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F-actin-rich contractile endothelial pores prevent vascular leakage during leukocyte diapedesis through local RhoA signaling

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**ABSTRACT**

During immune surveillance and inflammation, leukocytes exit the vasculature through transient openings in the endothelium without causing plasma leakage. However, the exact mechanisms behind this intriguing phenomenon are still unknown. Here, we report that maintenance of endothelial barrier integrity during leukocyte diapedesis requires local endothelial RhoA cycling. Endothelial RhoA depletion in vitro or Rho inhibition in vivo provokes neutrophil-induced vascular leakage that manifests during the physical movement of neutrophils through the endothelial layer. Local RhoA activation initiates the formation of contractile F-actin structures that surround emigrating neutrophils. These structures that surround neutrophil-induced endothelial pores prevent plasma leakage through actomyosin-based pore confinement. Mechanistically, we found that the initiation of RhoA activity involves ICAM-1 and the Rho GEFs Ect2 and LARG. Additionally, regulation of actomyosin-based endothelial pore confinement involves ROCK2b, but not ROCK1. Thus, endothelial cells assemble RhoA-controlled contractile F-actin structures around endothelial pores that prevent vascular leakage during leukocyte extravasation.
INTRODUCTION

The clinical signs of inflammation, redness, heat, swelling and pain are caused by the acute inflammatory response including increased vasodilatation, enhanced microvascular permeability and leukocyte recruitment. During inflammation the endothelial barrier becomes more permissive for large molecules, leading to local plasma proteins leakage and edema formation. Whether leukocyte transendothelial migration (TEM) directly causes increased microvascular permeability has been controversial for decades. Certain studies suggested leukocyte adhesion and transmigration to be the critical events leading to tissue damage and organ failure during inflammation and ischemia-reperfusion since neutrophil depletion or CD11/CD18 blocking antibodies have been shown to attenuate vascular injury under these circumstances. However, when microvascular permeability was measured simultaneously with leukocyte-endothelial interactions, local plasma leakage sites were often different from those of leukocyte adhesion or transmigration. Recently, it has been shown that intravenous injection of TNF-α caused significant leukocyte adhesion and transmigration but did not affect basal microvessel permeability. Moreover, several studies have shown that the timing of leukocyte adhesion and transmigration are not well linked with the evoked permeability change during acute inflammation. Most of the abovementioned studies are descriptive, molecular evidence for the uncoupling between leukocyte TEM and vascular permeability has been recently shown by Wessel and colleagues. They mechanistically uncoupled leukocyte extravasation and vascular permeability by showing that opening of endothelial junctions in those distinct processes are controlled by different tyrosine residues of VE-cadherin in vivo. However, how the endothelium maintains a tight barrier during leukocyte transendothelial migration is still unknown.

Here, we investigate the mechanism by which endothelial cells (ECs) prevent vascular leakage during leukocyte TEM. We examine the correlation between neutrophil extravasation and the evoked permeability changes during acute inflammation in vitro and in vivo. Spatiotemporal RhoA activation during leukocyte crossing is measured using a recently developed RhoA biosensor. In addition, we use fluorescently-tagged Lifeact and Lifeact-EGFP transgenic knock-in mice to investigate endothelial filamentous (F)-actin dynamics in remodeling junctions during neutrophil diapedesis in vitro and in vivo, respectively. We show that endothelial pore restriction limits vascular leakage during leukocyte extravasation which is driven by a basolateral actomyosin-based structure that requires local endothelial RhoA activation.
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Figure 1: Impaired endothelial RhoA function results in increased vascular leakage during leukocyte diapedesis in vivo. (a) Extravasation kinetics of calcein-red labelled neutrophils and FITC-dextran through TNF-α treated ECs cultured on 3µm pore permeable filters. Neutrophils transmigrated towards a C5a chemotactic gradient in the lower compartment. Four conditions were tested; RhoA depletion (EC) + neutrophils (Orange line), control + neutrophils (purple line), RhoA depletion (EC) only (red line) and control only (green line). (b) Quantification of FITC-dextran and neutrophil extravasation after 20 minutes of neutrophil transmigration. (c) Correlation analysis of dextran and neutrophil extravasation kinetics through control and RhoA depleted ECs. (d) Confocal intravital microscopy of 20-80 µm diameter cremasteric venulesin LysM-GFP mice (green neutrophils) immunostained in vivo for EC junctions by intrascrotal injections of fluorescent-labeled PECAM-1 (blue) and stimulated for four hours with IL-1β and TNF-α only, or with Rho-inhibitor (C3). A second dose of Rho inhibitor was given intrascrotally and TRITC-dextran (40 kDa) was injected intravenously at T = 2 hours. **
RESULTS

RhoA controls vascular leakage during leukocyte diapedesis

To investigate the molecular mechanism that controls endothelial barrier function during neutrophil TEM, we simultaneously measured neutrophil transmigration kinetics and FITC-dextran leakage across TNF-α-stimulated human umbilical vein endothelial cells (ECs) towards a C5a gradient, for 60 minutes. Neutrophil transmigration across control ECs was associated with minimal FITC-dextran leakage (Fig. 1a). Increasing neutrophil numbers in the upper compartment up to 10-fold did not induce FITC-dextran leakage, indicating that ECs maintained their barrier function, despite increased numbers of transmigrating neutrophils (Supplementary Fig. 1a). To investigate the functional role of RhoA in EC barrier maintenance during neutrophil TEM, we depleted RhoA using siRNA (Supplementary Fig. 1b). We found that endothelial RhoA depletion increased FITC-dextran leakage during neutrophil extravasation whereas minimal FITC-dextran leakage was measured during neutrophil crossing through control ECs (Fig. 1a,b). Correlation analysis showed that the increase in FITC-dextran leakage was highly correlated to neutrophil transmigration (Fig. 1c). Note that endothelial RhoA depletion did not alter FITC-dextran leakage under basal conditions which was comparable to control EC (Fig. 1a,b). Moreover, endothelial resistance measured under physiological flow conditions was significantly reduced during transmigration of neutrophils across Rho-inhibited endothelium (Supplementary Fig. 1c). We next investigated the role of RhoA in EC barrier maintenance during neutrophil TEM in vivo. Vessel permeability was monitored by TRITC-dextran leakage into the cremaster of C57BL/6 WT or LysM-GFP mice during IL-1β and TNF-α-stimulated neutrophil recruitment. Intrascrotal administration of anti-PECAM-1 labelling antibody resulted in a strong labelling of EC junctions in cremasteric venules (Fig. 1d). Administration of IL-1β and TNF-α and allowed to circulate until T = 4 hours. Scale bar 100µm. (e) Neutrophil extravasation in animals left unstimulated (control), stimulated with C3 alone, IL-1β/TNF-α treated, IL-1β/TNF-α treated + C3 or IL-1β/TNF-α treated + neutrophil depletion. (f) Correlation analysis of dextran and neutrophil extravasation kinetics in animals stimulated with IL-1β/TNF-α alone or with IL-1β/TNF-α treated + C3. *** P < 0.001 control versus RhoA depleted HUVEC (ANOVA) or P = 0.3504 control versus RhoA depleted HUVEC (Student’s t-test) (b). r = 0.2547 P = 0.359 (Pearson correlation) transmigrated neutrophils versus FITC-dextran leakage in control HUVECs or r = 0.6345 ** P < 0.01 (Pearson correlation) transmigrated neutrophils versus FITC-dextran leakage in RhoA depleted HUVECs (c). P = 0.4230 IL-1β/TNFα versus IL-1β/TNF-α + C3 (Student’s t-test) (e). r = 0.8258 ** P < 0.01 (Pearson correlation) transmigrated neutrophils versus FITC-dextran leakage in IL-1β/TNF-α + Rho inhibitor treated mice (f). Data are from three experiments (a-c) or are representative of five to thirteen (d-e) or nine (f) independent experiments (d-f; one mouse per experiment; error bars (a-c,e and f), s.e.m).
enhanced leakage of intravenous TRITC-dextran into the interstitium and neutrophil recruitment into the cremaster (Fig. 1d and Supplementary Fig. 1d). Rho inhibitor I (C3)-treated animals showed similar extravasated neutrophil levels, however TRITC-dextran leakage in those animals was highly increased compared to IL-1β and TNF-α administration alone (Fig 1d-e). Although no change in neutrophil extravasation was measured in the presence or absence of C3, we cannot exclude that the inhibitor affects other cells. Neutrophil extravasation and TRITC-dextran leakage in WT mice were not correlated in individual mice, although there was an overall association between extravasation and permeability, whereas the two processes in Rho-inhibited animals showed a highly significant correlation (Fig. 1f). Animals treated with C3 alone showed unaltered basal vascular permeability (Supplementary Fig. 1e). Thus, neutrophil extravasation and evoked changes in vascular permeability during inflammation are not correlated. However, when endothelial RhoA is inhibited, neutrophil diapedesis provokes vascular leakage, suggesting that endothelial RhoA is required to maintain a tight EC barrier during leukocyte diapedesis in vivo.

**Spatiotemporal RhoA activation during leukocyte diapedesis**

To examine spatiotemporal RhoA activation in ECs during EC barrier maintenance associated with neutrophil TEM, we used a recently developed FRET-based RhoA biosensors called the Dimerization Optimized Reporter for Activation (DORA) RhoA sensors (Fig. 2a) 19. DORA RhoA biosensors design were based on the published RhoA biosensor 20. The ON-state Fluorescence Resonance Energy Transfer (FRET) efficiency of the GTPase was improved through modelling of the fluorescent protein dimers and the GTPase-effector domain complexes. Stable α-helical repeats from ribosomal protein L9, rather than an unstructured linker, were inserted between the fluorescent proteins to disrupt dimerization and diminish FRET efficiency in the inactive state (Fig. 2a). As a control, DORA RhoA mutant Protein kinase N (PKN) was developed to report misalignment of Cerulean3 (Cer3) and Venus image before and after image registration, motion artefacts or pH changes affecting the sensors fluorescent proteins. Glutamine substitution for a leucine at position 59 in the PKN domain prevents PKN binding to activated RhoA 21. The characterizations of both DORA RhoA biosensors are described in online methods (Supplementary Fig. f,2 and 3a). From these validation experiments, we conclude that the DORA RhoA biosensor accurately reports RhoA dynamics in ECs downstream from endogenous stimuli such as thrombin (Fig. 2b and Supplementary Fig. 1f).

To study spatiotemporal RhoA activation in ECs during neutrophil TEM, we expressed the DORA RhoA biosensor in ECs and investigated
RhoA activation following neutrophil extravasation under physiological flow conditions. Important to note, Venus and Cer3 emission were simultaneously recorded utilizing a double camera system since sequential image acquisition resulted in motion artefacts induced by migrating leukocytes displacing fluorescent signals in ECs. We found unaltered RhoA biosensor activation during neutrophil rolling and crawling over the endothelium (Fig. 2c,g). Also RhoA activation during the initial opening of EC junctions was found to be unaltered (Fig. 2d). However, RhoA biosensor activity in the endothelium was locally increased at sites where neutrophils breeched the endothelium, between the first and second minute of neutrophil diapedesis (Fig. 2d-f, Supplementary Fig. 2d and Supplementary Movie 1). Based on the normalized ratiometric imaging and the relative displacement of the sensor, the data showed a 1.2-fold increase in FRET ratio upon diapedesis, comparable to what has been observed during RhoA activation after thrombin stimulation (Fig. 2h,i). The negative control DORA RhoA biosensor (mutant PKN) showed no change in FRET during leukocyte diapedesis (Supplementary Fig. 3b,c and Supplementary Movie 2). Importantly, expressing the DORA RhoA biosensors in ECs did not interfere with neutrophil TEM. Thus, endothelial RhoA is transiently and locally activated during the final stage of neutrophil diapedesis, but not during crawling or opening of endothelial junctions, indicating a role for local RhoA activity in EC barrier maintenance during the final stage of neutrophil extravasation.

