The Company of **Biologists**

COMMENTARY

Cell-stiffness-induced mechanosignaling – a key driver of leukocyte transendothelial migration

Antje Schaefer* and Peter L. Hordijk

ABSTRACT

The breaching of cellular and structural barriers by migrating cells is a driving factor in development, inflammation and tumor cell metastasis. One of the most extensively studied examples is the extravasation of activated leukocytes across the vascular endothelium, the inner lining of blood vessels. Each step of this leukocyte transendothelial migration (TEM) process is regulated by distinct endothelial adhesion receptors such as the intercellular adhesion molecule 1 (ICAM1). Adherent leukocytes exert force on these receptors, which sense mechanical cues and transform them into localized mechanosignaling in endothelial cells. In turn, the function of the mechanoreceptors is controlled by the stiffness of the endothelial cells and of the underlying substrate representing a positive-feedback loop. In this Commentary, we focus on the mechanotransduction in leukocytes and endothelial cells, which is induced in response to variations in substrate stiffness. Recent studies have described the first key proteins involved in these mechanosensitive events, allowing us to identify common regulatory mechanisms in both cell types. Finally, we discuss how endothelial cell stiffness controls the individual steps in the leukocyte TEM process. We identify endothelial cell stiffness as an important component, in addition to locally presented chemokines and adhesion receptors, which guides leukocytes to sites that permit TEM.

KEY WORDS: Adhesion, Endothelium, ICAM1, Mechanotransduction, Transmigration

Introduction

Single-cell organisms, circulating cells and cells in tissues respond to a wide range of stimuli with polarization and increased motility. In multicellular organisms, directional motility is essential for tissue morphogenesis, angiogenesis, wound healing and inflammation. Cell motility is efficiently induced when an agonist is presented in a gradient; cells can sense extremely small spatial differences in ligand concentration, down to differences of ∼2–5% and less across the length of the cell (Merlot and Firtel, 2003). The receptor-associated signaling machinery translates such shallow extracellular concentration differences into steep gradients of intracellular response. Various stimuli induce cell movement, including chemokines (inducing chemotaxis or chemokinesis), substrate-bound ligands (haptotaxis), and electric current (electrotaxis or galvanotaxis). Here, we focus on leukocyte migration in response to stiffness, a response known as durotaxis – the tendency of a cell to migrate toward regions of increased substrate stiffness (Plotnikov and Waterman, 2013). Biochemical differences (e.g. the presence of collagen versus fibronectin) may result in differences in stiffness together with altered outside-in signaling through integrins, whereas

Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, Swammerdam Institute of Life Sciences, University of Amsterdam, Amsterdam 1066 CX, The Netherlands.

*Author for correspondence (a.schaefer@sanquin.nl)

crosslinking or spatial variations in the concentration of a substrate can result in quantitative variations in stiffness. The response to a stiff substrate is the reorganization of cell adhesion accompanied by force generation and cell spreading, which precedes directional motility. This has been demonstrated for stem cells, cancer cells, neutrophils and platelets (Roca-Cusachs et al., 2013b). Thus, durotaxis is a generic cellular response that is relevant for migration in 2D and, most probably, in 3D environments.

In addition to sensing stiffness, cells can regulate their own stiffness, even on a subcellular level. Cellular stiffness does, amongst other things, depend on the stiffness of the nucleus, the osmotic pressure and the cellular coat (i.e. the glycocalyx) (Fels et al., 2014). However, the most dominant feature controlling cellular stiffness appears to be the F-actin cytoskeleton. Branched or linear F-actin filaments can be differentially crosslinked, with concomitant effects on their mechanical properties, which is reflected in differences in stiffness (Tseng et al., 2005). It follows that cytoskeletal dynamics, triggered through adhesion receptors or by soluble receptor ligands, regulate cellular stiffness in a highly dynamic and well-organized fashion.

The response of cells to mechanical cues presented by their environment is termed mechanotransduction (Humphrey et al., 2014). Mechanotransduction requires mechanosensing, a property attributed to various cell surface molecules, including growth factor receptors and transmembrane adhesion molecules (and their complexes), such as integrins, cadherins or immunoglobulin (Ig)-like cell adhesion molecules (CAMs). A fraction of the F-actin cytoskeleton is linked, through adaptors, to such transmembrane proteins (Schaefer et al., 2014; van Buul and Hordijk, 2009). This interaction is not static, but is regulated by actin dynamics and the binding to specific ligands. This may lead to ligand-induced conformational changes (i.e. increased affinity) and increased clustering (i.e. increased avidity). In addition, there can be regulation from within the cell through signaling-induced conformational changes of intracellular domains (termed inside-out signaling) (Lim and Hotchin, 2012; Ye et al., 2011). Thus, there is bidirectional communication between the cytoskeleton and membrane-integral adhesion molecules. Because transmembrane proteins can act as mechanosensors that detect substrate stiffness, it is clear that mechanosensing and -transduction are key to durotaxis.

One of the key events in development, inflammation and tumor cell metastasis is the breaching of cellular and structural barriers by migrating cells (Bravo-Cordero et al., 2012; Nourshargh et al., 2010). The most extensively studied example is the migration of leukocytes across the vascular endothelium (Fig. 1). Transendothelial migration (TEM) of leukocytes, also known as extravasation or diapedesis, is a hallmark of inflammation. It is prevalent in post-capillary venules but can also occur in arteries, driving atherogenesis. Briefly, TEM requires leukocytes to make transient and weak interactions with endothelial adhesion molecules of the selectin family. These low-affinity interactions allow
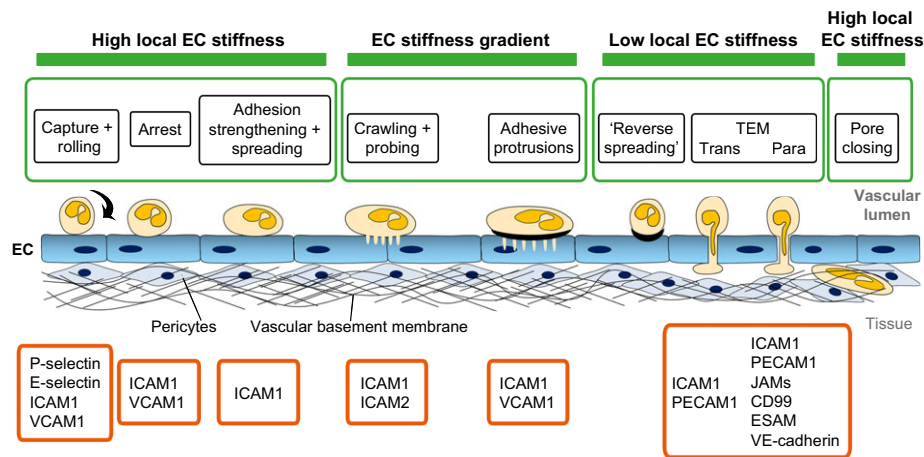
Fig. 1. Leukocyte TEM is regulated by EC stiffness. Leukocyte behavior during the different steps of TEM is regulated, as indicated, by distinct endothelial adhesion receptors, such as P-selectin, ICAM1 and PECAM1 (orange boxes) and relies on variations in EC stiffness (green boxes). Following capture, rolling and arrest, leukocytes adhere and spread on the endothelium. The function of endothelial adhesion receptors is promoted by their anchoring to the actomyosin network and increased EC stiffness. Next, crawling leukocytes scan the EC surface for sites that permit TEM during which both leukocytes and ECs form adhesive protrusions. In addition to chemotactic and haptotactic gradients, leukocytes are guided in forming contact with ECs by a durotactic gradient, the movement towards rigid regions, until they encounter areas that permit TEM ('hot spots'). Note that a gradient from lower relative stiffness at nuclear regions to higher stiffness towards the periphery exists even within a single EC (see Fig. 3.A,C for further details). Prior to the actual extravasation, leukocytes round up ('reverse spreading') and protrude their leading edge towards the abluminal EC side, which is facilitated by local soft junctional regions (TEM hot spots) (see also Fig. 3). Importantly, not every cell junction will be equally attractive for leukocytes to transmigrate as a consequence of a junctional heterogeneity in stiffness or density in adhesion receptors across the EC monolayer. Thus, EC stiffness is only one component among several others that determine the sites of TEM. Moreover, transcellular TEM also occurs, but to a lesser extent, and could be facilitated by a heterogeneous surface with local, soft regions across the cell body. TEM through cell–cell junctions (paracellular) or the cell body (transcellular) requires that the EC stiffness is balanced at these sites to allow both the formation of the transmigratory pore and the restriction of its size. Finally, closing the pore and restoring the EC barrier function requires an increase in cellular forces and EC stiffness.

leukocytes to roll over the endothelium, increasing their exposure to locally presented chemokines, which are produced in response to inflammatory cytokines (e.g. TNFα). These chemokines will activate signaling that feeds into leukocyte integrins, such as LFA-1, Mac-1, VLA-4 or VLA-5 (officially known as ITGAL, ITGAM, ITGA4 or ITGA5, respectively). Integrins, in turn, mediate strong binding to the endothelial ligands ICAM1 and vascular cell adhesion protein 1 (VCAM1). Additional endothelial adhesion molecules involved in TEM include platelet endothelial adhesion molecule 1 (PECAM1), endothelial cell selective adhesion molecule (ESAM), CD99 and members of the junctional adhesion molecules (JAMs) (Nourshargh et al., 2010; Sullivan and Muller, 2014; Vestweber et al., 2014). Adherent leukocytes spread on the endothelial cell (EC) surface and crawl towards sites that permit TEM (Phillipson et al., 2006; Schenkel et al., 2004). As cell spreading depends on the degree of substrate stiffness, it is logical to assume that EC stiffness regulates leukocyte spreading and crawling. In this Commentary, we will summarize mechanosensitive signaling events, including the RhoA–ROCK–actomyosin pathway, in leukocytes and ECs that are induced in response to substrate stiffness and variations thereof. We will further outline how EC stiffness guides leukocytes to sites that permit TEM and how EC stiffness regulates the various steps of the TEM process.

