase upon DLC-1-GD-GFP overexpression. Spreading of PMNs on top of ECs that are cultured on high, medium or low substrate stiffness (ctrl) or on top of ECs overexpressing DLC-1-GD-GFP cultured on high, medium or low substrate stiffness shown as PMN surface area (G) or circularity (H). TEM of PMNs through before mentioned conditions (G-H) is quantified as fold increase upon DLC-1-GD-GFP after normalization for high substrate stiffness (I). Fold increase >1 indicates more PMN transendothelial migration upon DLC-1-GD-GFP overexpression compared to wild-type ECs on the same substrate stiffness. Data are obtained from 3 independent experiments.
Substrate stiffness-induced ICAM-1 adhesome assembly depends on DLC-1 expression

Since PMNs displayed impaired spreading on ECs that are cultured on soft substrates, and the formation of the ICAM-1 adhesome is impaired under these conditions, we investigated if the defect in spreading of PMNs on ECs cultured on soft substrates can be rescued by DLC-1. DLC-1 overexpression was forced in HUVECs via transduction with DLC-1-GD-GFP lentivirus that expresses the construct under a CMV promotor, which showed no regulation by substrate stiffness, in contrast to endogenous DLC-1 (Figure 5D, right bottom two panels). ECs overexpressing DLC-1 were cultured on a high, medium or low stiffness substrate and subjected to ICAM-1 clustering. Expression of DLC-1-GD showed no further increase in recruitment of Filamin A, Filamin B or α-actinin-4 in ECs cultured on high stiffness substrates (Figure 5D middle lanes). However, overexpression of DLC-1-GD in ECs cultured on both medium or low stiffness substrates showed an increase in Filamin A, Filamin B and α-actinin-4 recruitment towards clustered ICAM-1 (Figure 5A). ICAM-1 clustering IP results were quantified as fold increase of adaptor protein recruitment upon DLC-1-GD overexpression over control cells on the same substrate stiffness. Filamin A, Filamin B and α-actinin-4 all showed an increase on both medium and low substrate stiffness, indicating restoration of actin adaptor protein recruitment (Figure 5E). Since cortactin expression is regulated by substrate stiffness, no conclusions on restoration of cortactin recruitment to ICAM-1 can be drawn.

To study the functionality of the restoration of actin adaptor protein recruitment on soft substrate stiffness by DLC-1-GD overexpression, we analyzed PMN spreading and transendothelial migration under the same conditions. Indeed, forcing DLC-1 expression in ECs cultured on soft substrates translates into increased surface area of PMNs (Figure 5G). In line, PMNs showed a decreased circularity index, indicating more spreading of the PMNs upon DLC-1 overexpression (Figure 5H). Consequently, the increased spreading resulted in increased TEM of PMNs through ECs cultured on soft substrates that express DLC-1 (Figure 5I). In conclusion, DLC-1, independent of its GAP function, regulates the stability of the ICAM-1 adhesome to allow PMNs to fully spread and promotes transmigration across ECs.
Discussion

This study shows for the first time the molecular mechanism how substrate stiffness regulates leukocyte spreading and diapedesis. Typically, prior to crossing of the vessel wall, leukocytes follow a cascade of events referred to as the multistep paradigm of leukocyte transendothelial migration. However, when leukocytes adhere to endothelial cells that are presented on soft substrates, leukocytes fail to efficiently cross the EC monolayer. Where previous studies only described these observation of decreased leukocyte transmigration rates, our work now shows that expression of endothelial DLC-1 forces leukocytes to spread and start crawling, resulting in leukocyte transendothelial migration. In line with several clinical indications that correlate vascular stiffness with increased leukocyte extravasation events, we found increased DLC-1 expression in ECs at human atherosclerotic lesion regions compared to ECs at non-plaque regions. Moreover, microvascular ECs isolated from lungs from PAH patients showed a significant increase in DLC-1 expression. In addition, the endothelium from aged mice showed increased DLC-1 expression levels compared to young mice. These data indicate the clinical importance of DLC-1 in cardiovascular diseases and the potential therapeutic options that our finding may have. Overall, our findings identify the endothelial Rho-GAP DLC-1 as a central mediator for inflammatory-based transendothelial migration of leukocytes under stiff and potentially diseased conditions.