**F-actin-rich endothelial pores during diapedesis**

To investigate endothelial F-actin dynamics during neutrophil diapedesis, we transfected ECs with GFP- and/or mCherry-tagged Lifeact. It is important to note that phalloidin staining to visualize F-actin cannot be used to investigate endothelial actin structures that are in close proximity of transmigrating leukocytes, since F-actin in both leukocytes and ECs are visualized by phalloidin staining, making it impossible to discriminate between the two (Supplementary Fig. 3d). Transmigrating neutrophils initiated small endothelial pores in the endothelial lining. To study those endothelial pores at high resolution, transmigrating neutrophils were fixed with formaldehyde when partly breeched the endothelium. Confocal microscopy imaging and 3-D reconstruction showed that ECs assembled F-actin-rich structures around endothelial pores through which neutrophils transmigrated, both during transcellular and paracellular migration (Fig. 3a and Supplementary Movie 3-5). During paracellular migration, the junctional protein VE-cadherin was distributed to the endothelial pore margins (Fig. 3a). Interestingly, using ECs expressing either Lifeact-GFP or Lifeact-mCherry, we found that paracellular pores were formed by at
least two ECs. At the structures apical site, filopodia-like protrusions were found whereas at the basolateral site, a cortical actin-ring appeared during leukocyte crossing (Fig. 3b-d). In contrast to VE-cadherin distributed to the pores margins, the junctional protein PECAM-1 was localized around the basolateral F-actin-ring and distributed to apical protrusions surrounding migrating leukocytes during trans- and paracellular migration.
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(Supplementary Fig. 3e,f). Moreover, we found that the adhesion molecule ICAM-1 was localized in the apical protrusions at endothelial pores (Supplementary Fig. 4a). We found that approximately 90% of all neutrophils and monocytes used the paracellular route, whereas approximately 10% migrated transcellular, in line with the migratory preference for neutrophils and monocytes found in vivo (Fig. 3e). Note that all these diapedesis events by either neutrophils or monocytes were associated with basolateral F-actin-ring formation around endothelial pores (Fig. 3e). Within the paracellular route of migration, leukocyte transmigration through a bi-cellular or a multi-cellular junction was approximately 50% (Supplementary Fig. 4b). In conclusion, ECs assemble F-actin-rich ring-like structures around endothelial pores through which neutrophils and monocytes transmigrate. This data indicates that maintenance of EC barrier function during leukocyte diapedesis involves actin cytoskeleton strengthening around endothelial pores. Basolateral F-actin ring formation may tighten the endothelial barrier during neutrophil crossing, making the leukocyte-induced endothelial pore impermeable for macromolecules.

**Figure 2** Spatiotemporal RhoA activation during neutrophil TEM. Endothelial RhoA is locally and transiently activated during neutrophil extravasation (a) Schematic illustration of the DORA RhoA sensor design containing Rho effector PKN (red), circular permutated Venus (yellow), structured linker protein L9 (green), circular permutated Cer3 (blue) and RhoA GTPase (green), left panel. Right panel shows the DORA RhoA mutant PKN biosensor that was developed as a negative control biosensor, the glutamine was substituted for the leucine at position 59 in the PKN domain. This mutation prevents binding of PKN to activated RhoA. (b) Time-lapse Venus/Cer3 ratio images of DORA RhoA biosensor simultaneously recorded with an epifluorescent microscope showing spatiotemporal RhoA activation upon thrombin treatment (1U per ml) in HUVECs. Filled arrows indicate RhoA activation. Scale bar, 10µm. Calibration bar shows RhoA activation (Red) relative to basal RhoA activity (Blue). (c) Epi-fluorescent live-cell imaging of HUVEC expressing the DORA RhoA biosensor during neutrophil adhesion under physiological flow conditions (0.8dyne per cm²). Red open arrows indicate adherent neutrophils. Scale bar 10µm. Calibration bar shows RhoA activation (red) relative to basal RhoA activity (blue). (d) Epi-fluorescent live-cell imaging of HUVECs expressing the DORA RhoA biosensor during neutrophil TEM. Time-lapse images of DIC (upper) Venus/Cer3 ratio images of DORA RhoA biosensor (middle) and Merge (bottom) during leukocyte diapedesis. Open arrows indicates adherent neutrophil at the apical side of the endothelium. Filled arrows indicate local RhoA activation during neutrophil diapedesis. Scale bar, 10µm. (e) Detailed zoom of RhoA activation during neutrophil diapedesis. (open arrows) prior diapedesis at time point t = 00:12 minutes. (f) Detailed zoom of local RhoA activation during neutrophil transmigration at time point t = 01:12 minutes. Filled arrows indicate local RhoA activation during neutrophil diapedesis. Scale bar, 10µm. (g) Quantification of temporal RhoA activation during multiple neutrophil adhesion events starting at time zero (arrow). (h) Quantification of temporal RhoA activation during multiple neutrophil transmigration events starting at time zero (arrow). (i) Quantification of DORA RhoA biosensor activation after thrombin treatment (1U per ml) in HUVEC. Asterisk indicates thrombin addition. Data represents mean and s.e.m of seven experiments (g) five experiments (h). ten experiments (i).
**Figure 3** ECs assemble F-actin rich ring-like structures around transmigrating leukocytes. (a) Confocal imaging of para- and transcellular migrating leukocytes through Lifeact-GFP expressing HUVECs. Filled arrows indicate EC F-actin (Lifeact in green) assembly around extravasating leukocytes. Open arrows indicate VE-cadherin (directly labeled Alexa-647 antibody in red) distribution to the F-actin structure sites during paracellular diapedesis. Asterisks indicate extravasating leukocyte (DAPI in blue) in DIC. Flow speed 0.8 dyne per cm². Scale bar 5µm. (b) Confocal imaging showing a Z-stack of Lifeact-GFP and Lifeact-mCherry positive endothelial membrane structures from apical to basal plane. Open arrows indicate filopodia-like protrusions at the apical site of the structure.
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**F-Actin-rich Endothelial Pores Are Confined in Size**

Electron microscopy studies showed that ECs maintain intimate contact with transmigrating neutrophils during the entire transmigration process \(^{15,24}\). To examine the dynamic contact between ECs and extravasating neutrophils, we examined F-actin-enriched endothelial pore shape and size in relation to neutrophil size. Real-time recordings of transmigrating neutrophils through ECs expressing GFP-tagged Lifeact showed increased F-actin assembly around endothelial pores (Supplementary Movie 6).

The kinetics of neutrophil diapedesis is on average two minutes and can be distinguished into early, mid and late diapedesis based on endothelial pore size and neutrophil morphology (Fig. 4a-c). Endothelial pore formation started when neutrophils partly breeched the endothelium, defined as early diapedesis. Following neutrophil diapedesis, most endothelial pores are maximal enlarged one minute after transmigration was initiated, defined as mid diapedesis (Fig. 4a-c). Subsequently, the endothelial pore is closed in conjunction with transmigrating neutrophils until completely under the endothelium, a stage defined as late diapedesis (Fig. 4a-c). Real-time imaging of neutrophil diapedesis under physiological flow conditions showed that neutrophil total surface area prior to TEM was roughly 100 µm\(^2\), which was reduced to less than 20 µm\(^2\) to fit the confined gap in the endothelium that had a maximal inner-surface area of 19 µm\(^2\) (Fig. 4b,c). To investigate the morphology of de novo formed F-actin-positive rings and F-actin-positive apical protrusions that surround endothelial pores during neutrophil TEM, we trapped neutrophils at different stages of diapedesis. Interestingly, de novo formed F-actin-positive rings surrounding endothelial pores were found throughout all diapedesis steps, but not during neutrophil adhesion or crawling steps (Fig. 4d and Supplementary Fig. 4c). Quantification of endothelial pore size showed significant larger pores during mid diapedesis than during early and late diapedesis when pores open and close respectively (Fig. 4d). We next measured, the pore-size width, length and height of F-actin-positive rings during different stages of diapedesis.
rich endothelial pores surrounding transmigrating neutrophils and monocytes. On average, endothelial pores are 4 µm wide, 6 µm in length and mostly oval-shaped for all leukocytes migrating through the cell-cell junctions (Supplementary Fig. 4d and 4f). Additionally, we found that only during diapedesis approximately 40% of the endothelial pores contained F-actin-rich apical protrusions (Fig. 4d). No such structures were detected during the crawling step. These structures reached a maximal height of 6-7 µm (Supplementary Fig. 4e). Transcellular pores were found to be more round or circular shaped and had an average circularity of about 1.3 according to the circularity index (Supplementary Fig. 4f). Endothelial pore sizes showed remarkably little variation, despite leukocyte size, type or transmigration route (Fig. 4e). Thus, endothelial pores induced by extravasating neutrophils and monocytes are confined in size and close directly behind transmigrated cells. Active endothelial pore confinement and pore closure corroborated earlier findings that showed intimate contact between neutrophils and ECs during the entire TEM process and provides an explanation for limited transendothelial escape of macromolecules during neutrophil crossing.

**Pore confinement and pore closure requires endothelial RhoA**

Our data showed that increased endothelial RhoA activity during neutrophil TEM corresponded to endothelial pore restriction and closure during mid and late diapedesis. To investigate whether RhoA regulates endothelial pore confinement, we silenced endothelial RhoA using siRNA. RhoA was successfully depleted as shown by Western blot analysis (Supplementary Fig. 5a). Confocal microscopy showed that RhoA depletion in ECs reduced Lifeact-GFP accumulation around endothelial pores, whereas Lifeact-GFP in the apical protrusions was still present (Fig. 5a). Basal F-actin rings in RhoA-depleted ECs were significantly reduced compared to control conditions (Fig. 5b). Endothelial RhoA depletion had no effect on the formation of F-actin-rich apical protrusions (Fig. 5b).