Mechanotransduction in leukocytes
Substrate stiffness determines leukocyte adhesion, spreading and crawling
Upon adhesion to the vascular wall, leukocytes spread and crawl on ECs to find sites that permit TEM (Fig. 1) (Nourshargh et al., 2010). By adapting their adhesion, spreading and crawling, leukocytes respond to forces exerted by ECs. As shown for human neutrophils, leukocytes show increased spreading and migrate slowly, but do so

more persistently on stiffer substrates (Oakes et al., 2009; Stroka and Aranda-Espinoza, 2009). Consequently, they migrate greater distances on stiffer substrates, which facilitates their search for sites that permit TEM. This suggests that leukocyte durotaxis is important for TEM. Leukocytes probe the surface of the substrate (i.e. ECs) and sense local EC stiffness through ventral filopodia or invadosome-like protrusions (Carman et al., 2007; Martinelli et al., 2014; Schaefer et al., 2014; Shulman et al., 2009). The mechanosensitive response of leukocytes to substrate stiffness is mediated by their integrins, which are force-generating mechanoreceptors (Roca-Cusachs et al., 2012; Ross et al., 2013). Integrin-derived forces are translated into (biochemical) signaling events within the leukocyte, including the recruitment of actin-binding or signaling proteins to the integrin cytoplasmic tail. This induces F-actin remodeling and myosin-based contraction. Leukocytes use actomyosin-based contraction (i.e. tugging) to sense and respond to substrate stiffness (Califano and Reinhart-King, 2010; Plotnikov et al., 2012; Plotnikov and Waterman, 2013). Neutrophils show greater tugging on stiffer surfaces that are coated with extracellular matrix (ECM) proteins or ECs (Oakes et al., 2009; Shin et al., 2010; Stroka et al., 2013). This promotes leukocyte adhesion, spreading and TEM. Leukocytes require an optimal substrate stiffness for maximal motility, which negatively correlates with the concentration of ECM proteins, such as fibronectin (Stroka and Aranda-Espinoza, 2009). As shown for human neutrophils adherent to inflamed human pulmonary microvascular ECs, adhesion increases leukocyte stiffening, which – in turn – further promotes adhesion and TEM, indicative for a positive-feedback loop (Wang et al., 2001). Indeed, pharmacological inhibition of F-actin dynamics in human neutrophils that impairs leukocyte stiffening reduces leukocyte TEM across inflamed human umbilical vein ECs (HUVECs) (Stroka et al., 2013).

Taken together, leukocytes sense mechanical forces that are induced by substrate (EC) stiffness through integrins; these are subsequently
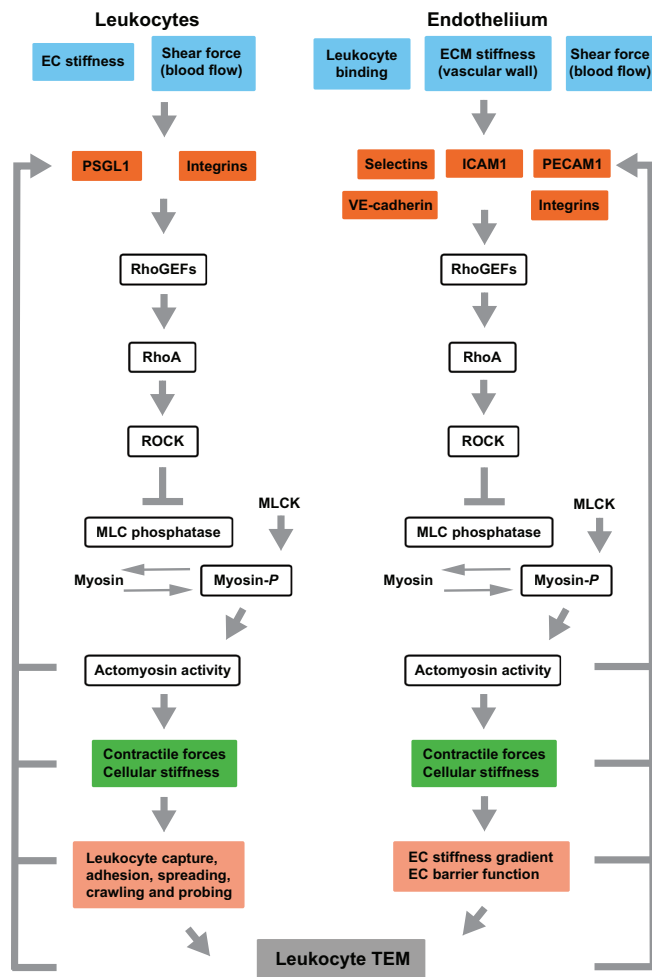
Fig. 2. The central role of the RhoA–ROCK–myosin pathway in leukocyte- and EC-mediated mechanotransduction. In leukocytes and ECs, mechanical forces (blue boxes) are sensed by various adhesion receptors (orange boxes). In both cell types, receptor activation and clustering induces RhoA-mediated ROCK activity, which inhibits the MLC phosphatase, resulting in myosin II activation (phosphorylation; myosin-*P*). Myosin II is also activated by MLCK. Increased actomyosin activity drives contractile forces and cellular stiffness that control leukocyte and EC signaling, which are both required for leukocyte TEM. Positive-feedback loops control the receptor function in an inside–out fashion.

transduced into cellular signaling, such as actin reorganization and generation of traction forces. This mechanotransduction allows leukocytes to change their shape from sphere-like to elongated and flattened, which promotes efficient adhesion, spreading and crawling.

Mechanosignaling in the uropod

The response of a leukocyte to EC stiffness by generating actomyosin-based forces is regulated in a spatiotemporal manner, as shown by traction force microscopy with human neutrophils (Shin et al., 2010). During TEM, large traction forces are generated in the uropod, the trailing end of a migrating leukocyte (Oakes et al., 2009; Shin et al., 2010; Stroka et al., 2013). Uropod retraction is required for efficient TEM of neutrophils, monocytes and T cells *in vitro* and *in vivo* (Hyun et al., 2012; Stroka et al., 2013). The mechanotransduction in the uropod is dependent on substrate stiffness because neutrophil crawling and TEM on stiff surfaces generate larger forces in the uropod than on softer surfaces (Oakes et al., 2009; Shin et al., 2010; Stroka et al., 2013). Human

neutrophils and T cells showed impaired uropod retraction during TEM across inflamed HUVECs when their contractile forces were blocked by inhibition of myosin II or its regulator myosin-light-chain-kinase (MLCK) (Hyun et al., 2012; Stroka et al., 2013). Similarly, genetic depletion of MLCK in murine monocytes impairs their TEM in an atherosclerotic mouse model (Sun et al., 2011). Moreover, polarity and chemotaxis of human neutrophils are dependent on myosin II and MLCK on soft and, even more so, on stiff surfaces (Shin et al., 2010). Localized activation of myosin II in the uropod of crawling and transmigrating leukocytes is induced by the small GTPase RhoA, which drives the spatiotemporal organization of contractile forces (Fig. 2) (Shin et al., 2010; Worthylake et al., 2001). This was confirmed by live-cell imaging using a RhoA fluorescence resonance energy transfer (FRET)-based biosensor in human T cells transmigrating across inflamed ECs (Heasman et al., 2010). Activated RhoA binds to its effector protein Rho-associated protein kinase (ROCK), which phosphorylates and inactivates the MLC phosphatase, thereby inhibiting the dephosphorylation of MLC (Fig. 2). This will activate myosin II and induce actomyosin-based contraction. Genetic depletion of RhoA in human T cells, and pharmacological inhibition of RhoA and ROCK in human monocytes inhibit uropod retraction and TEM across inflamed HUVECs (Heasman et al., 2010; Worthylake et al., 2001).

The Rho guanine exchange factor (GEF)-H1 is localized at the uropod of human T cells, where it activates RhoA, leading to ROCK–myosin-based contractility (Heasman et al., 2010). ROCK destabilizes microtubules, resulting in dissociation of microtubule-bound GEF-H1. Subsequently, GEF-H1 activates RhoA, representing a positive-feedback loop in the uropod to control TEM. Recently, GEF-H1 has been identified as a mechanosensitive RhoGTPase regulator. In response to applied tensional force on integrins or PECAM1, GEF-H1 activates RhoA to induce cellular stiffening (Collins et al., 2012; Guilluy et al., 2011). Whether GEF-H1 in the uropod has a similar function during TEM remains to be investigated.

In summary, the mechanotransduction in the leukocyte uropod is mediated by the RhoA–ROCK–myosin pathway, which results in localized actomyosin-based contractile forces (Fig. 2). These forces, which are dependent on EC stiffness, allow crawling and transmigrating leukocytes to retract their uropod for efficient TEM.