Mechanistically, we show that DLC-1 stabilizes the ICAM-1 adhesome: a protein complex that is initiated when ICAM-1 is clustered upon leukocyte binding. This adhesome includes the presence of actin adapter proteins such as Filamin A and Filamin B, α-actinin and cortactin. Through recruitment of the different actin adapter proteins, ECs are able to more accurately and locally regulate the amount of actin polymerization and crosslinking at the ICAM-1 adhesome. In turn, this actin network provides a proper surface to the leukocytes to spread and firmly adhere, followed by diapedesis. We showed that formation of a stable ICAM-1 adhesome requires stiffness-dependent expression of DLC-1 in order to recruit actin adaptor proteins and consequently induce local actin cytoskeletal remodeling.

Besides regulating actin organization directly, some of these actin adapter proteins are shown to act as scaffolds that could also bind the small Rho-GTPases Rac1 or RhoG and thereby could harbor a secondary actin regulating mechanism via activation of these GTPases. Here, we identified DLC-1 as a novel RhoGAP interactor for actin adaptor proteins. The GAP domain of DLC-1 possesses actin cytoskeleton regulatory capacities via inactivation of RhoGTPases, mainly RhoA, and is...
therefore the most studied function of DLC-1. However, our mechanistic studies with a GAP dead DLC-1 mutant resulted in formation of a stable ICAM-1 adhesome. This excludes direct RhoA regulation by DLC-1 and shows that the effects on actin adaptor proteins recruitment and consequently leukocyte spreading are independent of DLC-1 GAP activity. Taken together, these data argue that endothelial DLC-1 functions in a GAP-independent manner during leukocyte TEM.

There are several Rho-GAP-independent functions of DLC-1 described and more specifically GAP-independent actin cytoskeleton regulatory functions for DLC-1 through phospholipase C delta 1 (PLCδ1) signaling. The GAP-independent enhancement of PLCδ1 activity by DLC-1 is suggested to mainly modulate cell movement, as the two proteins interact at focal adhesions via co-localization with vinculin. Therefore our finding of DLC-1 interacting with actin adaptor proteins, and regulating recruitment towards clustered ICAM-1 in a GAP-independent manner describes a novel mechanism for regulation of actin dynamics by DLC-1. Recent studies on the different DLC isoforms reveal no redundancy between family members. The main evidence for non-redundancy of DLC-1 family members is obtained from gene deletion studies in mice, where DLC-1-deficient embryos die around 10.5 days post coitum, while mice deficient for DLC-2 are still viable. This suggests a specific role for endothelial DLC-1 in regulating leukocyte spreading but future studies on DLC-2 or DLC-3 in leukocyte TEM need to provide direct evidence.

Next to the novel molecular mechanism, this work on DLC-1 is also of interest to the field of mechano-signal transduction. Previous research on KLF2 showed that overexpression of this transcription factor mimics ECs responses to flow, resulting in many studies using KLF2 overexpression to study EC alignment, which is normally only induced after exposure to physiological flow for several days. Now, manipulating DLC-1 expression in a variety of cell types can be used to mimic substrate stiffness in vitro without culturing on those difficult and very expensive stiffness gels. Thus, by controlling the expression levels of DLC-1, we now identified the mechanism how ECs allow leukocytes to fully spread prior to diapedesis in a stiffness-dependent environment. Since aging is a major risk factor for the development of cardiovascular diseases and is correlated with increased vascular stiffness, discovering the molecular signaling pathways involved in stiffness-regulated leukocyte extravasation can contribute to the development of pharmacological treatments for matrix stiffness-related diseases like atherosclerosis and PAH or age-related increase in matrix stiffness. Previous studies unsuccessfully tried to inhibit or reverse matrix stiffening. Therefore, we believe that interfering with EC responses to increased matrix stiffness may be a successful way of decreasing one of the major risk factors of cardiovascular disease, namely aging. Here we discovered DLC-1 as the
master switch for regulating stiffness-dependent leukocyte diapedesis, making DLC-1 a potential novel and specific target for exploring treatments of age-related cardiovascular diseases that involve excessive leukocyte transmigration.
Experimental Procedures

DNA and RNA constructs
See Supplemental Experimental Procedures.