Quantification of endothelial pore size showed that in the absence of RhoA, endothelial pores were not only larger than endothelial pores formed in control ECs, but also did not close properly (Fig. 5c). Note that neutrophil adhesion and transmigration under physiological flow conditions were unaltered in RhoA-depleted ECs (Fig. 5d). To study if VE-cadherin signalling regulates endothelial pore size, we depleted VE-cadherin and analysed endothelial pore size. However, VE-cadherin depletion had no effect on endothelial pore size (Fig. 5e and Supplementary Fig. 5b-d). In conclusion, RhoA facilitates endothelial pore confinement and pore closure during leukocyte diapedesis.
Figure 4 Endothelial pores formed during para- and transcellular leukocyte transmigration are confined in size. (a) Epi-fluorescent live-cell imaging of ECs expressing Lifeact-GFP. Red open arrows indicate F-actin-rich endothelial pore formation during leukocyte diapedesis under physiological flow conditions (0.8 dyne per cm²). Filled arrows indicate extravasating leukocyte in DIC. Dashed lines indicate neutrophil localization under the endothelium. Scale bar, 10µm. (b, c) Quantification of size changes occurring in the neutrophil cell body and endothelial pore during neutrophil diapedesis. Endothelial pore size (red), neutrophil cell body apical (blue) and neutrophil cell body under EC (yellow), diapedesis starts at time zero. (d) Quantification of Neutrophil size, F-actin positive ring structures, F-actin positive apical protrusions and endothelial pore size. (e) Quantification of endothelial pore size for neutrophils and monocytes during paracellular migration. **** P < 0.0001 (ANOVA). Data are representative of four independent experiments (d, e) with 40 transmigration events per group. Data in (b, c) are representative of 10 transmigration events (error bars (b-e), s.e.m).
Figure 5 RhoA signalling is required for endothelial pore confinement. (a) Confocal imaging of paracellular migrating neutrophils through Lifeact-GFP expressing HUVECs after 72 hours transfection with control siRNA (upper panel) or RhoA siRNA (lower panel) under physiological flow conditions (0.8 dyne per cm²). Open arrows and filled arrows indicate filopodia-like protrusions at the apical site and the cortical F-actin-ring at the basolateral site of the endothelial pore, respectively. Asterisk indicates extravasating neutrophil (DAPI in blue). VE-cadherin (Red). Scale bar, 5µm. (b) Quantification of F-actin-positive ring structures and F-actin-positive apical protrusions in control versus RhoA depleted ECs. (c) Quantification of endothelial pore size during early, mid and late diapedesis. (d) Quantification of neutrophil adhesion and diapedesis through TNF-α treated ECs under physiological flow conditions after 72 hours transfection with control siRNA (open bar) or RhoA siRNA (filled bar). (e) Quantification of endothelial pore size in control versus VE-cadherin depleted HUVECs. **** P < 0.0001 (ANOVA). Data are representative of four independent experiments (a-e) with > 12 transmigration events per group (error bars (c, e), s.e.m).
Pore confinement is driven by actomyosin contractility

To investigate how RhoA regulates endothelial pore confinement during leukocyte diapedesis, we examined RhoA effector myosin II activation. To study myosin II activation we locally measured myosin light chain (MLC) phosphorylation on position Thr18 and Ser19. Immunofluorescent staining of pMLC showed an asymmetric phosphorylation pattern in F-actin-rich endothelial pores surrounding transmigrating neutrophils (Fig. 6a). MLC was particularly phosphorylated at cortical actin bundles as part of the F-actin ring (Fig. 6a and Supplementary Fig. 5e). Note that the uropod of the neutrophil is positive for MLC phosphorylation, most likely to retract its tail during transmigration. In contrast to local MLC phosphorylation in control ECs, endothelial pores in RhoA-deficient ECs were enlarged and negative for local phospho-MLC (Supplementary Fig. 5e). In addition, we quantified Lifeact-GFP distribution around endothelial pores and found asymmetric F-actin distribution around endothelial pores, indicative of increased tension (Fig. 6b). To corroborate our findings in vivo, we studied F-actin localization during leukocyte diapedesis in retinal vasculature of Lifeact-EGFP-transgenic knock-in mice. Lifeact-EGFP expression in the retinas of these mice is largely restricted to the endothelium and this allowed us to properly visualize F-actin in ECs in situ. We found that endothelial pores induced by transmigrating neutrophils (isolectin B4-positive) were surrounded by Lifeact-EGFP-positive rings in retinal ECs (Fig. 6c). Quantification of these rings showed that endothelial pore size in vivo was comparable to endothelial pores measured in the in vitro setup (compare Fig. 6d and 4e). Lifeact was present in the basolateral ring and in apical protrusions that surrounded transmigrating neutrophils (Supplementary Fig. 5f). These data showed that apical membrane protrusions in vivo are rich for F-actin and surround adherent leukocytes. Next, we examined local MLC phosphorylation in WT mice during IL-1β and TNF-α-induced neutrophil recruitment in cremasteric venules. PECAM-1 was used as a marker to visualize endothelial pores in vivo (Fig. 6e). In line with our in vitro findings, endothelial pores in mouse cremaster venules showed asymmetric MLC phosphorylation (Fig. 6f,g). In order to address the role of ROCK in endothelial pore confinement we depleted the ROCK isoforms ROCK1 and ROCK2b in endothelial cells and examined vascular permeability during neutrophil diapedesis. In line with RhoA inhibition, silencing ROCK1 and ROCK2b did not prevent the adhesion or transmigration of neutrophils through the endothelial monolayer (Supplementary Fig. 6a,b). Basal endothelial barrier function in ROCK1 or ROCK2b deficient ECs was not affected. However, neutrophil diapedesis through ROCK2b, but not ROCK1 deficient ECs elicited increased endothelial permeability up to a twofold (Supplementary Fig. 6a,b). These
Figure 6 Endothelial pore confinement is driven by actomyosin contractility. (a) Immunofluorescence analyses of MLC phosphorylation during neutrophil transmigration. Open and filled arrows indicate Lifeact-mCherry (red) and MLC phosphorylation (green) localization respectively during neutrophil transmigration under physiological flow conditions (0.8 dyne per cm$^2$). Asterisk indicates extravasating leukocyte in DIC. Scale bar, 10 µm. (b) Quantification of F-actin distribution in endothelial pores surrounding transmigrating neutrophils and monocytes. Maximum intensity projection of F-actin in the endothelial pore was used to quantify F-actin distribution surrounding transmigrating leukocytes. Distribution asymmetry is defined by the ratio of region of interest ROI-1 and ROI-2 corrected for background. Scale bar, 5µm. (c) Confocal imaging of F-actin dynamics during leukocyte diapedesis in retina vasculature of Lifeact-EGFP C57BL6 mice. Filled arrows indicate the vasculature of mice retina, highly expressing Lifeact-GFP. Zoom of ROI, open arrows indicate the Lifeact-EGFP (green)-positive endothelial pore, filled arrows
findings may indicate that endothelial pore confinement is mediated through ROCK2b but not ROCK1. Altogether, local accumulation of F-actin and MLC phosphorylation is associated with neutrophil diapedesis in vitro and in vivo, suggesting that endothelial pore confinement is driven by local actomyosin contractility.

**Endothelial Pore Confinement Requires ICAM-1, LARG and Ect2**

To investigate the signalling events upstream from RhoA, we focused on the involvement of guanine-nucleotide exchange factors (GEF) and performed a GEF screen that included: p115RhoGEF, Ect2 and LARG. Depletion of endothelial LARG together with Ect2 increased endothelial pore size, whereas depletion of LARG, p115 and Ect2 alone was not sufficient (Fig. 7a and Supplementary Fig. 6c). Quantification of endothelial pore size at different stages of diapedesis showed that endothelial pores in LARG- and Ect2-depleted endothelium were enlarged during early and mid-diapedesis but had no effect on endothelial pore closure (Fig. 7b). Under these conditions the number of F-actin-positive rings and F-actin-positive apical protrusions was unaltered (Fig. 7c). Enlarged endothelial pores in Ect2- and LARG-deficient ECs showed increased endothelial permeability during neutrophil diapedesis, whereas basal EC barrier function was not affected (Fig. 7d and Supplementary Fig. 6d-f). Neutrophil diapedesis through Ect2- and LARG-deficient ECs was slightly reduced (Fig. 7d and Supplementary Fig. 6d). To study LARG and Ect2 recruitment to the intracellular tail of PECAM-1 or ICAM-1 we performed clustering experiments induced by anti-ICAM-1 or anti-PECAM-1 coated beads. We found that LARG and Ect2 are recruited to the intracellular tail of ICAM-1 (Supplementary Fig. 6g). Whereas PECAM-1 recruited only LARG but not Ect2 to its intracellular tail upon clustering (Supplementary Fig. 6h). To investigate if ICAM-1 or PECAM-1 initiate and coordinate local RhoA activation and endothelial pore confinement during neutrophil diapedesis, we depleted ICAM-1 and PECAM-1 in ECs and indicate transmigrating neutrophil. Scale bar, 5µm. (d) Quantification of endothelial pore size in retina vasculature. (e) Confocal imaging of PECAM-1 in cremasteric venules during TNF-α and IL-1β induced neutrophil recruitment. Open arrows indicate PECAM-1 positive endothelial pores that surround extravasating neutrophils (filled arrows). Scale bar, 20µm. (f) IF analyses of MLC phosphorylation during neutrophil transmigration into the cremaster of C57BL6 mice. Filled and open arrows indicate phospo-MLC and PECAM-1 localization to endothelial pores, respectively. Scale bar, 5µm. (g) Quantification of pMLC and PECAM-1 localization in endothelial pores. We quantified MLC phosphorylation defined as distribution asymmetry. The distribution asymmetry uses the intensity of one ROI vs another ROI as indicated in Figure 6b. Because MLC may occur at different heights within the pore we used max projection for this analysis. Data are representative of three independent experiments (a-g) with > 12 transmigration events per group (error bars (b, d, g), s.e.m).
Figure 7 ICAM-1 regulates endothelial pore confinement through recruitment of the Rho GEFs LARG and Ect2. (a) Quantification of endothelial pore size in LARG, p115RhoGEF or Ect2 depleted ECs. (b) Quantification of endothelial pore size during early, mid and late diapedesis in control versus LARG + Ect2 depleted ECs. (c) Quantification of F-actin-positive ring structures and F-actin-positive apical protrusions in control versus LARG + Ect2 depleted ECs. (d) Quantification of FITC-dextran and neutrophil extravasation after 20 minutes of neutrophil transmigration through control and Ect2/LARG (d) or ICAM-1 deficient ECs (e). (f) Model of endothelial pore formation. Adherent leukocytes destabilizing cell-cell junctions subsequently insert small pseudopodia between transient openings in the endothelium (leukocyte propping) during early diapedesis. The next step (mid diapedesis) involves local
examined the extravasation of calcein-red-labeled-neutrophils and FITC-dextran across EC and measured endothelial pore size. We found that neutrophil transmigration through ICAM-1 deficient ECs compromised the endothelial barrier (Fig. 7e and Supplementary Fig. 7a-c), whereas PECAM-1 depletion did not alter endothelial pore size or vascular leakage (Supplementary Fig. 7d-e). ICAM-1 and PECAM-1 depletion alone had no effect on endothelial permeability (Fig. 7e and Supplementary Fig. 7d). In agreement with the existing literature endothelial ICAM-1 depletion significantly reduced the number of transmigrated neutrophils (Fig.7e). Neutrophil diapedesis through PECAM-1 deficient endothelial cells showed no reduction in transmigration numbers (Supplementary Fig. 7d). These data point out an important role for ICAM-1, Ect2 and LARG signalling in controlling RhoA-mediated endothelial pore confinement and EC barrier protection during neutrophil diapedesis.