Mechanotransduction in ECs
Substrate stiffness controls endothelial spreading and barrier function

Like many other types of cell, ECs show enhanced adhesion and spreading on stiffer substrates. This is mediated by an increase in integrin clustering and in the size and number of integrin-based focal adhesions (Califano and Reinhart-King, 2010; van Geemen et al., 2014). Integrins sense the stiffness of the underlying substrate and translate mechanical forces into endothelial signaling to induce F-actin remodeling and myosin-based contraction (Oakes and Gardel, 2014; Roca-Cusachs et al., 2012; Ross et al., 2013). Human ECs on stiff matrices show increased numbers of focal adhesions and F-actin stress fibers, similar to what was observed in rigid human arteries (van Geemen et al., 2014). Conversely, human ECs on soft surfaces have less focal adhesions and more cortical F-actin, a similar phenotype as in human veins. Moreover, culturing ECs on stiff substrates promotes the generation of RhoA–ROCK–myosin-based contractile forces (Califano and Reinhart-King, 2010; Huynh et al., 2011; Krishnan et al., 2011; Stroka and Aranda-Espinoza, 2011a). The greatest ROCK activity and contractile forces are observed at the cell edges of a single
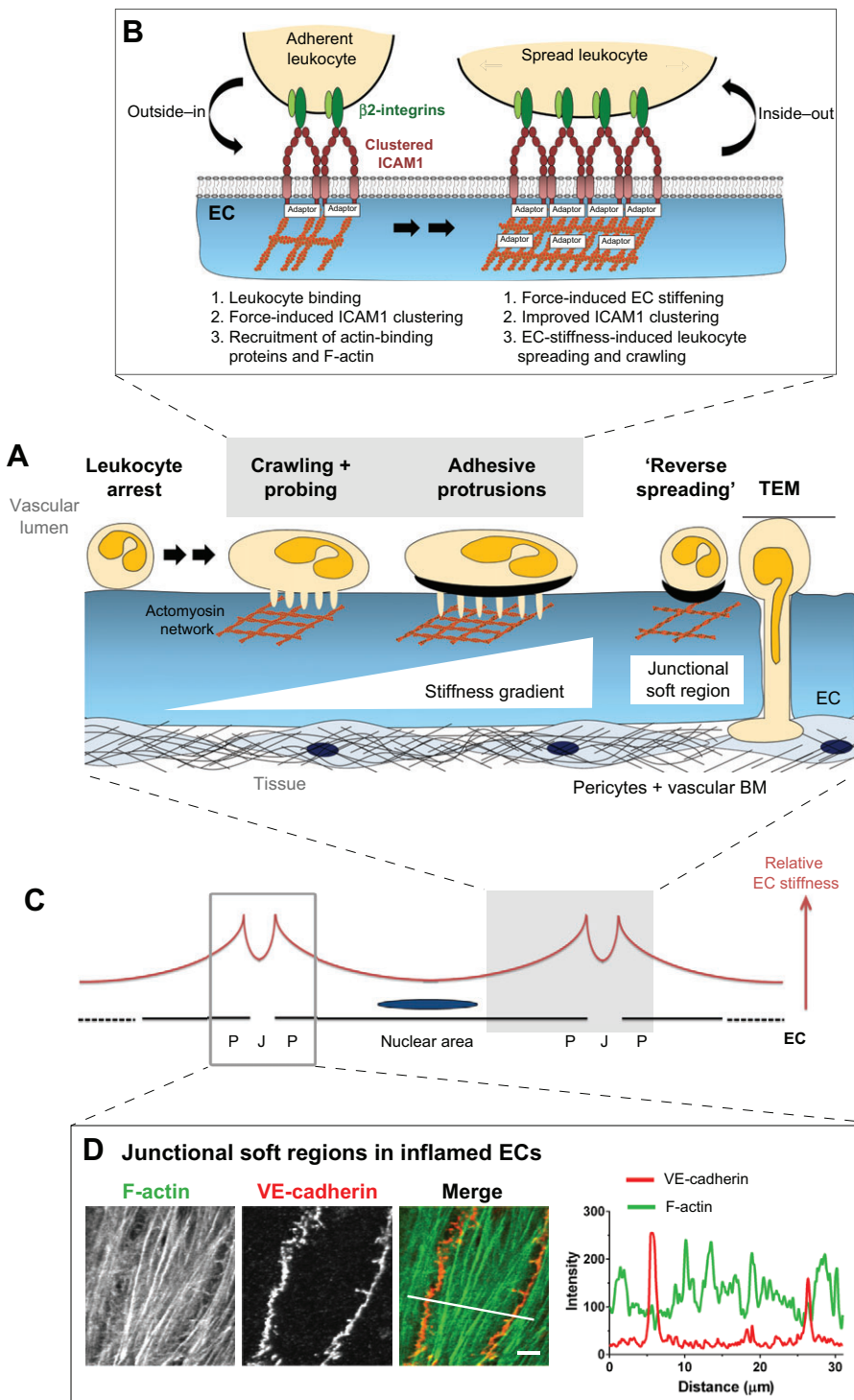
Fig. 3. A gradient in EC stiffness determines the search of leukocytes for sites that permit TEM. (A) Leukocytes sense EC stiffness through their ventral filopodia and crawl in a durotactic fashion in the direction of the periphery of a single EC. Adhesive protrusions formed by both ECs and leukocytes improve leukocyte adhesion. Prior to actual extravasation, leukocytes round up and protrude their leading edge into the endothelium at localized, soft junctional regions. EC stiffness is one of the factors that determine sites permitting TEM. (B) Binding of leukocyte integrins induces ICAM1 clustering in ECs. Clustering-induced recruitment of actin-binding proteins anchors ICAM1 to the F-actin cytoskeleton and promotes local EC stiffening (outside–in signaling). Local EC stiffening, in turn, further promotes ICAM1 clustering, which reinforces leukocyte adhesion and spreading (inside–out signaling). (C) Schematic representation of the stiffness gradient across a single EC within a monolayer (P, EC periphery; J, junction). (D) Immunofluorescence image of TNFα-treated HUVECs stained for F-actin and VE-cadherin; scale bar: 5 μm. The line scan (taken from the section indicated by the white line in the right image) indicates areas that are low in F-actin at narrow junctional regions, which suggest a reduced junctional stiffness that promotes 'reverse spreading' and leukocyte TEM. This is a modified version of an image that has been published previously (Schaefer et al., 2014).

cell or at cell–cell contacts in a monolayer (Califano and Reinhart-King, 2010; Krishnan et al., 2011; Rabodzey et al., 2008), which is indicative for the spatiotemporal regulation of subcellular forces.

In vivo, ECs form a stable monolayer, in which cell–cell contacts are mediated by adhesion receptors including VE-cadherin. Similar to integrins, VE-cadherin-mediated signaling is mechanosensitive (Heemskerk et al., 2014; Huveneers and de Rooij, 2013; Vestweber et al., 2014). Age-induced stiffening of the subendothelial matrix disrupts VE-cadherin-based cell–cell junctions and enhances the permeability of the endothelial monolayer in mouse thoracic aorta

(Huynh et al., 2011; Krishnan et al., 2011). This is determined by cell contractility because inflamed and non-inflamed HUVECs – as well as ECs from human brain microvasculature and bovine aortas – exert greater RhoA–ROCK–myosin-based contraction on stiffer surfaces, which destabilizes cell–cell junctions (Huynh et al., 2011; Krishnan et al., 2011; Stroka and Aranda-Espinoza, 2011b). Consequently, the cells form larger intercellular gaps, in particular upon stimulation with the barrier-disruptive agonist thrombin. These effects can be reversed through genetic and pharmacological inhibition of ROCK, as shown *in vitro* and in a mouse model

(Huynh et al., 2011; Krishnan et al., 2011). In line with this, blocking the RhoA–ROCK pathway by depletion of endothelial MCLK attenuates the barrier-reducing effects of thrombin *in vivo* (Sun et al., 2011).

Taken together, ECs respond to increased substrate stiffness by enhanced integrin-mediated adhesion and spreading, and generation of RhoA–ROCK–myosin-based contractile forces that control monolayer permeability and barrier function (Fig. 2). This mechanotransduction pathway is the same as that described for leukocytes, underscoring common force-dependent regulatory mechanisms in both cell types.

Substrate rigidity affects EC stiffness and leukocyte TEM
On ECs, different types of mechanical force are simultaneously exerted, such as blood flow, the mechanical properties of the vascular wall (i.e. ECM) and cell–cell interactions (Conway and Schwartz, 2013; Humphrey et al., 2014) that, together, drive vascular disease (Box 1). The stiffness of a complete, intact blood vessel is the same as that of the ECM of a vessel without any ECs (Peloquin et al., 2011), indicating that mechanical forces induced by substrate stiffness are directly reflected by the endothelium and transmitted to adherent leukocytes. ECM stiffening is induced by aging and inflammation (e.g. atherosclerosis), as shown by rigidity measurements of thoracic aortas of mice of different ages, and of murine and human atherosclerotic plaques (Chai et al., 2013; Huynh et al., 2011; Tracqui et al., 2011). *In vitro* studies confirm that culturing ECs on stiffer ECM substrates induces EC stiffening (Stroka and Aranda-

---

Box 1. Mechanotransduction in inflammatory disease

When discussing mechanotransduction and its relevance for vascular inflammation, it is important to discriminate localized versus systemic disease. Differences in blood flow due to bifurcations or stenosis may lead to local effects, such as the formation of atherosclerotic plaques or aneurysms. In addition to flow-induced signaling, the accompanying leukocyte adhesion will also trigger mechanotransduction in the vascular wall (see main text). In contrast to local events, systemic pathology is usually the result of increased blood pressure (i.e. hypertension), which can be associated with chronic (mild) inflammation, e.g. during aging (Jain et al., 2014).