Antibodies
See Supplemental Experimental Procedures.

Cell cultures and treatments
Pooled human umbilical vein endothelial cells (HUVECs) purchased from Lonza (P1012, Cat # C2419A), were cultured until passage 5 on fibronectin (FN)-coated dishes in EGM-2 medium, supplemented with singlequot (Lonza). Culturing on stiffness substrates was performed on high (plastic), medium (25-50 kPa) or low (1.5-2 kPa) 10 cm dishes (Matrigen) or glass bottom 35 mm dishes (Ibidi). Overnight pretreatment with 10 ng/ml recombinant TNF-α (Peprotech) before each experiment. Human Embryonic Kidney (HEK)-293T cells were maintained in DMEM (Invitrogen), containing 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin and streptomycin and 1x sodium pyruvate (all Invitrogen). All cells were cultured at 37°C and 5% CO2. Lentiviral constructs were packaged into lentivirus in HEK-293T cells by means of third generation lentiviral packaging plasmids (Dull et al., 1998; Hope et al 1990). Lentivirus containing supernatant was harvested on day 2 and 3 after transfection. Lentivirus was concentrated by Lenti-X concentrator (Clontech, Cat# 631232). Cells were transfected with the expression vectors according to manufacturer’s protocol with Trans- IT-LT1 (Myrus) and transduced target cells were used for assays after 72 hours.

Immunohistochemistry for DLC-1 in human atherosclerotic plaques
Immunohistochemistry was performed on 7µm thick formalin-fixed paraffin embedded iliac artery sections from an organ donor (female, 41 years old, smoker). Sections were first deparaffinized in xylene followed by rehydration. To perform antigen retrieval, the sections were boiled in citrate buffer pH6 for 10 min. Thereafter the sections were washed in TBS and incubated overnight at room temperature in a mix of two polyclonal rabbit antibodies against DLC-1 in 0.1% BSA in TBS. Control sections were treated with 0.1% BSA in TBS. The following day the slides were incubated for 1h with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin polymer (Immunologic) diluted 1:1 with TBS followed by development with DAB (Immunologic). The sections were counterstained very briefly with Gill’s hematoxylin and mounted with glycergel (DAKO).
PAH patient and control cells
Lobectomy tissue was used for control microvascular EC isolations. Peri-
pheral microvascular tissue for the isolation of PAH EC was obtained from
patients with clinically well-characterized PAH group I (familial, associated,
and idiopathic PAH cases). Cell isolation was based on the previously publis-
hed protocol 47. The study was approved by the institutional review board of
the VU University Medical Center (VUmc, Amsterdam, the Netherlands), and
consent was given.

Mouse experiments
C57Bl/6 mice were purchased from Janvier (Le Genest Saint-Isle, France)
and animal experiments were approved by the Regional Board of the State
of Hessen, Germany. Lung endothelial cells were isolated as described 48
and immediately lysed using QIAzol (Qiagen). RNA was isolated using the
miRNeasy RNA isolation kit (Qiagen).