**DISCUSSION**

Leukocytes that cross the endothelium induce large endothelial gaps without provoking leakage of plasma into the underlying tissue. However, the mechanisms behind this intriguing phenomenon are unclear. The present study shows how ECs limit vascular leakage during leukocyte TEM. We found that RhoA-mediated F-actin rings contribute to endothelial pore confinement that maintains endothelial barrier integrity during leukocyte diapedesis. Neutrophil diapedesis through ICAM-1, Ect2/LARG and RhoA-deficient ECs provokes vascular leakage that was highly correlated with neutrophil breeching events. Mechanistically, we found that the initiation of RhoA activity involves ICAM-1 and the Rho GEFs Ect2 and LARG. Additionally, we found that the regulation of actomyosin-based endothelial pore confinement involves ROCK2b, but not ROCK1. Our work identifies a novel mechanism that maintains endothelial barrier integrity during leukocyte extravasation which is driven by a basolateral actomyosin-based structure that requires spatiotemporal RhoA cycling (Fig. 7f).

and transient RhoA activation that mediates endothelial pore confinement through the formation of a *de novo* basolateral F-actin ring and actomyosin contractility. Finally, persistent actomyosin contractility closes the endothelial pore behind transmigrating leukocytes. ICAM-1 is involved in the regulation of endothelial pore confinement through recruitment of the Rho GEFs LARG and Ect2. Basolateral F-actin ring formation and actomyosin contractility tightens the endothelial barrier during leukocyte diapedesis, making the leukocyte-induced endothelial pore impermeable for macromolecules.**** $P < 0.0001$ (ANOVA) (a-c),** $P < 0.01$ (ANOVA) (b) * $P < 0.05$ (ANOVA) (e,d).* $P < 0.05$ control versus siLARG/shEct2 (d) ** $P < 0.01$ control versus siICAM-1 (e) (Student’s t-test). Data are representative of three independent experiments (a-e) with > 12 transmigration events per group (a-c) (error bars (a-e), s.e.m).
Inflammation-driven leukocyte recruitment and vascular permeability are separable events. In line with these observations, we discovered that during the TEM process endothelial RhoA plays a central role in EC barrier maintenance but is redundant for leukocyte transmigration. In agreement with previous reports, blocking RhoA activity or depleting RhoA in ECs did not perturb adhesion or transmigration. In contrast to the general concept that RhoA activation is required for leukocyte adhesion and opening of endothelial junctions, we found that endothelial RhoA was locally and transiently activated during the diapedesis step and not during neutrophil crawling, firm adhesion or opening of endothelial junctions prior to leukocyte extravasation. These processes require a separate, RhoA-independent mechanism that allows leukocyte-EC adhesion or opening of endothelial junctions. In agreement with our findings, for both transmigration routes, endothelial pore opening is in part mediated by mechanical forces that are generated by migrating leukocytes. Polarized actin polymerization in the leukocyte elicits pulling and pushing forces that support the movement of immune cells through the confined endothelial pore. ICAM-1 is known to mediate leukocyte-EC interactions, and crosslinking ICAM-1 using ICAM-1-coated beads or ICAM-1 antibodies results in increased RhoA activation suggesting a role for ICAM-1-mediated RhoA activation in leukocyte adhesion. However, based on the spatiotemporal activation of RhoA, we suggest that ICAM-1-mediated RhoA signaling specifically occurs during the diapedesis step, in agreement with our data that shows ICAM-1 enrichment only at diapedesis sites. PECAM-1 was also detected at sites of diapedesis, for either paracellular or transcellular migration. Recently, mechanical tension exerted on ICAM-1 and also PECAM-1 enhanced RhoA activation and MLC phosphorylation in ECs that was dependent on the recruitment of the RhoGEF LARG and ICAM-1 clustering. Our work shows that ICAM-1 clustering indeed promotes the recruitment of LARG and we additionally found Ect2 to be recruited upon ICAM-1 clustering. Depletion of ICAM-1- and Ect2/LARG in ECs compromised the endothelial barrier during neutrophil diapedesis. Indicating that the ICAM-1-LARG/Ect2 signaling axis is likely to be activated upstream from RhoA activation, in order to regulate de novo F-actin rearrangements, endothelial confinement and barrier protection during neutrophil crossing. Together these data suggest that leukocytes exert mechanical forces on endothelial adhesion molecules that modulate endothelial F-actin cytoskeleton through mechanotransduction that may cause endothelial confinement.

The relationship between Ect2 and actomyosin contractility has been clearly established by several studies. For instance, Ect2 has been described to be involved in RhoA activation and contractile ring formation.
F-actin-rich contractile endothelial pores prevent vascular leakage during cytokinesis. In addition, it has been shown that the molecular pathways that regulate local RhoA activation during cytokinesis are also used to control RhoA dynamics at the zonula adherens in interphase cells. Although no proof for a role of Ect2 in endothelial junction regulation has been described we can speculate that Ect2 mediates similar functions in ECs, regulating actomyosin contractility around the pore. The latter is probable, since depletion of LARG and Ect2 simultaneously results in larger pores without affecting the number of F-actin rings. In agreement with this hypothesis overall endothelial pore size in RhoA-deficient endothelial cells was increased due to the lack of basal F-actin ring formation. In addition we observed that RhoA-deficient endothelial cells were unable to phosphorylate MLC near endothelial pores. Surprisingly, the length-to-width aspect ratio between paracellular and transcellular pores was found to be different, however this was not due a difference in nuclear size, shape or composition between neutrophils and monocytes. We speculate that in case of paracellular migration the amount of VE-cadherin disassembly at the pores margins may regulate pore size, which may affect the length/width ratio or circularity. In case of transcellular migration mechanical forces from the endothelium might counteract leukocyte induced forces from all directions forcing a circular passageway. A physical explanation for circular transcellular passages may also explained by cellular dewetting. Despite different length-to-width ratio of the pores, the overall pore size is constant independent of leukocyte type, or transmigration route. This may indicate that the contractile forces generated during endothelial pore formation are high enough to counteract the mechanical forces generated by transmigrating leukocytes. Alternatively, the RhoA-induced basolateral F-actin ring itself may also add as a limiting factor for pore confinement on top of the actomyosin based contractility. Endothelial pore confinement is probably not restricted to neutrophil and monocyte diapedesis but may also occur during the diapedesis of other immune cells such as T-lymphocytes. Additional research is required to investigate this hypothesis. Surprisingly, we found that VE-cadherin depletion did not affect endothelial pore integrity, despite the prominent VE-cadherin localization at the pores margins. It is known that other junctional molecules such as N-cadherin may take over the function of VE-cadherin when VE-cadherin is depleted. The fact that we see unaltered pore morphology in VE-cadherin deficient cells makes it conceivable that other molecules like N-cadherin take over the function of VE-cadherin controlling the integrity of the endothelial pore at its margins. It is evident that VE-cadherin plays a dominant role in endothelial barrier formation and regulation of leukocyte traffic through the endothelial barrier. For instance, locking VE-cadherin junctions reduces the number emigrating
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leukocytes and the phosphorylation of VE-cadherin at Y731 induced by adherent leukocytes prior diapedesis is a necessity for junctional destabilization and paracellular diapedesis. We cannot exclude a supportive role for VE-cadherin in endothelial pore integrity, but we can exclude a direct role for VE-cadherin as a signaling molecule being involved in controlling and coordinating of endothelial pore confinement. Whether other junctional proteins such as JAM-A or CD99, that act distally from ICAM-1, and signal to RhoA to prevent leakage is currently unknown. We found that many F-actin rings comprise apical membrane protrusions. These projections, also known as "docking structures" or "transmigratory cups", have been suggested to anchor endothelial adhesion receptors and therefore control leukocyte adhesion. However, the biological function of these structures is still under debate. Interestingly, we found F-actin rings associated with leukocyte diapedesis that contained no apical protrusions suggesting that directional neutrophil diapedesis can occur through other mechanisms than "apical projection"-guidance for instance transendothelial migration-promoting endothelial chemokines that are locally released within the endothelial pore. In agreement with studies showing that apical projection assembly requires RhoG and Rac1 but not RhoA activity, we still observed apical membrane protrusions around migrating leukocytes upon RhoA depletion, whereas the F-actin rings were significantly decreased. Suggesting that the basolateral F-actin ring and not the apical protrusions in the endothelial pore contribute to vascular leakage prevention during TEM. Interestingly, in drosophila, similar actomyosin networks have been found to rapidly close multicellular-wounds by actomyosin contraction. Studies that investigated the mechanisms by which ECs repair gaps in the endothelial monolayer, show that mechanical induced micro-wounds in the endothelium are healed by ventral lamellipodia, a mechanism that may also be involved in the closure of leukocyte induced endothelial pores. Our data shows that RhoA-mediated contractile force generation responsible for endothelial pore restriction precedes ventral lamellipodia formation. Moreover, RhoA-mediated pore constriction in ECs seems to be specific for the closure of leukocyte induced endothelial pores, whereas ventral lamellipodia are also observed in maintenance of basal junctional integrity. Based on electron microscopy studies, it has been suggested that the intimate contact between neutrophils and ECs during the entire transendothelial migration process limits leakage of plasma proteins. Moreover, several studies showed that ECs reseal the endothelial barrier prior to or in conjunction with neutrophils penetrating the basal lamina. In agreement with these ultra-structural studies we found that endothelial pores closed prior to or in conjunction with neutrophils that fully breached the endothelial
lining. In the context of EC barrier maintenance it is well conceivable that endothelial pore confinement and closure directly prevents vascular leakage during leukocyte diapedesis whereas ventral lamellipodia restore junctional homeostasis after leukocyte crossing. Endothelial LSP1 has been implicated in a role for “dome” formation and controlling permeability during TEM \(^5^8\) and has been found to be activated downstream from ICAM-1 clustering \(^5^9\). Together, this may open up the possibility that ICAM-1 clustering activates RhoA through LSP1. However, future experiments should show if this signaling axis indeed is operational during TEM.

In conclusion, we have discovered that local RhoA-mediated F-actin rings in the endothelial lining prevent vascular leakage during leukocyte diapedesis. Elucidating the molecular and cellular mechanisms of barrier maintenance during leukocyte diapedesis may have implications for the development of new therapies to restore normal homeostatic junctional remodeling to counteract vascular dysfunction during chronic inflammation.
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METHODS

DNA AND RNA CONSTRUCTS

The Dimerization Optimized Reporter for Activation (DORA) RhoA and DORA RhoA mutant PKN biosensors were a very kind gift of Yi Wu (University of Connecticut Health centre, Farmington, USA). Briefly, circular permutated Protein kinase N (PKN) effector of RhoA coupled to dimeric circular permutated Venus is linked via a ribosomal protein-based linker (L9H) with dimeric Cerulean3 (Cer3) coupled to RhoA. The DORA RhoA sequence within a pTriEx-HisMyc backbone is cpPKN(S69-H97-GSG-S14-R68)-Kpnl-GS-dcpVen-L9Hx3-BamHI-dCer3(G229)-NheI-RhoA-WT-HindIII. The DORA RhoA mutant PKN sequence within a pTriEx-HisMyc backbone is cpPKN (S69-H97-GSG-S14-R68, L59Q)-Kpnl-GS-dcpVen-L9Hx3-BamHI-dCer3(G229)-NheI-RhoA-WT-HindIII. The Leucine (L) on position 59 in the PKN domain of the RhoA control biosensor is substituted for a glutamine (Q). The H1R, p63-RFP and mRFP-RhoGDI-α (pcDNA 3.1) were a kind gift of Joachim Goedhart (Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands). pLenti-Lifeact-mCherry, pLenti-Lifeact-GFP, were a kind gift of Stephan Huveneers (Sanquin, Amsterdam, the Netherlands). shRNA in pLKO.1 targeting VE-cadherin (12) B6 (TRCN 54090), GEF-H1 (TRCN 3174), GEF-H1 (TRCN 3175), p115RhoGEF (TRCN 33567), and Ect2 (TRCN 47686) were purchased from sigma Aldrich mission library. siRNA targeting RhoA (sc-29471) (working concentration 50 nM), ICAM-1 sc-29354 (50 nM), PECAM-1 sc-29445 (50 nM), LARG sc-41800 (50 nM), Rock-1 (sc-29473) (50 nM), Rock-2b (sc-29474) (50 nM), and scrambled non-silencing siRNA were purchased from (Santa Cruz Biotechnology, Santa Cruz, CA).