The mechanical forces induced by blood flow, be it laminar or turbulent, are sensed and transduced by the vascular endothelium. Turbulent or discontinuous flow triggers various forms of mechanosignaling, most of which feed into the RhoA–ROCK-mediated activation of the myosin light chain kinase (MLCK) (Conway and Schwartz, 2013). This leads to increased actomyosin-based contraction and stiffening. This is important because stiffer areas in the vascular wall promote leukocyte adhesion and extravasation, and are thus prone to inflammation (Huynh et al., 2011). Beyond the endothelium, altered mechanical properties of the vascular wall, including fibronectin deposition, stiffening of the ECM or of smooth muscle cells, reduced NO production and local calcification, will also all further enhance EC stiffening and inflammation. From a clinical perspective, it is important to note that vascular (i.e. arterial) stiffening precedes hypertension (Kaess et al., 2012). Consequently, reduction of vascular wall stiffening by inhibition of the Rho–ROCK pathway reduces vascular inflammation as well as hypertension. The ROCK inhibitor Y27632 as well as (hydroxy)fasudil reduce blood pressure and aortic stiffness , in both animal models as well as in clinical trials, (Shi and Wei, 2013). Similarly, statins, which alter cholesterol metabolism with the consequent inhibition of Rho lipidation and -function, reduce arterial stiffness and (chronic) inflammation (Jain et al., 2014). Therefore, mechanotransduction is a key aspect of inflammatory disease, in particular in the vasculature, and its targeting appears to be a sensible therapeutic strategy.

Espinoza, 2011b). It is well established that areas of increased ECM and vascular stiffening, such as atherosclerotic plaques, are characterized by enhanced leukocyte extravasation (reviewed in (Döring et al., 2015). Furthermore, the extravasation of human neutrophils across inflamed HUVECs on stiff surfaces is stimulated as a consequence of enhanced endothelial myosin-based contractile forces that destabilize cell–cell contacts in the EC monolayer (Huynh et al., 2011; Stroka and Aranda-Espinoza, 2011b). Blocking these contractile forces by pharmacological inhibition of ROCK, MLCK or myosin II in inflamed HUVECs cultured on stiff surfaces normalizes the effects of increased substrate rigidity and, therefore, reduces neutrophil TEM.

Of note, the EC monolayer forms a heterogeneous surface for circulating leukocytes. Even a single EC within the monolayer shows a remarkable surface heterogeneity with regard to the distribution of, for instance, adhesion receptors. This results in the formation of preferred regions ('hot spots') for leukocyte TEM across the endothelium (Nourshargh and Alon, 2014). As we discuss here, substrate-induced mechanical forces determine EC stiffness in a spatiotemporal manner and ECs present a pre-existing stiffness gradient (a durotactic gradient) towards leukocytes, which may well be another factor that creates TEM hot spots (Fig. 3A). Atomic force microscopy (AFM) studies revealed that a single cell in a monolayer of HUVECs or bovine aortic ECs, either inflamed or non-inflamed, is stiffer at the cell periphery compared to the cell center and the nucleus (Sato et al., 2000; Schaefer et al., 2014; Stroka and Aranda-Espinoza, 2011a). However, it has also been shown that non-inflamed HUVECs have a softer cell periphery compared to their nucleus and that non-inflamed ECs from human pulmonary aortas show no stiffness gradient (Birukova et al., 2009; Mathur et al., 2001). These discrepancies might be due to the differences in cytokine stimulation, the cell type used, and experimental conditions, such as the size and geometry of the AFM probe.

Taken together, mechanical forces induced by age- and inflammation-mediated ECM stiffening promote EC stiffness, which impairs barrier function and stimulates leukocyte TEM (Fig. 2). Even a single EC can present an adherent leukocyte with a gradient in cellular stiffness that might contribute to the formation of leukocyte TEM hot spots.

EC stiffness during leukocyte TEM
Capture, rolling and arrest
Leukocyte TEM is initiated by capture and rolling of circulating leukocytes on the EC surface. This is mediated by transient interactions between endothelial E- and P-selectin and leukocyte P-selectin glycoprotein ligand 1 (PSGL-1), and the formation of leukocyte slings and tethers (Nourshargh and Alon, 2014; Sundd et al., 2012). The function of E- and P-selectin is regulated through shear force that is induced by blood flow (McEver and Zhu, 2010). High shear force initially stabilizes ligand binding (so-called catch bonds) and, thus, leukocyte contact; but as a maximum of binding events is reached and the force increases, the lifetime of these interactions are reduced (so-called slip bonds), which ensures efficient leukocyte rolling. Finally, slow rolling activates the leukocyte LFA-1 integrin and allows leukocytes to sense chemokine gradients on the EC surface that drive leukocyte arrest (Nourshargh and Alon, 2014).

Antibody- or leukocyte-induced clustering of selectins in inflamed HUVECs promotes the recruitment of proteins to the cytoplasmic region of the selectin molecules, including F-actin and the actin-binding proteins filamin and α-actinin (Lorenzon et al.,

1998; Yoshida et al., 1996), which control EC stiffening (Razinia et al., 2012; Schaefer et al., 2014). This recruitment induces a local increase in the density of the F-actin network at the selectin clusters, which is likely to result in local EC stiffening and, thus, provides an improved platform for anchoring of selectins to the F-actin network in order to resist shear force, similar to what has been described for integrins (Roca-Cusachs et al., 2012; Ross et al., 2013). By contrast, destabilization the endothelial F-actin network with cytochalasin B inhibits clustering und function of selectins, which is indicative for a positive-feedback loop (Wojciak-Stothard et al., 1999). These findings suggest that selectin function is regulated by shear force and EC stiffness in a cooperative fashion, which might promote leukocyte capture and rolling.

How leucocytes find sites that permit TEM

Following arrest, leukocytes adhere and crawl on the EC surface to search for sites that permit TEM. This requires a tight mechanosensitive signaling crosstalk between leukocytes and ECs, and is driven by different types of gradient. To scan the EC surface for sites that permit TEM, leukocytes use ventral filopodia or invadosome-like protrusions (Carman et al., 2007; Shulman et al., 2009). These F-actin-rich membrane protrusions are induced by shear force and endothelial chemokines. Leukocytes use these structures to sense EC stiffness (Martinelli et al., 2014; Schaefer et al., 2014) and chemokine vesicles that are located directly beneath the EC surface and are docked to F-actin fibers (Shulman et al., 2012). Although direct proof is still missing, EC stiffness might control the location of these vesicles, which would reflect a cooperation between different gradients (chemokines, cellular stiffness) to control leukocyte TEM.
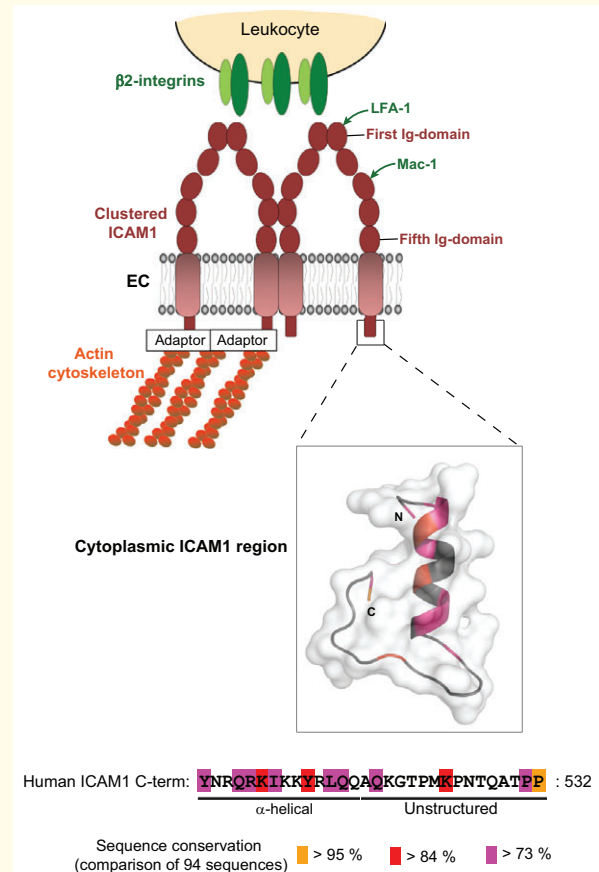
Leukocytes not only sense forces exerted by EC stiffness but their adhesion also induces forces in the endothelium and, therefore, alters mechanotransduction in ECs. This includes F-actin recruitment, generation of traction forces and local EC stiffening, as shown in inflamed HUVECs and human pulmonary microvascular ECs (Liu et al., 2010; Rabodzey et al., 2008; Wang et al., 2001). Pharmacological inhibition of actin dynamics reduces the stiffening response of human pulmonary microvascular ECs, indicating a positive-feedback loop (Wang et al., 2001). The highest traction forces and EC stiffness are measured in close proximity to adherent leukocytes (Liu et al., 2010).