PMN isolation
Polymorphonuclear cells (PMNs) were isolated from whole blood derived
from healthy donors. Whole blood was diluted (1:1) with 5% (v/v) TNC in
PBS. Diluted whole blood was pipetted carefully on 12.5 ml Percoll (room
temperature) 1.076 g/ml. Tubes were centrifuged (Rotanta 96R) at 2000
rpm, slow start, low brake for 20 minutes. After erythrocyte lysis in an ice-cold
isotonic lysis buffer (155 mM NH4CL, 10 mM KHCO3, 0.1 mM EDTA, pH7.4
in Milli-Q (Millipore), PMNs were centrifuged at 1500 rpm for five minutes at
4°C, incubated once with lysis buffer for 5 minutes on ice, centrifuged again
at 1500 rpm for five minutes at 4°C, washed once with PBS, centrifuged
again at 1500 rpm for five minutes at 4°C and resuspended in HEPES medi-
um pH7.4 (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM CaCL2, 1 mM
MgSO4, 1.2 mM K2HPO4, 5 mM glucose (all from Sigma-Aldrich), and 0.4
% (w/v) human serum albumin (Sanquin Reagents) and kept at room tempe-
rature for not longer than four hours until use. PMN counts were determined
by cell counter (Casey).

PMN TEM under physiological flow
150.000 HUVECs were cultured per channel in a FN-coated Ibidi µ-slide VI0.4
(Ibidi) the day before the experiment was executed and stimulated overnight
with 10 ng/ml TNFα (Peprotech). Freshly isolated PMNs were resuspended
at 1*106 cells/ml in HEPES medium pH7.4 and were activated for 30 minutes
at 37°C. Cultured HUVECs in Ibidi flow chambers were connected to a per-
fusion system and exposed to 0.5 ml/minute HEPES medium pH7.4 shear
flow for 5 minutes (0.8 dyne/cm2). PMNs were subsequently injected into the
perfusion system and leukocyte-endothelial interactions were recorded for
20 minutes at 0.2 frames/second by a Zeiss Observer Z1 microscope. All live
imaging was performed at 37°C in the presence of 5% CO2. Transmigrated PMNs were distinguished from those adhering to the apical surface of the endothelium by their transition from bright to phase-dark morphology, spread PMNs were distinguished from rolling by displaying small protrusions. Number of adhesive, percentage transmigrated and percentage spread vs. round phenotype PMNs were manually quantified using the ImageJ plug-in Cell Counter. Migration distance, migration speed, duration from adhesion until start of diapedesis and duration actual diapedesis were quantified using the ImageJ plug-in Manual Tracking.

Western blotting
For total cell lysates, cells were washed once with PBS+/+ (1mM CaCl and 0.5 mM MgCl), and lysed with 95°C SDS-sample buffer containing 4% β-me-capto-ethanol. Samples were boiled at 95°C for 5-10 minutes to denature proteins. Proteins were separated on 12.5% SDS running gel in running buffer (200 mM Glycine, 25 mM Tris, 0.1% SDS (pH8.6)), transferred to nitrocellulose membrane (Thermo Scientific Cat#26619) in blot buffer (48 nM Tris, 39 nM Glycine, 0.04% SDS, 20% MeOH) and subsequently blocked with 5% (w/v) milk (Campina) in Tris-buffered saline with Tween 20 (TBST) for 45 minutes. The immunoblots were analyzed using primary antibodies incubated overnight at 4°C and secondary antibodies linked to horseradish peroxidase (HRP) (Dako, Aligent Technologies) or IR800/IR680 (LI-COR biosciences), after each step immunoblots were washed 4x with TBST. HRP signals were visualized by enhanced chemiluminescence (ECL) (Thermo Scientific) and light sensitive films. IR800/IR680 signals were scanned with Odyssey.

Confocal laser scanning microscopy/FRAP
HUVECs were cultured on FN-coated (5µg/ml) 8 well labteck dishes and transduced as indicated and stimulated with TNFα (10ng/ml)(Peprotech) o/n for ICAM-GFP FRAP. FRAP experiments (Figure S3D-H) were performed on a Leica TCS SP8 confocal microscope using a 63x NA 1.4 oil immersion objective and the LAS-AF FRAP application. Regions of interest (four times 4x4 µm) were photobleached by 5 iterations using the argon laser at 488 nm at maximal power (77 mWatt).