ANTIBODIES

Rabbit antibody against GEF-H1 (55B6) (Cat #4076) (1:1000 for WB), phosho Myosin light chain Thr18/Ser19 (Cat #3674) (1:100 for IF), p115RhoGEF (D25D2) (Cat #3669) (1:1000 for WB), RhoA (67B9) (Cat #2117X) (1:1000 for WB) and CD31 (PECAM-1) (89C2) (Cat #3528) (1:1000 for WB) were purchased from Cell signaling (BIOKE). Polyclonal rabbit antibody against Ect2 (Cat# 07-1364) (1:1000 for WB) was purchased from Millipore. Polyclonal goat antibody against LARG (Cat#AF4737) (1:1000 for WB) was purchased from R&D systems. Alexa Fluor 405 Phalloidin (1:100 for IF) was purchased from Promokine (Cat# PK-PF405-7-01). Polyclonal goat antibody against VE-cadherin (C-19) (Cat# SC-6458) (1:1000 for WB), Rock-2 (C-20) sc-1851 (1:1000 for WB), Rock-1 (H-85) sc-5560 (1:1000 for WB) were purchased from Santa Cruz (Bio-Connect). Polyclonal rabbit antibody against ICAM-1 (Cat #SC-7891) (1:1000 for WB) was purchased.
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from Santa Cruz Biotechnology. Monoclonal mouse antibody against Filamin A (Cat #MCA464S) (1:1000 for WB) was purchased from Serotec. Monoclonal mouse Alexa Fluor 647 VE-cadherin (55-7H1) (Cat# 560411) (1:100 for IF) and Alexa Fluor 488 PECAM-1 (Cat# 555445) (1:100 for IF) were purchased from Becton Dickinson. Monoclonal mouse antibody against Actin (AC-40) (Cat# A3853) (1:1000 for WB) was purchased from Sigma. The Alexa Fluor 405 goat anti-rabbit IgG (Cat# A31556) (1:100 for IF), Alexa Fluor 647 chicken anti-goat IgG (Cat# A21469) (1:100 for IF), Alexa Fluor 488 chicken anti-rabbit IgG (Cat# A21441) (1:100 for IF) and Texas red 568 Phalloidin (Cat #T7471) (1:100 for IF) were purchased from Invitrogen. Secondary HRP-conjugated goat anti-mouse, swine anti-rabbit antibodies (1:3000 for WB) were purchased from Dako (Heverlee, Belgium). Hoechst 33342 (H-1399) (1:50 for IF) was purchased from Molecular probes (Life Technologies). All antibodies were used according to manufacturer’s protocol.

**CELL CULTURES AND TREATMENTS**

Pooled Human umbilical vein ECs (HUVECs) purchased from Lonza (P938, Cat # C2519A), were cultured on fibronectin-coated dishes in EGM-2 medium, supplemented with singlequots (Lonza, Verviers, Belgium) HUVECs were cultured until passage 9. HEK-293T were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Breda, The Netherlands) containing 10% (v/v) heat-inactivated fetal calf serum (Invitrogen, Breda, The Netherlands), 300 mg/ml L-glutamine, 100 U/ml penicillin and streptomycin and 1x sodium pyruvate (Invitrogen, Breda, The Netherlands). HeLa cells (American Tissue Culture Collection: Manassas, VA, USA) were cultured using Dulbecco’s Modified Eagle Medium (DMEM) supplied with Glutamax, 10% FBS, Penicillin (100 U/ml) and Streptomycin (100µg/ml). Cells were cultured at 37°C and 5% CO₂. HUVECs were treated with 1U/ml thrombin (Sigma-Aldrich, St. Louis, USA) for periods as indicated, pre-treated with 10 ng/ml recombinant Tumor-Necrosis-Factor (TNF)-α (PeproTech, Rocky Hill, NJ) 24 hours before each leukocyte TEM experiment, For Rho inhibition cells were pre-incubated with cell-permeable Rho inhibitor I (C3) (Cytoskeleton, Cat# CT04) for 3 hours. Cells were transfected with the expression vectors according to the manufacturer’s protocol with Trans IT-LT1 (Myrus, Madison, WI, USA). Lentiviral constructs were packaged into lentivirus in Human embryonic kidney (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). Transduced target cells were used for assays after 72 hours. Cells were transfected with siRNA according to manufacturer’s protocol using INTERFERin (Polyplus).
HeLa cells were transfected with Lipofectamine and imaged the next day. HeLa cells were treated with 100 µM histamine (Sigma-Aldrich, St. Louis, USA) and 10 µM Pyrilamine (mepyramine) (Sigma-Aldrich, St. Louis, USA) for periods as indicated.

**NEUTROPHIL AND MONOCYTE ISOLATION**

Polymorphonuclear (PMN) neutrophils and monocytes were isolated from whole blood derived from healthy donors and were conducted under the rules and legislation in place within the Netherlands. The rules and legislations are based on The Declaration of Helsinki (informed consent for participation of human subjects in medical and scientific research) and guidelines for Good Clinical Practice. 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. Ring fraction containing lymphocytes and monocytes was collected and further processed as indicated below*. 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), neutrophils 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 medium (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), pH7.4) and kept at room temperature for not longer than four hours until use. *Ring fraction was washed three times with MACS buffer (0.5% (v/v) BSA in PBS, 2mM EDTA in PBS). Ring fraction was centrifuged at 1700 rpm for 7 minutes at 4°C and low break, and resuspended in 100 µl MACS buffer and incubated with 5 µl CD14 microbeads (Miltenyi biotec, # 130-050-201) for 30 minutes at 4°C and subsequently washed with 5 ml MACS buffer, centrifuged and resuspended in 1 ml MACS buffer. LS columns (Miltenyi biotec, # 130-042-401) were placed in QuadroMACS separator (Miltenyi biotec, # 130-090-976) and cells were subsequently washed three times with 1 ml MACS buffer. Column was removed from the QuadroMACS separator and monocytes were collected in a collection tube. Neutrophil and monocyte counts were determined by cell counter (Casey).

**FITC-DEXTRAN PERMEABILITY ASSAY**

200,000 ECs were cultured in fibronectin (FN) treated 24 well cell culture inserts (Corning FluoroBlok, Falcon, 3.0 µm pore size # 351151)
and treated with TNF-α overnight. 30 µg FITC-dextran (70 kDa) (Sigma) in HEPES medium (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), pH7.4) was added to the upper and 0.1 nM C5a (Sigma C-5788) in HEPES medium was added to the lower compartment. FITC-dextran and calcein red-orange (Molecular probes C34851) labeled neutrophil (200,000 cells) extravasation was monitored simultaneously for a period of 60 minutes with an interval of 1 minute by an Infinite F200 pro plate reader (TECAN) at 37 °C. EX BP 485/9 and EM BP 535/20 was used to measure FITC-dextran kinetics. EX BP 535/9 and EM BP 595/20 was used to measure neutrophil (calcein red-orange) transmigration kinetics.

**Neutrophil and monocyte TEM under physiological flow**
HUVECs were cultured to 70% confluence in FN-coated 6 well plate, and transfected with different expression vectors (e.g. DORA RhoA biosensor) according to the manufacturer’s protocol with Trans IT-LT1 (Myrus, Madison, WI, USA) for 24 hours or transduced with expression vectors in lentivirus (e.g. pLenti-Lifeact-mCherry) for 72 hours. HUVECs were cultured in a FN-coated ibidi µ-slide VI 0.4 (ibidi, Munich, Germany) the day before the experiment was executed and stimulated overnight with TNF-α (10 ng/ml). Freshly isolated neutrophils and monocytes were resuspended at 1x10⁶ cells/ml in HEPES medium and were incubated for 30 minutes at 37°C. Cultured HUVECs in ibidi flow chambers were connected to a perfusion system and exposed to 0.5 ml/minute HEPES shear flow for 10 minutes (0.8 dyne/cm²). Actual levels of injected neutrophils ranged between 0.5-2*10^6 dependent on the donor and activity of the neutrophils. Actual levels of monocytes ranged between 1.5-2.2*10^6. Neutrophils or monocytes were subsequently injected into the perfusion system and real-time leukocyte-endothelial interactions were recorded for 20 minutes by a Zeiss Observer Z1 microscope using a 40x NA 1.3 oil immersion objective or samples were fixed for immunofluorescent staining and subsequent analysis. All live imaging was performed at 37°C in the presence of 5% CO₂. Transmigrated neutrophils were distinguished from those adhering to the apical surface of the endothelium by their transition from bright to phase-dark morphology. Percentage adherent or transmigrated neutrophils were manually quantified using the ImageJ plug-in Cell Counter (type 1, adherent cells, type 2, transmigrated cells).

**Confocal laser scanning microscopy**
Cells were cultured in FN-coated ibidi µ-slide VI 0.4 (ibidi, Germany) and transfected or stimulated as indicated. After treatment, cells were washed
with cold PBS, containing 1 mM CaCl₂ and 0.5 mM MgCl₂, and fixed in 4% (v/v) formaldehyde for 10 minutes. After fixation, cells were permeabilized in PBS supplemented with 0.5% (v/v) Triton X-100 for 10 minutes followed by a blocking step in PBS supplemented with 2.5% (w/v) BSA. Cells were incubated with primary and secondary antibodies and after each step washed with PBS. Z-stack image acquisition was performed on a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss Micro-Imaging) using a voxel size of 0.06x0.06x0.48 µm and a 63x NA 1.4 oil immersion objective. Following acquisition, the sequences of Z-stack images were analyzed off-line using Imaris which renders the optical sections into 3D models enabling analysis of leukocyte-endothelial interaction dynamics.

**Quantification Endothelial Pore Structures**

Real-time endothelial pore dynamics in HUVECs expressing Lifeact-GFP was analyzed by ImageJ. Three parameters were scored; the area of the neutrophil cell body (luminal site), the area of the pore, and the area of the neutrophil cell body under the endothelium (abluminal site). The area (µm²) of each parameter was measured manually by drawing a ROI resembling the parameter (e.g. pore size) and measured in ImageJ using analyze measure. Endothelial pore sizes of fixed HUVECs were measured by drawing a ROI resembling various parameters (width, length, height, circularity, and pore size). Length of the endothelial pore was defined as the total distance of the pore parallel to the junction and width was defined as the total distance perpendicular to the junction. Circularity of the pore is defined by the length over width ratio. Pore size was defined as the total surface area of endothelial pore. Total F-actin in the pore (sum intensity Z-slices) was divided in two equal ROI. Distribution asymmetry was defined by ROI (1)-BG over ROI (2)-BG ratio. Leukocytes migrating through the EC body not interrupting junctional VE-cadherin were scored as transcellular migration, whereas leukocyte migrating between ECs interrupting junctional VE-cadherin were scored as paracellular migration.