Leukocyte adhesion and crawling is mediated by endothelial ICAM1 and ICAM2, which bind to leukocyte LFA-1 and Mac-1, as well as by endothelial VCAM1, a ligand for leukocyte VLA-4 (Halai et al., 2014; Heemskerk et al., 2014; Nourshagh and Alon, 2014). Leukocyte binding induces clustering of ICAM1 and VCAM1, which leads to the formation of higher-order clusters and nanodomains, thereby forming adhesive platforms and membrane protrusions that surround adherent leukocytes and support TEM (Barreiro et al., 2002; Carman and Springer, 2004; Phillipson et al., 2008; van Buul et al., 2007). Endothelial ICAM1 plays a central role in leukocyte extravasation because it is involved in leukocyte adhesion, crawling and TEM through the cell body (transcellular TEM) or junctions (paracellular TEM) (Heemskerk et al., 2014; Lyck and Enzmann, 2015; Nourshargh et al., 2010). ICAM1 consists of five extracellular domains, a transmembrane domain and a short cytoplasmic region (Box 2). Antibody binding or leukocyte adhesion induces ICAM1 clustering and, through its cytoplasmic region, downstream signaling in ECs to ensure successful leukocyte TEM. For instance, the intracellular region associates with actin-binding proteins that anchor ICAM1 to the F-actin cytoskeleton (Fig. 3B). Destabilization or stabilization of the actin network, respectively, blocks or promotes ICAM1 clustering in inflamed HUVECs, thereby

---

Box 2. A structural look at ICAM1

Endothelial ICAM1 is a mechanosensitive adhesion receptor involved in leukocyte TEM. Human ICAM1 contains five extracellular glycosylated Ig-like domains (482 residues), a transmembrane domain (22 residues) and a short cytoplasmic region (28 residues) without any binding motif (Lyck and Enzmann, 2015). ICAM1 forms a homodimer through the first and the fourth extracellular Ig-domain. The dimer has a higher affinity for integrin than the monomer (Chen et al., 2007). Leukocyte LFA-1 interacts with the first Ig-domain, whereas Mac-1 binds to the third domain. Integrin binding induces conformational changes in the Ig-domains, the transmembrane region and the cytoplasmic tail. These changes will affect both the extent of higher-order clustering and protein binding to the intracellular region (outside–in signaling), and vice versa (inside–out signaling).

ICAM1 clustering initiates the recruitment of many cytoplasmic proteins. Some of them, including filamin-B (280 kDa) and α-actinin-4 (100 kDa), interact directly with the cytoplasmic ICAM1 region and respond to forces by conformational changes (Ehrlicher et al., 2011; Schaefer et al., 2014). Because the 3D-structure of the cytoplasmic region of ICAM1 has not yet been published, we recently modelled this domain of human ICAM1 (Schaefer et al., 2014) (see Figure). Combined with a sequence alignment of 94 homologues from different species (Ashkenazy et al., 2010), the results suggest that the α-helical membrane-proximal part is highly conserved. In contrast, the distal region is less conserved and unstructured, as confirmed by using a database to predict disordered proteins (Oates et al., 2013). The α-helix is required for surface expression of ICAM1 and its clustering, whereas the unstructured region mediates adaptor binding (Oh et al., 2007; Schaefer et al., 2014). The intrinsically disordered region might become structured upon adaptor binding, possibly in an adaptor-specific manner (Schaefer et al., 2014; Wright and Dyson, 2014). This mechanism ensures the recognition of diverse binding partners and might reflect the spatiotemporal regulation of ICAM1 function. A similar mechanism was described for the cytoplasmic region of PECAM1 and of E-cadherin, whose endothelial homologue VE-cadherin also controls TEM (Huber et al., 2001; Paddock et al., 2011).

Human ICAM1 C-term: YNRQRKIKKYRLQQAQKGTPMKPNTQATPP : 532
α-helical   Unstructured

Sequence conservation
(comparison of 94 sequences)   > 95 %   > 84 %   > 73 %

regulating neutrophil TEM (Schaefer et al., 2014; van Buul et al., 2010). Furthermore, using beads coated with anti-ICAM1 antibodies to induce ICAM1 clustering in human pulmonary artery ECs was sufficient to increase cellular traction forces (Liu et al., 2010), indicating that ICAM1 is a force-sensing receptor that initiates mechanosensitive signaling events (Fig. 3B). These include recruitment of actin-binding and scaffolding proteins (e.g. α-actinin-4), increased formation of F-actin stress fibers, RhoA activity and ROCK–myosin-based contraction forces (Heemskerk et al., 2014). ICAM1 clustering induces force-dependent positive feedback-signaling in inflamed ECs, resulting in the amplification of ICAM1 signaling towards the F-actin cytoskeleton and improved leukocyte adhesion (Lessey-Morillon et al., 2014; Schaefer et al., 2014). Accordingly, the application of tension on ICAM1 in order to mimic leukocyte pulling forces further promotes RhoA activity, myosin-based contractile forces and EC stiffening (Fig. 2) (Lessey-Morillon et al., 2014). Tensional force activates the RhoGEF LARG in inflamed HUVECs and microvascular ECs, which in turn increases RhoA activity to induce cellular stiffening. This mechanotransduction stimulates neutrophil crawling, which is essential for their extravasation *in vitro* and *in vivo* (Phillipson et al., 2006; Schenkel et al., 2004). Together these studies clearly identify ICAM1 as a mechanosensitive receptor.

Recently, we have shown that ICAM1 forms mechanosensitive, functionally distinct complexes with α-actinin-4, filamin-B or cortactin that control, in an adaptor-specific manner, the neutrophil search for sites that permit TEM (Schaefer et al., 2014). Depletion of endothelial α-actinin-4 resulted in the most pronounced defect in neutrophil spreading and adhesion as it reduces ICAM1 clustering and EC stiffness. Our AFM studies demonstrated that α-actinin-4 and – to a lesser extent – cortactin but not filamin-B, regulate a cell-autonomous stiffness gradient from the nucleus (with low stiffness) to the cell periphery (showing high stiffness) in inflamed HUVECs (Schaefer et al., 2014). In line with these results, increased EC stiffness, such as in human and murine atherosclerotic plaques, in murine arteries (compared to veins) or in inflamed ECs on stiffer surfaces, stimulates expression of α-actinin-4 and its binding to ICAM1, thereby facilitating leukocyte TEM (Schaefer et al., 2014). Here, α-actinin-4 functions as a force-transmitting element that promotes maturation of the ICAM1 complex, similar to the role it has in integrin complexes (Roca-Cusachs et al., 2013a). These findings suggest that leukocytes are guided by the low-to-high EC stiffness gradient that exists within an individual EC towards the EC periphery (Fig. 3A,C). A local rigid surface ensures improved anchoring of the ICAM1 complex, which will resist and further promote pulling forces that are exerted through the leukocyte β2 integrins and, finally, promotes leukocyte adhesion, spreading and crawling (Fig. 3B).

Intriguingly, upon their durotactic guidance towards the periphery of a single EC and, thus, prior to actual extravasation, crawling leukocytes round up and protrude their leading edge towards the abluminal side of the endothelium (Manes and Pober, 2014; Schaefer et al., 2014). This 'reverse spreading' might be linked to a local reduction in the stiffness at junctional regions (Fig. 3A,C). Our immunofluorescence microscopy data, indeed, indicate that F-actin levels are relatively low in the narrow junctional regions of inflamed HUVECs (Fig. 3D), pointing to lesser EC stiffness, although we were unable to directly measure reduced junctional stiffness in our recent AFM work, probably owing to the experimental settings (Schaefer et al., 2014). We determined the stiffness of a single EC within a monolayer with AFM by using a 10-μm bead, the same size as a neutrophil (Schaefer et al., 2014). In

agreement with this, another recent study indicated that high barrier function and EC stiffening are linked predominantly to transcellular T-cell migration, whereas destabilization of cell–cell contacts and reduced EC stiffness promote TEM through junctions (Martinelli et al., 2014). There, a 600–nm tip was used that mimics the leukocyte invadosome-like protrusion (see above) to measure EC stiffness in response to changes in endothelial barrier function. Thus, the authors suggest that leukocytes prefer to transmigrate at regions with the lowest F-actin density and stiffness because the resistance exerted by a rigid endothelial F-actin network, compared with a soft surface, impairs the invasion of invadosome-like protrusions of T cells, which reduces their ability to crawl along the ECs. Of note, it is known that the F-actin cytoskeleton generates tension at VE-cadherin-based junctions, which is necessary to maintain cell–cell contacts; however, too much force will destroy these contacts (Huveneers and de Rooij, 2013; Vestweber et al., 2014). A low F-actin density and concomitantly reduced EC stiffness might, therfore, not only stabilize cell–cell adhesion but also promote leukocyte TEM at junctional regions. Importantly, EC stiffness is only one of several components that determine the sites of TEM. We suggest that not all cell–cell junctions will be equally soft in an EC monolayer and, thus, that stiffer junctions or suboptimal density of EC adhesion molecules may restrict leukocyte TEM.

Together, these studies show that leukocytes probe the EC surface to find sites that permit extravasation, which is at least partly determined by EC stiffness. We propose that leukocytes are guided by a durotactic gradient in the direction of the periphery of an individual EC (Fig. 3A,C). Soft junctional regions (of low F-actin density) allow leukocytes to round up there and to protrude their leading edge towards the abluminal side of the EC in order to transmigrate. Although the vast majority of leukocytes (70–90%) transmigrate in a paracellular fashion, both *in vitro* and *in vivo* (Vestweber et al., 2014; Woodfin et al., 2011) transcellular TEM does occur and could be facilitated through a heterogeneous EC surface with localized soft regions. Moreover, transcellular TEM is important for leukocyte TEM across brain vascular ECs, owing to their tight junctional contacts that restrict paracellular TEM (Carman, 2009; Engelhardt and Ransohoff, 2012).