Immunofluorescent staining was in general performed on HUVECS cultured on 12 mm glass coverslips coated with 5 µg/ml FN and treated with or without o/n TNFα (10ng/ml) (Peprotech), washed with PBS+/-+(supplemented with 1mM CaCl2 and 0.5mM MgCl2), fixed in 4% PFA (Merck) or 100% icecold MeOH (Merck), blocked for 30 min with 2% BSA (Affimetric) and mounted in Mowiol4-88/DABCO solution. ICAM-1 clustering was induced by adding 2 µl of mouse-anti-ICAM-1 (Cat#BBA4) (R&D) coupled to a 2.5% suspension of polystyrene microparticles (Polysciences) for 30-60 minutes. Z-stack image acquisition was performed on a confocal laser scan-
ning microscope (Leica SP8) using a 40x NA 1.3 or 63x NA 1.4 oil immersion objective.

**Flow cytometry**

For flow cytometry analysis HUVECs transduced as indicated were collected with Accutase cell detachment solution (GE healthcare), washed 1x in PBS containing 5% FCS (Bodinco) and 0.1 mM EDTA (Merck), incubated with anti-surface E-selecting-APC (Cat#551144) (1:50) from BD Biosciences, P-selectin-PE (Cat#M1706) (1:50) from Sanquin, ICAM-1-AF647 (Cat#MCA1615A647T) (1:400) from Serotec or VCAM-1-PE (Cat#CLB206P) (1:400) from Chemicon Europe for 30 min at 4 °C, washed 2x with PBS containing 5% FCS (Bodinco) and 0.1 mM EDTA (Merck) and subsequently analysed on a FACS LSR II SORP (Becton Dickinson).

FACS cell sorting of shDLC-1-tRFP and DLC-1-FL-GFP or DLC-1-GD-GFP transduced HUVECs were collected with Trypsin-EDTA (Sigma) and sorted based on RFP and GFP expression levels as indicated with gates on a FACS ARIA III or ARIA IIIu (Becton Dickinson).

**ICAM-1 IP after clustering**

Volume of 1.2 mg/ml Dynabeads® M-280 Streptavidin (Invitrogen) per condition were washed ones with 1ml buffer 1 containing PBS+ 2mM EDTA and 0.1% BSA (Millipore) using a magnetic holder. Dynabeads were coated with 3 ng a-ICAM-1 CD54 (BBIG-I1/IIC81) -biotin, R&D systems (Cat #BBA9) antibodies per condition and incubated head-over-head at 4 °C for 45 min. The beads were washed twice using buffer 1 and resuspended in PBS+/+ containing 0.5 MgCl2 and 1mM CaCl2. Overnight TNF-α treated HUVEC (2–5 million cells) were incubated with 1.2 mg/ml dynabeads per condition to cluster ICAM-1 for 30 min. Cells were washed once on ice using PBS+/+. Next, cells were lysed in 1ml icecold pH7.4 RIPA buffer containing 50mM Tris, 100mM NaCl, 10mM MgCl2, 1% NP40, 10% glycerol, 0.1% SDS, 1% DOC (Sigma-Aldrich), DNAse inhibitor and protease phosphatase inhibitor cocktail for 5min. Cells were scraped together and lysates were transferred to an eppendorf. Then, ICAM-1 coated dynabeads were added to nonclustered- control cell lysates. 50 µl whole-cell lysate was taken from all conditions. Beads and cell-lysates were subsequently incubated head-over-head for 1–2 h at 4 °C. Next beads were washed twice with Ripa buffer and three times with NP-40 lysis buffer. Beads were resuspended in 45 µl 2x SDS-sample buffer and assessed by western blotting.

**Statistics**

Data are represented as mean ± SEM. Comparisons between the indicated conditions were made in Prism Graph-Pad using unpaired T-test. P values for results are: n.s. P>0.05, * P≤ 0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001.
Chapter 6

Author contributions

LS and JDvB designed the research. LS, MvdS, AvS, AdL, MH, ST, RS, HJB, PH, RB, VdW performed the experiments. LS, VdW and JDvB analyzed the data. LS and JDvB wrote and edited the manuscript with input from SH and VdW.