**Characterization of DORA RhoA Biosensor**

The design of the DORA RhoA sensor is based on the published RhoA biosensor. Importantly, the GTPase is placed at the C-terminus of the construct enabling the DORA RhoA biosensor to localize at the plasma membrane similar to endogenous RhoA (Fig. 2a). FRET efficiency of the ON-state of the GTPase is improved through modelling of the fluorescent protein dimers and the GTPase-effector domain complexes. Repeats of stable α-helix from ribosomal protein L9, rather than an unstructured linker, is inserted between the fluorescent proteins to disrupt dimerization and diminish FRET efficiency in the inactive state.
In ECs, the DORA RhoA sensor accurately reported thrombin induced RhoA activation, showing a fast and transient RhoA activation (Fig. 2b and Supplementary Fig. 1f and Supplementary Movie 7). Quantification of 10 independent experiments showed reproducible RhoA activation patterns upon thrombin treatment (Supplementary Fig. 1f). As a control, DORA RhoA mutant PKN was generated to report misalignment of Cer3 and Venus image before and after image registration, motion artefacts or pH changes affecting the sensors fluorescent proteins. The substitution of glutamine for the leucine at position 59 in PKN prevents the binding of PKN to activated RhoA, in the DORA RhoA mutant PKN. The DORA RhoA mutant PKN showed no change in Venus/Cer3 ratio after thrombin stimulation (Supplementary Fig. 2a and Supplementary Movie 8). Quantification of 10 independent experiments showed no change in Venus/Cer3 ratio after thrombin addition in ECs that expressed the DORA RhoA mutant PKN biosensor (Supplementary Fig. 2a). In addition, Hela cells that over-expressed the histamine receptor, histamine stimulation induced a fast and sustained RhoA activation (Supplementary Fig. 2b and Supplementary Movie 9). Subsequent addition of Pyrilamine reduced RhoA activity to baseline levels, showing that the sensor dynamically reports RhoA activation and inactivation. The DORA RhoA mutant PKN showed no change in Venus/Cer3 ratio after histamine stimulation (Supplementary Fig. 2c and Supplementary Movie 10). To show that the sensor is cycling between an active and inactive, Rho-GDI-bound form, we co-expressed Rho-GDI and found a reduced FRET ratio, illustrative for binding to Rho-GDI (Supplementary Fig. 3a). In conclusion, the DORA RhoA biosensor accurately reports RhoA dynamics in ECs. Important to note, Venus and Cer3 emission were simultaneously recorded utilizing a double camera system since sequential image acquisition results in motion artefacts induced by migrating leukocytes displacing fluorescent signals in the ECs.

Characterization of DORA FRET RhoA-sensor

The intrinsic single-chain nature of the DORA RhoA biosensor rules out the possibility of unequal Cer3 and Venus localization within the EC. Therefore, DORA RhoA sensor translocation always results in a paired translocation of Cer3 and Venus emission. In case the biosensor does translocate (e.g. in response to EC shape alterations induced by migrating neutrophils lowering the local Venus and Cer3 emission dramatically) sequential imaging may result in erroneous measurements. The delay in the acquired Cer3 and Venus emission image of around 400-800 milliseconds may therefore contain an intrinsic local CFP-YFP pixel shift that cannot be solved by registration software. Therefore, quality of the obtained Venus/Cer3 Ratio acquired by sequential image acquisition is dependent
on the transmigration speed of the neutrophil. To control for erroneous measurements during sequential image acquisition one could change the acquisition order of sequential image acquisition from Cer3-Venus to Venus-Cer3, the DORA RhoA activity status will change from high to a low activity pattern. If the activity pattern alters, it indicates that an intrinsic local CFP-YFP pixel shift is present in the sequentially acquired Cer3 and Venus dataset. To circumvent motion artefacts a dual camera setup was used to acquire Cer3 and Venus emission images simultaneously. In our setup photo bleaching-rates of Cer3 and Venus are more or less equivalent. Photo bleaching (average ± s.d.) was determined by the reduction in fluorescence (1-F/F0) in living cells after 30 minutes illumination with HXP V at 20%, 800ms illumination with an interval of 5 seconds. dCer3 photo bleaching was 16.3% ±7.6%) and dcpVenus bleaching was 13.3% ±7.9%). All ratio-metric images and normalized intensity graphs were not corrected for photo bleaching, since photobleaching kinetics may change when FRET changes. Cer3 bleed through into the Venus channel is 57%. All normalized intensity graphs were corrected for bleed through using the equation (YFP-BG)-(0.57*(CFP-BG).

**DORA FRET RhoA-sensor analysis**

We use a Zeiss Observer Z1 microscope with 40x NA 1.3 oil immersion objective, a HXP 120 V excitation light source, a Chroma 510 DCSP dichroic splitter, and two Hamamatsu ORCA-R2 digital CCD cameras (2x2 binning) for simultaneous monitoring of Cer3 and Venus emissions. Image acquisition was performed using Zeiss Zen 2011 microscope software. The lowest achievable HXP excitation power, through a FRET filter cube (Exciter ET 436/20x, and 455 DCLP dichroic mirror (Chroma), the emission filter is removed) was used to excite the Cer3 donor. The emission is directed to the left side port by a 100% mirror, to an attached dual camera adaptor (Zeiss) controlling a 510 DCSP dichroic mirror. Emission wavelengths between 455-510 nm are directed to an emission filter (ET 480/40, Chroma) and then captured by the ‘straight’ Hamamatsu ORCA-R2 camera resulting in Cer3 image acquisition. The Emission wavelength 510 nm and higher are directed to a six positions LEP filter wheel (Ludl Electronic Products) placed in front of the second ‘rear’ Hamamatsu ORCA-R2 camera. Position 1 in the emission filter wheel is equipped with an ET 540/40m used for venus image acquisition. Position 2 is left empty to allow mCherry image acquisition (EX BP 572/25, BS FT 590, EM BP 629/62, Zeiss). The LEP filter wheel is controlled by the MAC 6000 controller system (Ludl Electronic Products). Exposure time of DIC image was set to 76 ms and exposure time of simultaneous CFP and YFP acquisition was set to +/-800 ms, images were subsequently recorded.
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every 5 seconds for periods as indicated. Offline ratio analysis between
Cer3 and Venus images was done utilizing the MBF ImageJ collection
(Tony Collins). Individual images in the raw Cer3 and Venus image stacks
were background (BG) corrected using the plug-in ‘ROI, BG subtraction
from ROI’. An region of interest (ROI) in the image were no cells were
present was selected as the background. Next, the Cer3 and Venus stacks
were aligned using the registration plug-in ‘Registration, MultiStackReg’.
A smooth filter was applied to both image stacks to improve image quality
by reducing the noise. The smooth filter (mean filter) was applied over
the entire image and replaces each pixel with the average of its 3 × 3
neighborhood. Next, both image stacks were converted to a 32-bit image
format, required for subsequent masking. A user defined threshold was
applied exclusively to the Venus image stack, converting the background
pixels to ‘not a number’ (NaN). It allows elimination of artifacts in ratio
image stemming from the background noise. Finally the Venus/Cer3
ratio was calculated using the plug-in ‘Ratio Plus’, and a custom lookup
table was applied to generate a color-coded image illustrating the high
‘red’ and low ‘blue’ activities. Note that some of the plug-ins, namely
MultiStackReg, and Ratio Plus are not included in the basic MBF ImageJ
collection and should be downloaded from the plug-in page in the ImageJ
intensity graphs; the intensity of an manually selected ROI of interest and
the BG of the raw Cer3 and Venus image stacks were measured using the
plug-in ROI, Multi Measure in ImageJ. The raw Cer3 and Venus intensities
were BG subtracted using equation Cer3 = (Cer3 raw - BG), subsequently
Venus only was corrected for bleed through using the equation VenusC
= (Venus)-(0.5748*(Cer3)). Normalization of the individual intensity traces
was done by dividing the data by the average over acquisition 3 to 13. The
Venus/Cer3 ratio was calculated from the normalized data using Excel.

**Confocal intravital microscopy of mouse cremaster muscles**

To investigate local myosin light chain phosphorylation during
leukocyte diapedesis, whole-mounted cremaster muscles of mice
expressing endogenously labeled GFP-leukocytes (*Lys-EGFP-ki, CX3CR1-
EGFP-ki, Tie-2Cre*) or C57BL6 mice were fixed, immunostained and
analyzed by confocal microscopy. Inflammation in mouse cremaster
muscles was induced by intrascrotal injection (i.s.) of IL-1β/TNF-α (50 ng
IL-1β, 300 ng TNF-α in 400µl saline) or saline (400 µl) for 2 hours. EC
junctions were labeled through co-administration of Alexa Fluor-555 or
647-labeled anti-PECAM-1 mAb (clone 390; 3µg i.s.). Anti-PECAM mAb
clone 390 was conjugated to Alexa Fluor-555 using a commercially
available kit (Invitrogen, Paisley, UK). The cremasters were surgically
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Exteriorized and TEM was analyzed by intravitral microscopy. Straight post-capillary venules of 20-40 µm in diameter were selected for analysis of leukocyte-vessel wall interactions. Z-stacks of images were captured by confocal microscopy as described by Abigail Woodfin. The tissues were fixed with 4% paraformaldehyde for 30 minutes at a time point with several paracellular pores visible in the PECAM-1 labelling. Subsequently, tissues were permeabilised with 0.5% Triton and blocked with 10% BSA and 10 µg/µl FC block for 2 hours. Tissues were incubated with anti-phospho Myosin light chain Thr18/Ser19 (Cat #3674) purchased from Cell signaling (BIOKE) antibody for 2 days and a further 2 days with the secondary antibody or IgG isotype control (1:100). Transmigration induced permeability was examined by looking at fluorescent TRITC-dextran leakage into the cremaster of C57BL/6 WT or LysM-GFP mice during IL-1β and TNF-α stimulated neutrophil recruitment. Four different groups were defined; unstimulated, Rho inhibitor I (C3) alone, IL-1β/TNF-α treated, IL-1β/TNF-α treated + C3. IL-1β (50 ng), TNF-α (300 ng), Rho-inhibitor (1 µg) and anti-PECAM-1 labelling antibody (4 µg) were given intrascrotally at T = 0 hours. A second dose of C3 (1 µg) was given intrascrotally and TRITC-dextran (40 kDa, 200 µl/40 µM) was injected intravenously at T = 2 hours and allowed to circulate until T = 4 hours. At T = 4 hours animals were sacrificed and tissues were subjected to vascular wash-out by perfusion of 10 ml PBS via the left ventricle. Tissues were examined by confocal microscopy (Leica SP5, 20X objective) and the level of TRITC-dextran fluorescence by region of interest in the surrounding tissue was quantified using the LAS-AF Lite software. Neutrophil extravasation per 5x10⁴ µm² tissue area was quantified by endogenous GFP fluorescence in the neutrophils of LysM-GFP mice, or by fixation and labelling of neutrophils with anti-MRP14-alexa647 in WT mice after analysis of TRITC-dextran leakage in unfixed tissues.

F-actin visualization in retinal vasculature of Lifeact-GFP mice

Lifeact-EGFP transgenic mice have been previously described. All experiments with mice were performed in accordance to German guidelines and regulations. Mice were analysed at p7 and gender was randomly picked. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Ludwig-Maximilians University Munich.

Whole retina immunohistochemistry

Dissection and labelling of retinas was performed as previously described. Briefly, retinas were fixed for 2 hours on ice in 4% paraformaldehyde (PFA), incubated in 1% BSA and 0.3% Triton X-100, washed two times in
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Pblec (1% Triton X-100, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2 PBS [pH 6.8]), and incubated overnight with isolectin-B4 (1:50) and antibodies diluted in Pblec. Images were acquired and processed using a Leica TCS SP5 II microscope, LAS Montage Imaging software (Leica) and the IMARIS Digital Imaging software (Biplane). Biotinylated *Griffonia simplicifolia* lectin I (IB4), (B-1205) was purchased from Vector Laboratories.

**WESTERN BLOTTING**

Cells were washed twice with PBS, and lysed with 95°C SDS-sample buffer containing 4% β-mercapto-ethanol. Samples were boiled at 95°C for 4 minutes to denature proteins. Proteins were separated on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen), transferred to Immobilon-PVDF transfer membranes (Millipore Corp., Billerica, MA) and subsequently blocked with 2.5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) for 60 minutes. The immunoblots were analyzed using primary antibodies incubated overnight at 4°C and secondary antibodies linked to horseradish peroxidase (HRP) (GE Healthcare, UK), after each step immunoblots were washed 6x with TBST. Signals were visualized by enhanced chemiluminescence and light sensitive films (GE Healthcare, UK).