TEM and pore closing

Compared to leukocyte adhesion, the actual extravasation step in paracellular or transcellular TEM induces three-fold higher traction forces in the EC, which are propagated to adjacent ECs as shown in HUVECs and human pulmonary arterial ECs (Liu et al., 2010; Rabodzey et al., 2008). Leukocyte guidance to junctions is not only mediated by endothelial ICAM1 and VE-cadherin, but also by a number of other adhesion molecules, including PECAM1, ICAM2, CD99, ESAM and JAM proteins (Fig. 1), whose role in leukocyte TEM has been well established (Nourshargh and Alon, 2014; Sullivan and Muller, 2014; Vestweber et al., 2014). In contrast to VE-cadherin and ICAM1, however, less is known regarding their role in mechanotransduction. Recently, PECAM1 has been identified as a mechanosensor for shear force and shown to mediate flow-induced RhoA activity, cytoskeletal remodeling and stiffening in ECs (Collins et al., 2012, 2014; Privratsky and Newman, 2014; Tzima et al., 2005). Given the role of PECAM1 in leukocyte TEM and flow-induced EC mechanotransduction, and the relevance for shear force in TEM, PECAM1 might well be involved in EC mechanosignaling during TEM. However, this remains to be investigated. *In vivo* studies showed that PECAM1, ICAM2 and JAM-A function in a sequential manner to promote neutrophil extravasation (Woodfin et al., 2009). It will be interesting

to investigate how EC stiffness regulates the crosstalk between these receptors.

Closure of the endothelial pore after the leukocyte has passed is necessary to maintain the vascular barrier (Vestweber et al., 2014). A recent study suggests that ventral lamellipodia generated from pre-existing actin filaments close these small, micrometer-scale pores (Martinelli et al., 2013). Barrier disruption is recognized by the endothelium as the localized loss of isometric tension and force unloading that induces F-actin remodeling, which depends on Rac1, Arp2/3 and reactive oxygen species, and the formation of membrane protrusions in order to close the pore. Different types of primary human EC have been shown to use such a loss-of-tension-based mechanism to restore their barrier function (Martinelli et al., 2013; Mooren et al., 2014).

Taken together, extravasation of leukocyte induces high traction forces and stiffening in ECs, underscoring the importance of cellular forces and stiffness to regulate leukocyte breaching of the endothelial barrier. A variety of endothelial adhesion molecules regulate TEM, particularly through the paracellular route (Fig. 1). Although, so far, only ICAM1, PECAM1 and VE-cadherin have been identified as mechanosensitive receptors, it is clear that the endothelium also senses force changes to initiate closure of transmigratory pores, further reinforcing the concept of an interplay between stiffness, and forces created and sensed in leukocytes and ECs to guide TEM.

Conclusions and perspectives

An increasing number of studies have shown that the mechanosensitive response of leukocytes and ECs to substrate stiffness drives leukocyte TEM across the vascular endothelium. Mechanotransduction in both ECs and leukocytes includes the RhoA–ROCK–actomyosin pathway (Fig. 2). Leukocytes respond to mechanical forces exerted by EC stiffness, which is in turn determined by the stiffness of the underlying substrate. A combination of chemotactic, haptotactic and durotactic gradients might ensure efficient leukocyte trafficking and TEM. *In vivo*, the endothelium is surrounded by a heterogeneous distribution of pericytes (Fig. 1) that, indirectly, determine the guidance of leukocytes to sites that permit TEM (Ayres-Sander et al., 2013; Proebstl et al., 2012; Stark et al., 2013). Future work is required to show whether EC stiffness, maybe in a tissue-specific manner, is affected by this heterogeneity.

The individual steps of leukocyte TEM that are mediated by distinct endothelial adhesion (mechano-) receptors, such as ICAM1, PECAM1 and VE-cadherin, are differently regulated by EC stiffness (Fig. 1), and the molecular mechanisms controlling mechanosensitive complexes and adaptor-specific functions are only beginning to be elucidated. It will be interesting to investigate how other adhesion receptors and their crosstalk are regulated by EC stiffness to ensure the spatiotemporal regulation of leukocyte guidance on and across the endothelium. FRET-based tension sensors (Chew et al., 2002; Conway et al., 2013; Meng and Sachs, 2011; Nakamura et al., 2014) might enable researchers to elucidate force-dependent protein interactions within ECs in time and space, and to monitor the generation and propagation of EC stiffness during leukocyte TEM in live cells. However, to translate any *in vitro* findings into the *in vivo* environment, for example by using tension sensors *in vivo*, will be a great challenge of future studies. A detailed understanding of force-induced molecular mechanisms will not only increase our knowledge of stimulus-, tissue- and leukocyte-specific regulation of TEM, but may also help to discover new therapeutic targets against inflammatory disease.

References
Ashkenazy, H., Erez, E., Martz, E., Pupko, T. and Ben-Tal, N. (2010). ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res.* **38**, W529-W533.

Ayres-Sander, C. E., Lauridsen, H., Maier, C. L., Sava, P., Pober, J. S. and Gonzalez, A. L. (2013). Transendothelial migration enables subsequent transmigration of neutrophils through underlying pericytes. *PLoS. ONE* **8**, e60025.

Barreiro, O., Yanez-Mo, M., Serrador, J. M., Montoya, M. C., Vicente-Manzanares, M., Tejedor, R., Furthmayr, H. and Sanchez-Madrid, F. (2002). Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. *J. Cell Biol.* **157**, 1233-1245.

Birukova, A. A., Arce, F. T., Moldobaeva, N., Dudek, S. M., Garcia, J. G. N., Lal, R. and Birukov, K. G. (2009). Endothelial permeability is controlled by spatially defined cytoskeletal mechanics: atomic force microscopy force mapping of pulmonary endothelial monolayer. *Nanomedicine* **5**, 30-41.

Bravo-Cordero, J. J., Hodgson, L. and Condeelis, J. (2012). Directed cell invasion and migration during metastasis. *Curr. Opin. Cell Biol.* **24**, 277-283.

Califano, J. P. and Reinhart-King, C. A. (2010). Substrate stiffness and cell area predict cellular traction stresses in single cells and cells in contact. *Cell. Mol. Bioeng.* **3**, 68-75.

Carman, C. V. (2009). Mechanisms for transcellular diapedesis: probing and pathfinding by 'invadosome-like protrusions'. *J. Cell Sci.* **122**, 3025-3035.

Carman, C. V. and Springer, T. A. (2004). A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J. Cell Biol.* **167**, 377-388.

Carman, C. V., Sage, P. T., Sciuto, T. E., de la Fuente, M. A., Geha, R. S., Ochs, H. D., Dvorak, H. F., Dvorak, A. M. and Springer, T. A. (2007). Transcellular diapedesis is initiated by invasive podosomes. *Immunity* **26**, 784-797.

Chai, C.-K., Akyildiz, A. C., Speelman, L., Gijsen, F. J. H., Oomens, C. W. J., van Sambeek, M. R. H. M., van der Lugt, A. and Baaijens, F. P. T. (2013). Local axial compressive mechanical properties of human carotid atherosclerotic plaques-characterisation by indentation test and inverse finite element analysis. *J. Biomech.* **46**, 1759-1766.

Chen, X., Kim, T. D., Carman, C. V., Mi, L.-Z., Song, G. and Springer, T. A. (2007). Structural plasticity in Ig superfamily domain 4 of ICAM-1 mediates cell surface dimerization. *Proc. Natl. Acad. Sci. USA* **104**, 15358-15363.

Chew, T.-L., Wolf, W. A., Gallagher, P. J., Matsumura, F. and Chisholm, R. L. (2002). A fluorescent resonant energy transfer-based biosensor reveals transient and regional myosin light chain kinase activation in lamella and cleavage furrows. *J. Cell Biol.* **156**, 543-553.

Collins, C., Guilluy, C., Welch, C., O'Brien, E. T., Hahn, K., Superfine, R., Burridge, K. and Tzima, E. (2012). Localized tensional forces on PECAM-1 elicit a global mechanotransduction response via the integrin-RhoA pathway. *Curr. Biol.* **22**, 2087-2094.

Collins, C., Osborne, L. D., Guilluy, C., Chen, Z., O'Brien, E. T., III, Reader, J. S., Burridge, K., Superfine, R. and Tzima, E. (2014). Haemodynamic and extracellular matrix cues regulate the mechanical phenotype and stiffness of aortic endothelial cells. *Nat. Commun.* **5**, 3984.

Conway, D. E. and Schwartz, M. A. (2013). Flow-dependent cellular mechanotransduction in atherosclerosis. *J. Cell Sci.* **126**, 5101-5109.

Conway, D. E., Breckenridge, M. T., Hinde, E., Gratton, E., Chen, C. S. and Schwartz, M. A. (2013). Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. *Curr. Biol.* **23**, 1024-1030.

Döring, Y., Drechsler, M., Soehnlein, O. and Weber, C. (2015). Neutrophils in atherosclerosis: from mice to man. *Arterioscler. Thromb. Vasc. Biol.* **35**, 288-295.

Ehrlicher, A. J., Nakamura, F., Hartwig, J. H., Weitz, D. A. and Stossel, T. P. (2011). Mechanical strain in actin networks regulates FilGAP and integrin binding to filamin A. *Nature* **478**, 260-263.

Engelhardt, B. and Ransohoff, R. M. (2012). Capture, crawl, cross: the T cell code to breach the blood-brain barriers. *Trends Immunol.* **33**, 579-589.

Fels, J., Jeggle, P., Liashkovich, I., Peters, W. and Oberleithner, H. (2014). Nanomechanics of vascular endothelium. *Cell Tissue Res.* **355**, 727-737.