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References

Chapter 6


Figure S1. Effect of substrate stiffness on HUVEC morphology and GEFs and GAPs protein expression

Confocal immunofluorescent images of HUVECs cultured on stiff (glass) or soft (2 kPa) substrates with and without overnight TNFα stimulation, showing F-actin by Phalloidin staining, VE-cadherin, Vinculin and a merge of F-actin, VE-cadherin and Hoechst (A). Relative DLC-1 expression in mouse lung endothelial cells from 13 weeks and 20 months old mice measured by qPCR (B). Westernblot of total cell lysates of HAECs (C) and HUVECs (D) cultured on stiff (plastic) or soft (2 kPa) substrates showing no differences in expression levels of a panel of GEFs and GAPs upon substrate stiffness or TNFα treatment and quantification showing no significant difference of PDZ-GEF1, P190RhoGEF, P115RhoGEF, Ect2, ELMO1 or ASEF (E).
Figure S2. DLC-1 silencing does not affect actin and microtubule organization
Confocal microscopy of immunofluorescent staining for F-actin with phalloidin, VE-cadherin and Hoechst on control and DLC-1 kd HUVECs without (A) and with (B) TNFα treatment. Microtubule formation visualized by staining for α-tubulin and Hoechst on control and DLC-1 depleted HUVECs without (C) and with (D) TNFα treatment.

Figure S3. Endothelial adhesion molecules surface expression and mobility
Expression of endothelial adhesion receptors is not comprised upon DLC-1 depletion. Total cell lysates show no differences at protein levels on Western blot of E-selectin, ICAM-1 and VCAM-1 (A). Immunofluorescent staining of surface expression of E-selectin and ICAM-1 on TNFα treated ECs shows heterogeneous expression in both control and DLC-1 silenced ECs (B) and no difference in ICAM-1 filopodia formation (zoom). Surface expression of E-selectin (C), P-selectin (D), ICAM-1 (E) and VCAM-1 (F) measured by FACS in untreated cells, TNFα treated -control and -DLC-1 depleted cells with 2 different shRNAs shows no significant difference due to knockdown of DLC-1. ICAM-1 surface mobility and recruitment towards anti-ICAM-1 coated beads. Stills from live IF imaging of ICAM-1-GFP overexpression FRAP in control HUVEC (G) and DLC-1 depleted cells (H) after TNFα treatment showing both junctional and apical surface regions being bleached at time 0 sec and the recovery during first 60 seconds. Quantified recovery of ICAM-1-GFP fluorescent intensity in bleached areas within both junctional (I) or apical surface (J) ICAM-1 shows no difference between control and DLC-1 silenced ECs. Recovery
time of clustered ICAM-1 around anti-ICAM-1 coated beads does not differ in DLC-1 depleted cells compared to control cells (K). Data are obtained from 3 independent experiments.
Figure S4. FACS sorting of DLC-1 rescue cells
HUVECS transduced simultaneously with shDLC-1-tagRFP and DLC-1-FL-GFP or DLC-1-GD-GFP were selected on puromycin and FACS sorted. Gating based on EC size, FSC-A and SSC-A (A), and single cells (B) and subsequently on GFP and RFP expression levels. RFP-/GFP- cells served as control, RFP+/GFP- were considered DLC-1 kd cells and RFP+/GFP+ are either DLC-1-FL rescue or DLC-1-GD rescue cells (C).
Supplemental experimental procedures