**PULL-OUT ASSAY AND IMMUNOPRECIPITATION**

1.2 mg/ml dynabeads goat-x-ms IgG (Dynal, Invitrogen) per condition were washed ones with 1 ml buffer 1 containing PBS + 2 mM EDTA and 0.1% BSA (Millipore) using a magnetic holder. Dynabeads were coated with 1.6 µg α-ICAM-1 CD54 (BBIG-I1/IIC81, R&D systems (Cat #BBA9) antibodies per condition and incubated head-over-head at 4ºC for 45 min. The beads were washed twice using buffer 1 and resuspended in PBS containing 0.5 MgCl2 and 1 mM CaCl2. Overnight TNF-α treated (10 ng per ml) HUVEC (2-5 million cells) were pre-treated with 1 µM Ionomycin (Invitrogen, I-24222) for 10 min. 1.2 mg per ml dynabeads per condition were used to cluster ICAM-1 for 10-30 minutes. Cells were washed once on ice using PBS containing 0.5 MgCl2 and 1 mM CaCl2. Next, cells were lysed in 1 ml cold pH7.4 RIPA buffer containing 50 mM Tris, 100 mM NaCl, 10 mM MgCl2, 1% NP40, 10% glycerol, 0.1% SDS, 1% DOC (Sigma-Aldrich), DNAse inhibitor and protease phosphatase inhibitor cocktail for 5 min. Cells were scraped together and lysates were transferred to a new tube. Then, ICAM-1 coated dynabeads were added to non-clustered-control cells. 50 µl whole cell lysate was taken from all conditions. Beads and cell-lysates were subsequently incubated head-over-head for 1-2h at 4º C. Next cells were washed twice with Ripa buffer and three times with NP-40 lysis buffer. Beads were resuspended in 30 µl 2x SDS-sample buffer and assessed by Western blotting.
**Electric cell-substrate impedance sensing under flow**

Endothelial monolayer integrity during leukocyte diapedesis was determined by measuring the electrical resistance using Electric Cell-substrate Impedance Sensing (ECIS). Flow chamber electrode arrays (8F10E; Applied Biophysics, Troy, NY) were pretreated with 10 mM L-cysteine (Sigma-Aldrich) for 15 minutes at 37°C, subsequently washed twice with 0.9% NaCl and coated with FN (Sanquin) in 0.9% NaCl for 1 hour at 37°C. Cells were seeded at 300,000 cells per slide (2.5 cm²) and grown to confluence. Continuous resistance measurements were performed at 37°C at 5% CO₂ with the ECIS Zθ (Theta) system controller (Applied Biophysics). After forming a stable monolayer, HUVECs were subjected to flow (0.8 dynes/cm²) for periods as indicated.

**Statistics**

Endothelial pore analyses between leukocyte subtypes was tested using a one-way ANOVA assuming no matching or pairing, comparing the mean of each column with the mean of every other column, that were corrected for multiple comparisons by Tukey multiple comparisons test, with a single pooled variance. Statistical comparison between experimental groups was performed by the student T-test. A two-tailed p-value of < 0.05 was considered significant. Correlation analysis of dextran and neutrophil extravasation kinetics was performed by Pearson’s correlation. Unless otherwise stated, a representative experiment out of at least three independent experiments is shown.

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**Author contributions**

NH, LS, CO, JvR, TY, BM, JvU, BP, SH, JG, YW, EM, AW, JDvB performed the experiments; NH, SH, JG, YW, EM, AW and JDvB analyzed and interpreted the data; NH and JDvB did study conception and design; NH and JDvB wrote the manuscript.

**Competing Financial Interest**

The authors declare that they have no competing financial interest
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Supplementary Figure 1 Neutrophil transmigration through ECs is associated with minimal FITC-dextran leakage. (a) Quantification of FITC-dextran (70 kDa) leakage during neutrophil diapedesis through TNF-α treated HUVECs cultured on 3µm pore permeable filters. Neutrophils transmigrated towards a C5a chemotactic gradient in the lower compartment. (b) Endothelial RhoA depletion by siRNA. Immunoblot analysis of protein extracts prepared from HUVEC treated with TNF-α overnight. Extracts were prepared 72 hours after transfection with control and RhoA siRNA duplexes. Extracts were probed with antibodies directed against RhoA and actin. Quantification of RhoA depletion, presented as ‘quantum level’ (sum of grayness of each pixel in blot) minus background (QL-Bg), normalized to that of actin. (c) Normalized resistance of endothelial monolayer during leukocyte diapedesis through HUVECs treated with or without C3 inhibitor measured with ECIS in real-time under flow conditions. Green and red line indicates untreated and C3-pretreated ECs, respectively. Lines represent average of eight wells. Numbers: 1: Onset of flow (0.8 dyne per cm²); 2: addition of neutrophils (approximately 1*10⁶ per mL); 3: addition of thrombin (1U per mL). (d) Confocal intravital microscopy of 20 µm diameter cremasteric venules in LysM-GFP mice (green neutrophils) immunostained in vivo for EC junctions by intrascrotal injections of fluorescent-labeled PECAM-1 (blue) and stimulated for four hours with IL-1β and TNF-α only, or with Rho inhibitor (C3). A second dose of Rho inhibitor was given intrascrotally and TRITC-dextran (40 kDa) was injected intravenously at T = 2 hours and allowed to circulate until T = 4 hours. Scale bar 100µm. (e) Quantification of TRITC-dextran extravasation in animals left unstimulated (control), stimulated with C3 alone or IL-1β/TNF-α. (f) Characterization of DORA RhoA biosensor and DORA RhoA mutant PKN biosensor. Time-lapse Venus/Cer3 ratio images of DORA RhoA biosensor after thrombin treatment in HUVECs. Filled arrows indicate RhoA activation. Scale bar, 10µm. Calibration bar shows RhoA activation (Red) relative to basal RhoA activity (Blue). Graph on the right shows quantification of DORA RhoA biosensor ** P < 0.01 (ANOVA) (a) ** P < 0.01 control versus RhoA depletion (Student’s t-test) (b). * P < 0.05 Saline versus IL-1β/TNF-α (Student’s t-test) (e). Data are representative of three independent experiments (a-c) 10 experiments (f) (error bars (a-e), s.e.m).
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Supplemental Figure 1.

a

Supplemental Figure 1a-c

b

Supplemental Figure 1b

RhoA

Actin

siControl

siRhoA

**

RhoA

Signal (QL-Bg) x 10^4

Figure 1a-c

0.6

0.8

1.0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

0

2

4

6

8

10

Extravascular TRITC-dextran fluorescence (a.u.)

Venule diameter of 20 µm

Before + Thrombin

Venus/Cer3

DORA RhoA biosensor

1. Flow

2. Neutrophils

3. Thrombin

C3

**

ns

**

Venus

Cer3

Venus/Cer3

Before + Thrombin

DORA RhoA biosensor

Normalized intensity

Time (min)

0

1

2

3

4

5

6

7

8

9

10

0.5

0.6

0.7

0.8

0.9

1.0

1.1

1.2

1.3

1.4

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Supplementary Figure 2 Characterization of DORA RhoA biosensor and DORA RhoA mutant PKN biosensor. (a) Time-lapse Venus/Cer3 ratio images of DORA RhoA mutant PKN biosensor after thrombin treatment in HUVECs. Scale bar, 10µm. Calibration bar shows RhoA activation (Red) relative to basal RhoA activity (Blue). Graph on the right shows quantification of DORA RhoA mutant PKN biosensor after thrombin treatment (asterisk) in HUVECs. (b) Time-lapse Venus/Cer3 ratio images of DORA RhoA biosensor or DORA RhoA mutant PKN biosensor (c) after histamine and subsequent antagonist pyrilamine stimulation in Hela cells expressing the histamine receptor. Filled arrows indicate RhoA activation. Graph on the right shows quantification of DORA RhoA activation. Asterisk indicate addition of histamine; second asterisk shows addition of pyrilamine. (d) Epi-fluorescent live-cell imaging of HUVECs expressing the DORA RhoA biosensor during neutrophil adhesion under physiological flow conditions (0.8dyne per cm²). Open arrows indicate adherent neutrophil at the apical side of the endothelium. Filled arrows indicate local RhoA activation during neutrophil diapedesis. Scale bar 10µm. Calibration bar shows RhoA activation (red) relative to basal RhoA activity (blue). Data are representative of 10 experiments (a-c).
Supplemental Figure 2.

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a

b

c

d

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**Supplementary Figure 3** F-actin rich endothelial pores. (a) Quantification of basal DORA RhoA biosensor ratios in Hela cells with and without RhoGDI overexpression. Box plot shows median with 95% confidence interval (notches) and the limits of the box indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. The Venus/Cer3 ratios of individual cells are plotted as open circles. The lower FRET ratio in presence of RhoGDI reflects reduced GTP-loading, which is in agreement with regulation by RhoGDIs. (b) Time-lapse Venus/Cer3 ratio images of DORA RhoA mutant PKN biosensor during neutrophil transmigration. (c) Quantification of DORA RhoA mutant PKN biosensor activation during neutrophil TEM. First asterisk indicates leukocyte adhesion; second asterisk shows leukocyte diapedesis. (d) Confocal imaging of extravasating neutrophils through Lifeact-GFP expressing HUVECs under physiological flow conditions, cells were fixed and stained for phalloidin. Open and filled arrows indicate F-actin visualized using phalloidin or Lifeact-GFP, respectively. Scale bar, 20µm. PECAM-1 localization in para-and transcellular endothelial pores. (e) Paracellular migration of monocytes. Open and filled arrows indicate PECAM-1 localization to the endothelial pore at the basolateral or apical site, respectively. Lifeact (red), VE-cadherin (green) and neutrophil nuclei (blue). Asterisk indicates transmigrating neutrophil in DIC. Merge shows all colours. Scale bar, 5µm. (f) Transcellular migration of monocytes. Open and filled arrows indicate PECAM-1 localization to the endothelial pore at the basolateral or apical site, respectively. Lifeact (red), VE-cadherin (green) and neutrophil nuclei (blue). Asterisk indicates transmigrating neutrophil in DIC. Merge shows all colours. Scale bar, 5µm. Data are representative of ten independent experiments (a) seven experiments (c) > 20 transmigration events (e, f) (error bars (a,c), s.e.m).
Supplemental Figure 3.

**a**
FRET ratio [a.u.]

**b**
00:00 06:40 21:40 min

**c**
Normalized Venus/Cer3 emission ratio

**d**
Phalloidin-405 Lifeact-GFP

**e**
DIC PECAM-1 Lifeact VE-cad DAPI Merge

**f**
DIC PECAM-1 Lifeact VE-cad DAPI Merge
Supplementary Figure 4  Endothelial pore morphology at distinct stages of neutrophil diapedesis. (a) Confocal imaging of paracellular migrating neutrophils through ICAM-1-GFP and Lifeact-mCherry expressing HUVEC under physiological flow conditions. Open and filled arrows indicate ICAM-1-GFP and Lifeact-mCherry localization to endothelial pore, respectively. Asterisk indicates extravasating neutrophil (DAPI in blue). VE-cadherin (white). Scale bar, 5µm. (b) Quantification of paracellular migration route through bi-cellular (i.e. junctions of two adjacent ECs) or multi-cellular (i.e. junctions formed by three or more ECs). (c) Confocal imaging of Lifeact-GFP-positive endothelial pores at distinct stages of neutrophil diapedesis, defined as adhesion, early, mid, and late diapedesis. Open arrows indicate the cortical actin-ring at the basolateral site that appeared during leukocyte crossing. Asterisk indicates extravasating leukocyte (DAPI in blue). Scale bar, 10µm. (d) Quantification of pore length and pore width defined as the distance parallel and perpendicular to the cell-cell junction, respectively. (e) Quantification of F-actin rich apical protrusions height for neutrophils and monocytes. (f) Quantification of endothelial circularity for para- and transcellular migrating neutrophils and monocytes according to the circularity index, Circle = 1 and oval = 2. Diagram on the left indicates the orientation of the pore for the paracellular route. Statistical significance was tested using a student’s t-test. Data are representative of four independent experiments (a-f) with 40 transmigration events per group (error bars (d-f), s.e.m).
Supplemental Figure 4.