Guilluy, C., Swaminathan, V., Garcia-Mata, R., O'Brien, E. T., Superfine, R. and Burridge, K. (2011). The Rho GEFs LARG and GEF-H1 regulate the mechanical response to force on integrins. *Nat. Cell Biol.* **13**, 722-727.

Halai, K., Whiteford, J., Ma, B., Nourshargh, S. and Woodfin, A. (2014). ICAM-2 facilitates luminal interactions between neutrophils and endothelial cells in vivo. *J. Cell Sci.* **127**, 620-629.

Heasman, S. J., Carlin, L. M., Cox, S., Ng, T. and Ridley, A. J. (2010). Coordinated RhoA signaling at the leading edge and uropod is required for T cell transendothelial migration. *J. Cell Biol.* **190**, 553-563.

Heemskerk, N., van Rijssel, J. and van Buul, J. D. (2014). Rho-GTPase signaling in leukocyte extravasation: an endothelial point of view. *Cell Adh. Migr.* **8**, 67-75.

Huber, A. H., Stewart, D. B., Laurents, D. V., Nelson, W. J. and Weis, W. I. (2001). The cadherin cytoplasmic domain is unstructured in the absence of beta-catenin. A possible mechanism for regulating cadherin turnover. *J. Biol. Chem.* **276**, 12301-12309.

Humphrey, J. D., Dufresne, E. R. and Schwartz, M. A. (2014). Mechanotransduction and extracellular matrix homeostasis. *Nat. Rev. Mol. Cell Biol.* **15**, 802-812.

Huveneers, S. and de Rooij, J. (2013). Mechanosensitive systems at the cadherin-F-actin interface. *J. Cell Sci.* **126**, 403-413.

Huynh, J., Nishimura, N., Rana, K., Peloquin, J. M., Califano, J. P., Montague, C. R., King, M. R., Schaffer, C. B. and Reinhart-King, C. A. (2011). Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. *Sci. Transl. Med.* **3**, 112ra122.

Hyun, Y.-M., Sumagin, R., Sarangi, P. P., Lomakina, E., Overstreet, M. G., Baker, C. M., Fowell, D. J., Waugh, R. E., Sarelius, I. H. and Kim, M. (2012). Uropod elongation is a common final step in leukocyte extravasation through inflamed vessels. *J. Exp. Med.* **209**, 1349-1362.

Jain, S., Khera, R., Corrales-Medina, V. F., Townsend, R. R. and Chirinos, J. A. (2014). "Inflammation and arterial stiffness in humans". *Atherosclerosis* **237**, 381-390.

Kaess, B. M., Rong, J., Larson, M. G., Hamburg, N. M., Vita, J. A., Levy, D., Benjamin, E. J., Vasan, R. S. and Mitchell, G. F. (2012). Aortic stiffness, blood pressure progression, and incident hypertension. *JAMA* **308**, 875-881.

Krishnan, R., Klumpers, D. D., Park, C. Y., Rajendran, K., Trepat, X., van Bezu, J., van Hinsbergh, V. W. M., Carman, C. V., Brain, J. D., Fredberg, J. J. et al. (2011). Substrate stiffening promotes endothelial monolayer disruption through enhanced physical forces. *Am. J. Physiol. Cell Physiol.* **300**, C146-C154.

Lessey-Morillon, E. C., Osborne, L. D., Monaghan-Benson, E., Guilluy, C., O'Brien, E. T., Superfine, R. and Burridge, K. (2014). The RhoA guanine nucleotide exchange factor, LARG, mediates ICAM-1-dependent mechanotransduction in endothelial cells to stimulate transendothelial migration. *J. Immunol.* **192**, 3390-3398.

Lim, J. and Hotchin, N. A. (2012). Signalling mechanisms of the leukocyte integrin alphaMbeta2: current and future perspectives. *Biol. Cell* **104**, 631-640.

Liu, Z., Sniadecki, N. J. and Chen, C. S. (2010). Mechanical forces in endothelial cells during firm adhesion and early transmigration of human monocytes. *Cell. Mol. Bioeng.* **3**, 50-59.

Lorenzon, P., Vecile, E., Nardon, E., Ferrero, E., Harlan, J. M., Tedesco, F. and Dobrina, A. (1998). Endothelial cell E- and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. *J. Cell Biol.* **142**, 1381-1391.

Lyck, R. and Enzmann, G. (2015). The physiological roles of ICAM-1 and ICAM-2 in neutrophil migration into tissues. *Curr. Opin. Hematol.* **22**, 53-59.

Manes, T. D. and Pober, J. S. (2014). Polarized granzyme release is required for antigen-driven transendothelial migration of human effector memory CD4 T cells. *J. Immunol.* **193**, 5809-5815.

Martinelli, R., Kamei, M., Sage, P. T., Massol, R., Varghese, L., Sciuto, T., Toporsian, M., Dvorak, A. M., Kirchhausen, T., Springer, T. A. et al. (2013). Release of cellular tension signals self-restorative ventral lamellipodia to heal barrier micro-wounds. *J. Cell Biol.* **201**, 449-465.

Martinelli, R., Zeiger, A. S., Whitfield, M., Sciuto, T. E., Dvorak, A., Van Vliet, K. J., Greenwood, J. and Carman, C. V. (2014). Probing the biomechanical contribution of the endothelium to lymphocyte migration: diapedesis by the path of least resistance. *J. Cell Sci.* **127**, 3720-3734.

Mathur, A. B., Collinsworth, A. M., Reichert, W. M., Kraus, W. E. and Truskey, G. A. (2001). Endothelial, cardiac muscle and skeletal muscle exhibit different viscous and elastic properties as determined by atomic force microscopy. *J. Biomech.* **34**, 1545-1553.

McEver, R. P. and Zhu, C. (2010). Rolling cell adhesion. *Annu. Rev. Cell Dev. Biol.* **26**, 363-396.

Meng, F. and Sachs, F. (2011). Visualizing dynamic cytoplasmic forces with a compliance-matched FRET sensor. *J. Cell Sci.* **124**, 261-269.

Merlot, S. and Firtel, R. A. (2003). Leading the way: directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J. Cell Sci.* **116**, 3471-3478.

Mooren, O. L., Li, J., Nawas, J. and Cooper, J. A. (2014). Endothelial cells use dynamic actin to facilitate lymphocyte transendothelial migration and maintain the monolayer barrier. *Mol. Biol. Cell* **25**, 4115-4129.

Nakamura, F., Song, M., Hartwig, J. H. and Stossel, T. P. (2014). Documentation and localization of force-mediated filamin A domain perturbations in moving cells. *Nat. Commun.* **5**, 4656.

Nourshargh, S. and Alon, R. (2014). Leukocyte migration into inflamed tissues. *Immunity* **41**, 694-707.

Nourshargh, S., Hordijk, P. L. and Sixt, M. (2010). Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. *Nat. Rev. Mol. Cell Biol.* **11**, 366-378.

Oakes, P. W. and Gardel, M. L. (2014). Stressing the limits of focal adhesion mechanosensitivity. *Curr. Opin. Cell Biol.* **30**, 68-73.

Oakes, P. W., Patel, D. C., Morin, N. A., Zitterbart, D. P., Fabry, B., Reichner, J. S. and Tang, J. X. (2009). Neutrophil morphology and migration are affected by substrate elasticity. *Blood* **114**, 1387-1395.

Oates, M. E., Romero, P., Ishida, T., Ghalwash, M., Mizianty, M. J., Xue, B., Dosztanyi, Z., Uversky, V. N., Obradovic, Z., Kurgan, L. et al. (2013). D(2)P(2): database of disordered protein predictions. *Nucleic Acids Res.* **41**, D508-D516.

Oh, H.-M., Lee, S., Na, B.-R., Wee, H., Kim, S.-H., Choi, S.-C., Lee, K.-M. and Jun, C.-D. (2007). RKIKK motif in the intracellular domain is critical for spatial and dynamic organization of ICAM-1: functional implication for the leukocyte adhesion and transmigration. *Mol. Biol. Cell* **18**, 2322-2335.

Paddock, C., Lytle, B. L., Peterson, F. C., Holyst, T., Newman, P. J., Volkman, B. F. and Newman, D. K. (2011). Residues within a lipid-associated segment of the PECAM-1 cytoplasmic domain are susceptible to inducible, sequential phosphorylation. *Blood* **117**, 6012-6023.

Peloquin, J., Huynh, J., Williams, R. M. and Reinhart-King, C. A. (2011). Indentation measurements of the subendothelial matrix in bovine carotid arteries. *J. Biomech.* **44**, 815-821.

Phillipson, M., Heit, B., Colarusso, P., Liu, L., Ballantyne, C. M. and Kubes, P. (2006). Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J. Exp. Med.* **203**, 2569-2575.

Phillipson, M., Kaur, J., Colarusso, P., Ballantyne, C. M. and Kubes, P. (2008). Endothelial domes encapsulate adherent neutrophils and minimize increases in vascular permeability in paracellular and transcellular emigration. *PLoS. ONE* **3**, e1649.

Plotnikov, S. V. and Waterman, C. M. (2013). Guiding cell migration by tugging. *Curr. Opin. Cell Biol.* **25**, 619-626.

Plotnikov, S. V., Pasapera, A. M., Sabass, B. and Waterman, C. M. (2012). Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration. *Cell* **151**, 1513-1527.

Privratsky, J. R. and Newman, P. J. (2014). PECAM-1: regulator of endothelial junctional integrity. *Cell Tissue Res.* **355**, 607-619.