DNA and RNA constructs
shRNA in pLKO.1 targeting DLC-1 (TRCN47864 and TRCN47866), control shRNA (shC002) were purchased from sigma Aldrich mission library. ShCTRL-tRFP was created by inserting shC002 into a modified version of pLKO.1 backbone between the Agel and EcoRI restriction sites. shDLC-1-3’UTR-tRFP was created by allowing to oligo’s containing the shRNA sequence (5’-GGAGTGTAGGAATTGACTATA-3’) to selfligate and subsequently insert into a modified version of the pLKO.1 backbone between Clal and EcoRI restriction sites. Full-length human DLC-1 fused at its N terminus to a GFP tag was amplified by PCR from a pEGFP-C1-DLC-1 vector (provided by Xiaolan Qian) and cloned into a self-inactivating lentiviral pLV-CMV-Ires-Puro vector between the SnaBI and Xbal restriction sites. An arginine to glutamic acid mutation at amino acid position 603 in the DLC-1 GAP domain was generated by site-directed mutagenesis to generate pLV-DLC-1-[R603E]-GFP, expressing DLC-1-gap-dead-GFP.

Antibodies
Monoclonal mouse antibodies against actin (Cat#A3853) (1:1000 for WB) and against α-Tubulin (Cat#T6199) (1:500 for IF) were purchased from Sigma. Monoclonal mouse antibodies against VE-cadherin AF647 (Cat#561567) (1:200 for IF), against cortactin (Cat#610050) (1:1000 for WB) and against DLC-1 (Cat#612021) (1:1000 for WB) were purchased from BD Biosciences. Monoclonal mouse antibody against ICAM-1 (Cat#BBA4) (1:250 for IF and 300μg/ml for coupling to polystyrene beads), monoclonal mouse antibody against ICAM-1 Biotinylated (Cat#BBA9) (3ng/IP) and polyclonal goat antibody against E-selectin (Cat#BBA18) (1:100 for IF) were purchased from R&D systems. Alexa Fluor 555 Phalloidin (Cat#PHDH1) (1:500 for IF) was purchased from Cytoskeleton. Hoechst 33342 (Cat#H-1399) (1:25000 for IF) was purchased from Molecular probes. Chicken anti-mouse AF674 (Cat#A21463) (1:200 for IF) and chicken anti-goat AF647 (Cat#A21469) (1:200 for IF) were purchased from Invitrogen. Polyclonal rabbit antibodies against Filamin B (Cat#A301-726A) (1:1000 for WB) and against PDZ-GEF1 (Cat# A301-966A) (1:1000 for WB) and against p190RhoGEF (Cat# A303-930A) (1:1000 for WB) was purchased from Bethyl Laboratories. Monoclonal mouse antibody against Filamin A (Cat#MCA464S) (1:1000 for WB) was purchased from Serotec. Polyclonal rabbit antibody against α-actinin-4 (Cat#ALX-210-356) (1:1000 for WB) was purchased from Enzo Life Science. Monoclonal rabbit antibody against p115RhoGEF (Cat# 3669S) (1:1000 for WB) was purchased from Cell Signaling. Polyclonal rabbit antibody against Ect2 (Cat# 07-1364) (1:1000 for WB) was purchased from Millipore. Poly-
clonal rabbit antibodies against ELMO1 (Cat# SC-20965) (1:1000 for WB) and against ASEF (Cat# Sc-33218) (1:1000 for WB) and against DLC-1 (Cat#Sc-32931) (1:200 for IHC) was purchased from Santa Cruz. Polyclonal rabbit antibody against DLC-1 (Cat#C119221) (1:200 for IHC) was purchased from LS Bio.

Secondary HRP-conjugated goat anti-mouse (Cat#P0447) (1:5000 for WB), swine anti-rabbit (Cat#P0399) (1:5000 for WB) antibodies were purchased from Dako. Secondary IR800- or IR680-conjugated donkey anti-mouse (Cat#926-32212) donkey anti-rabbit (Cat#926-32223) or donkey anti-goat (Cat#926-32214) (all 1:5000) were from LiCOR Westburg. All antibodies were used according to manufacturer’s protocol.
Graphical abstract

Leukocyte
Docking
Spreading
Transmigration

Endothelial cell

Stiff substrate
Soft substrate

ICAM1 clustering

ICAM1
DLC1 Actin adaptors
Actin

DLC-1 and the ICAM-1 adhesome