**a** Paracellular diapedesis

DIC VE-cad Lifeact ICAM-1 GFP DAPI Merge

**b** F-actin abundant %

Bi-cellular Junction
Multi-cellular Junction

**c** Neutrophil adhesion

DIC Lifeact VE-cad DAPI Merge

**d** Pore length

Pore width

F-Actin rich pore
Cell-Cell Junction

**e** Height of EC protrusions

**f** Circularity index

Endothelial pore circularity

Endothelial pore length

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Supplementary Figure 5 RhoA signalling is required for local MLC phosphorylation and endothelial pore confinement during neutrophil transmigration. Endothelial RhoA and VE-cadherin depletion by siRNA. (a) Immunoblot analysis of protein extracts prepared from HUVEC treated with TNF-α overnight. Extracts were prepared 72 hours after transfection with control and RhoA siRNA duplexes. Extracts were probed with antibodies directed against RhoA and actin. Quantification of RhoA depletion, presented as ‘quantum level’ (sum of grayness of each pixel in blot) minus background (QL-Bg), normalized to that of actin. (b) Confocal imaging of paracellular migrating neutrophils through Lifeact-GFP expressing HUVEC after 72 hours transduction with control shRNA (left panel) or VE-cadherin shRNA (right panel) under physiological flow conditions. Open and filled arrows indicate PECAM-1 (white) and VE-cadherin (green) localization to the endothelial pore, respectively. Open and filled arrows indicate Lifeact-GFP localization to endothelial pore after 72 hours transduction with control shRNA (left panel) or VE-cadherin shRNA, respectively. Asterisks indicate extravasating neutrophils in DIC. VE-cadherin (green) and DAPI (blue). Merge shows all colours. Scale bar, 5µm. (c) Quantification of endothelial pore length, width, height, circularity of the endothelial pore in HUVECs after 72 hours transfection with control shRNA or VE-cadherin shRNA under physiological flow conditions. (d) Immunoblot analysis of protein extracts prepared from HUVECs treated with TNF-α overnight. Extracts were prepared 72 hours after transduction with control and VE-cadherin shRNA duplexes. Extracts were probed with antibodies directed against VE-cadherin and actin. (e) RhoA signalling is required for local MLC phosphorylation and endothelial pore confinement during neutrophil transmigration. Confocal imaging of paracellular migrating neutrophils through Lifeact-mCherry (red) expressing HUVECs after 72 hours transfection with control siRNA (upper panel) or RhoA siRNA (lower panel) under physiological flow conditions (0.8 dyne/cm²). Open arrows in the apical and basal plane indicate filopodia-like protrusions at the apical site and the cortical F-actin-ring at the basolateral site of the endothelial pore, respectively. Filled arrows indicate MLC phosphorylation (green) localization during neutrophil transmigration (0.8 dyne per cm²). Asterisk indicates extravasating leukocyte in DIC. Scale bar, 10 µm. (f) Confocal imaging of F-actin dynamics during leukocyte diapedesis in retina vasculature of Lifeact-EGFP mice. Upper panel shows x-y and z-x projection of Lifeact-EGFP surrounding isolectin B4 (IB4) positive neutrophil. Open and filled arrows indicate Lifeact-EGFP localization to the endothelial pore at the basolateral or apical site, respectively. Asterisk indicates transmigrating neutrophil. Scale bar, 2µm. ** P < 0.01 and * P < 0.05 siCTRL vs siRhoA (Student’s t-test) (a). Data are representative of four independent experiments (a-d) with >12 transmigration events per group (c) (error bars (a), s.e.m, (c), SD).
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Supplementary Figure 5

(a) Western blot analysis of RhoA and Actin expression in siCTRL and siRhoA samples. RhoA band is at -21 kDa, while Actin is at -42 kDa.

(b) Immunofluorescence images showing differences in endothelial pore morphology in shCTRL and shVE-cadherin samples. DIC, PECAM-1, Lifeact, VE-cadherin, and DAPI staining.

(c) Statistical analysis of endothelial pore length, width, height, and circularity in siCTRL and siRhoA samples, with ns indicating no significant difference and * indicating significant difference.

(d) Western blot analysis of VE-cadherin and Actin expression in TR-CNS and pDC-3H2 samples. VE-cadherin band is at -130 kDa, while Actin is at -42 kDa.

(e) Immunofluorescence images showing endothelial pore morphology in control, siRhoA, and pDC-3H2 samples. DIC, Lifeact, and pMLC staining.

(f) Immunofluorescence images showing endothelial pore morphology in control and siRhoA samples, with DIC, Lifeact, and pMLC staining.
Supplementary Figure 6 LARG and Ect2 are recruited to intercellular tail of ICAM-1 upon clustering (a) Quantification of FITC-dextran and neutrophil extravasation after 20 minutes of neutrophil transmigration through control and ROCK1 or ROCK2b deficient ECs (b) Immunoblot analysis of protein extracts prepared from ECs treated with TNF-α overnight. Extracts were prepared 72 hours after transfection with control, ROCK1 or ROCK2b siRNA duplexes. Extracts were probed with antibodies directed against ROCK1, ROCK2b and actin. (c) Immunoblot analysis of protein extracts prepared from ECs treated with TNF-α overnight. Extracts were prepared 72 hours after transduction with control, Ect2, p115RhoGEF shRNA or transfection with LARG siRNA duplexes. Extracts were probed with antibodies directed against LARG, Ect2, p115RhoGEF and actin. (d) Extravasation kinetics of calcein-red labelled neutrophils and FITC-dextran through TNF-α treated ECs cultured on 3µm pore permeable filters. Neutrophils transmigrated towards a C5a chemotactic gradient in the lower compartment. Four conditions were tested; LARG/Ect2 deficient ECs + neutrophils (Orange line), control + neutrophils (purple line), LARG/Ect2 deficient ECs only (red line) and control only (green line). (e) Correlation analysis of dextran and neutrophil extravasation kinetics through control and LARG/Ect2 deficient ECs. (f) Endothelial LARG/Ect2 depletion by RNAi. Immunoblot analysis of protein extracts prepared from HUVEC treated with TNF-α overnight. Extracts were prepared 72 hours after transfection with control and Ect2 shRNA or transfection with control and LARG siRNA duplexes. Extracts were probed with antibodies directed against LARG, Ect2 and actin. Quantification of LARG/Ect2 depletion, presented as ‘quantum level’ (sum of grayness of each pixel in blot) minus background (QL-Bg), normalized to that of actin. (g) ICAM-1 clustering using beads coated with anti-ICAM-1 antibodies pre-and post-membrane lysis. Extracts were probed with antibodies directed against ICAM-1, LARG, Ect2, GEF-H1 and Filamin A. (h) PECAM-1 clustering using beads coated with anti-PECAM-1 antibodies pre-and post-membrane lysis. Extracts were probed with antibodies directed against PECAM-1, LARG, Ect2, GEF-H1 and Filamin A. r = 0.1994 P = 0.5345 (Pearson correlation) transmigrated neutrophils versus FITC-dextran leakage in control HUVECs or r = 0.7734 ** P < 0.01 (Pearson correlation) transmigrated neutrophils versus FITC-dextran leakage in LARG/Ect2 depleted ECs (b). * P < 0.05 siCTRL vs siLARG (Student’s t-test) * P < 0.05 shCTRL vs shEct2 (Student’s t-test) (f). Data are representative for three independent experiments (d-h) (error bars (d-f), s.e.m).
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Supplemental Figure 6.

(a) FITC-Dextran 70kDa Leakage Fold Increase

(b) Transmigrated neutrophils Fold Increase

(c) ROCK1 ROCK2b Actin

(d) FITC-Dextran 70kDa Leakage Fold Increase

(e) Transmigrated neutrophils Fold Increase

(f) Transmigrated neutrophils Fold Increase

(g) ICAM-1 Clustering Total Cell Lysate

(h) PECAM-1 Clustering Total Cell Lysate
Supplementary Figure 7 ICAM-1, but not PECAM-1 depletion elicits vascular permeability during neutrophil diapedesis. (a) Extravasation kinetics of calcein-red labelled neutrophils and FITC-dextran through TNF-α treated ECs cultured on 3µm pore permeable filters. Neutrophils transmigrated towards a C5a chemotactic gradient in the lower compartment. Four conditions were tested: ICAM-1 depletion (EC) + neutrophils (Orange line), control + neutrophils (purple line), ICAM-1 depletion (EC) only (red line) and control only (green line). (b) Correlation analysis of dextran and neutrophil extravasation kinetics through control and ICAM-1 depleted ECs. (c) Endothelial ICAM-1 depletion by siRNA. Immunoblot analysis of protein extracts prepared from HUVEC treated with TNF-α overnight. Extracts were prepared 72 hours after transfection with control or ICAM-1 siRNA duplexes. Extracts were probed with antibodies directed against ICAM-1 and actin. Quantification of ICAM-1 depletion, presented as ‘quantum level’ (sum of grayness of each pixel in blot) minus background (QL-Bg), normalized to that of actin. (d) Quantification of FITC-dextran and neutrophil extravasation after 20 minutes of neutrophil transmigration through control and PECAM-1 deficient ECs. (e) Immunoblot analysis of protein extracts prepared from HUVECs treated with TNF-α overnight. Extracts were prepared 72 hours after transduction with control and PECAM-1 siRNA duplexes. Extracts were probed with antibodies directed against PECAM-1 and actin and quantification of endothelial pore size in control and PECAM-1 deficient ECs. \( r = 0.5688 \) \( P = 0.053 \) (Pearson correlation) transmigrated neutrophils versus FITC-dextran leakage in control HUVECs or \( r = 0.6078 \) \( * P < 0.05 \) (Pearson correlation) transmigrated neutrophils versus FITC-dextran leakage in ICAM-1 depleted ECs. ** \( P < 0.01 \) siCTRL vs siICAM-1 (Student’s t-test) (c). Data are from three experiments (a-d) or are representative of > 20 transmigration events error bars (a-e) are s.e.m.
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Supplemental Figure 7.

(a) Time course of FITC-dextran (70kDa) leakage fold increase after stimulation with siCTRL or siICAM-1 and transmigration of neutrophils fold increase upon PMN stimulation. siICAM-1-transfected cells showed a significant reduction in FITC-dextran leakage fold increase compared to siCTRL-transfected cells.

(b) Neutrophil diapedesis fold increase was significantly reduced in siICAM-1-transfected cells compared to siCTRL-transfected cells. Similar results were observed for FITC-dextran leakage fold increase.

(c) ICAM-1 and Actin protein expression levels were determined by Western blotting. siICAM-1-transfected cells showed a significant reduction in ICAM-1 expression compared to siCTRL-transfected cells. Similar results were observed for Actin expression.

(d) Endothelial pore size was measured in siCTRL and siICAM-1-transfected cells. siICAM-1-transfected cells showed a significant reduction in endothelial pore size compared to siCTRL-transfected cells.

(e) Results were confirmed by immunofluorescence staining for PECAM-1 and Actin. siICAM-1-transfected cells showed a significant reduction in PECAM-1 expression compared to siCTRL-transfected cells. Similar results were observed for Actin expression.