Proebstl, D., Voisin, M.-B., Woodfin, A., Whiteford, J., D'Acquisto, F., Jones, G. E., Rowe, D. and Nourshargh, S. (2012). Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. *J. Exp. Med.* **209**, 1219-1234.

Rabodzey, A., Alcaide, P., Luscinskas, F. W. and Ladoux, B. (2008). Mechanical forces induced by the transendothelial migration of human neutrophils. *Biophys. J.* **95**, 1428-1438.

Razinia, Z., Mäkelä, T., Ylänne, J. and Calderwood, D. A. (2012). Filamins in mechanosensing and signaling. *Annu. Rev. Biophys.* **41**, 227-246.

Roca-Cusachs, P., Iskratsch, T. and Sheetz, M. P. (2012). Finding the weakest link: exploring integrin-mediated mechanical molecular pathways. *J. Cell Sci.* **125**, 3025-3038.

Roca-Cusachs, P., del Rio, A., Puklin-Faucher, E., Gauthier, N. C., Biais, N. and Sheetz, M. P. (2013a). Integrin-dependent force transmission to the extracellular matrix by alpha-actinin triggers adhesion maturation. *Proc. Natl. Acad. Sci. USA* **110**, E1361-E1370.

Roca-Cusachs, P., Sunyer, R. and Trepat, X. (2013b). Mechanical guidance of cell migration: lessons from chemotaxis. *Curr. Opin. Cell Biol.* **25**, 543-549.

Ross, T. D., Coon, B. G., Yun, S., Baeyens, N., Tanaka, K., Ouyang, M. and Schwartz, M. A. (2013). Integrins in mechanotransduction. *Curr. Opin. Cell Biol.* **25**, 613-618.

Sato, M., Nagayama, K., Kataoka, N., Sasaki, M. and Hane, K. (2000). Local mechanical properties measured by atomic force microscopy for cultured bovine endothelial cells exposed to shear stress. *J. Biomech.* **33**, 127-135.

Schaefer, A., te Riet, J., Ritz, K., Hoogenboezem, M., Anthony, E. C., Mul, F. P. J., de Vries, C. J., Daemen, M. J., Figdor, C. G., van Buul, J. D. et al. (2014). Actin-binding proteins differentially regulate endothelial cell stiffness, ICAM-1 function and neutrophil transmigration. *J. Cell Sci.* **127**, 4470-4482.

Schenkel, A. R., Mamdouh, Z. and Muller, W. A. (2004). Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat. Immunol.* **5**, 393-400.

Shi, J. and Wei, L. (2013). Rho kinases in cardiovascular physiology and pathophysiology: the effect of fasudil. *J. Cardiovasc. Pharmacol.* **62**, 341-354.

Shin, M. E., He, Y., Li, D., Na, S., Chowdhury, F., Poh, Y.-C., Collin, O., Su, P., de Lanerolle, P., Schwartz, M. A. et al. (2010). Spatiotemporal organization, regulation, and functions of tractions during neutrophil chemotaxis. *Blood* **116**, 3297-3310.

Shulman, Z., Shinder, V., Klein, E., Grabovsky, V., Yeger, O., Geron, E., Montresor, A., Bolomini-Vittori, M., Feigelson, S. W., Kirchhausen, T. et al. (2009). Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. *Immunity* **30**, 384-396.

Shulman, Z., Cohen, S. J., Roediger, B., Kalchenko, V., Jain, R., Grabovsky, V., Klein, E., Shinder, V., Stoler-Barak, L., Feigelson, S. W. et al. (2012).

Transendothelial migration of lymphocytes mediated by intraendothelial vesicle stores rather than by extracellular chemokine depots. *Nat. Immunol.* **13**, 67-76.

Stark, K., Eckart, A., Haidari, S., Tirniceriu, A., Lorenz, M., von Brühl, M.-L., Gärtner, F., Khandoga, A. G., Legate, K. R., Pless, R. et al. (2013). Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs. *Nat. Immunol.* **14**, 41-51.

Stroka, K. M. and Aranda-Espinoza, H. (2009). Neutrophils display biphasic relationship between migration and substrate stiffness. *Cell Motil. Cytoskeleton* **66**, 328-341.

Stroka, K. M. and Aranda-Espinoza, H. (2011a). Effects of morphology vs. cell-cell interactions on endothelial cell stiffness. *Cell. Mol. Bioeng.* **4**, 9-27.

Stroka, K. M. and Aranda-Espinoza, H. (2011b). Endothelial cell substrate stiffness influences neutrophil transmigration via myosin light chain kinase-dependent cell contraction. *Blood* **118**, 1632-1640.

Stroka, K. M., Hayenga, H. N. and Aranda-Espinoza, H. (2013). Human neutrophil cytoskeletal dynamics and contractility actively contribute to trans-endothelial migration. *PLoS. ONE* **8**, e61377.

Sullivan, D. P. and Muller, W. A. (2014). Neutrophil and monocyte recruitment by PECAM, CD99, and other molecules via the LBRC. *Semin. Immunopathol.* **36**, 193-209.

Sun, C., Wu, M. H. and Yuan, S. Y. (2011). Nonmuscle myosin light-chain kinase deficiency attenuates atherosclerosis in apolipoprotein E-deficient mice via reduced endothelial barrier dysfunction and monocyte migration. *Circulation* **124**, 48-57.

Sundd, P., Gutierrez, E., Koltsova, E. K., Kuwano, Y., Fukuda, S., Pospieszalska, M. K., Groisman, A. and Ley, K. (2012). 'Slings' enable neutrophil rolling at high shear. *Nature* **488**, 399-403.

Tracqui, P., Broisat, A., Toczek, J., Mesnier, N., Ohayon, J. and Riou, L. (2011). Mapping elasticity moduli of atherosclerotic plaque in situ via atomic force microscopy. *J. Struct. Biol.* **174**, 115-123.

Tseng, Y., Kole, T. P., Lee, J. S. H., Fedorov, E., Almo, S. C., Schafer, B. W. and Wirtz, D. (2005). How actin crosslinking and bundling proteins cooperate to generate an enhanced cell mechanical response. *Biochem. Biophys. Res. Commun.* **334**, 183-192.

Tzima, E., Irani-Tehrani, M., Kiosses, W. B., Dejana, E., Schultz, D. A., Engelhardt, B., Cao, G., DeLisser, H. and Schwartz, M. A. (2005). A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* **437**, 426-431.

van Buul, J. D. and Hordijk, P. L. (2009). Endothelial adapter proteins in leukocyte transmigration. *Thromb. Haemost.* **101**, 649-655.

van Buul, J. D., Allingham, M. J., Samson, T., Meller, J., Boulter, E., Garcia-Mata, R. and Burridge, K. (2007). RhoG regulates endothelial apical cup assembly downstream from ICAM1 engagement and is involved in leukocyte trans-endothelial migration. *J. Cell Biol.* **178**, 1279-1293.

van Buul, J. D., van Rijssel, J., van Alphen, F. P. J., Hoogenboezem, M., Tol, S., Hoeben, K. A., van Marle, J., Mul, E. P. J. and Hordijk, P. L. (2010). Inside-out regulation of ICAM-1 dynamics in TNF-alpha-activated endothelium. *PLoS. ONE* **5**, e11336.

van Geemen, D., Smeets, M. W. J., van Stalborch, A.-M. D., Woerdeman, L. A. E., Daemen, M. J. A. P., Hordijk, P. L. and Huveneers, S. (2014). F-actin-anchored focal adhesions distinguish endothelial phenotypes of human arteries and veins. *Arterioscler. Thromb. Vasc. Biol.* **34**, 2059-2067.

Vestweber, D., Wessel, F. and Nottebaum, A. F. (2014). Similarities and differences in the regulation of leukocyte extravasation and vascular permeability. *Semin. Immunopathol.* **36**, 177-192.

Wang, Q., Chiang, E. T., Lim, M., Lai, J., Rogers, R., Janmey, P. A., Shepro, D. and Doerschuk, C. M. (2001). Changes in the biomechanical properties of neutrophils and endothelial cells during adhesion. *Blood* **97**, 660-668.

Wojciak-Stothard, B., Williams, L. and Ridley, A. J. (1999). Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. *J. Cell Biol.* **145**, 1293-1307.

Woodfin, A., Voisin, M.-B., Imhof, B. A., Dejana, E., Engelhardt, B. and Nourshargh, S. (2009). Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. *Blood* **113**, 6246-6257.

Woodfin, A., Voisin, M.-B., Beyrau, M., Colom, B., Caille, D., Diapouli, F.-M., Nash, G. B., Chavakis, T., Albelda, S. M., Rainger, G. E. et al. (2011). The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat. Immunol.* **12**, 761-769.

Worthylake, R. A., Lemoine, S., Watson, J. M. and Burridge, K. (2001). RhoA is required for monocyte tail retraction during transendothelial migration. *J. Cell Biol.* **154**, 147-160.

Wright, P. E. and Dyson, H. J. (2014). Intrinsically disordered proteins in cellular signalling and regulation. *Nat. Rev. Mol. Cell Biol.* **16**, 18-29.

Ye, F., Kim, C. and Ginsberg, M. H. (2011). Molecular mechanism of inside-out integrin regulation. *J. Thromb. Haemost.* **9** Suppl. 1, 20-25.

Yoshida, M., Westlin, W. F., Wang, N., Ingber, D. E., Rosenzweig, A., Resnick, N. and Gimbrone, M. A. Jr. (1996). Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton. *J. Cell Biol.* **133**, 445-455.