Leukocytes breach endothelial barriers by insertion of nuclear lobes and disassembly of endothelial actin filaments

Abstract:

The endothelial cytoskeleton is a barrier for leukocyte transendothelial migration (TEM). Mononuclear and polymorphonuclear leukocytes generate gaps of similar micron-scale size when squeezing through inflamed endothelial barriers in vitro and in vivo. To elucidate how leukocytes squeeze through these barriers, we co-tracked the endothelial actin filaments and leukocyte nuclei in real time. Nuclear squeezing involved either preexistent or de novo generated lobes inserted into the leukocyte lamellipodia. Leukocyte nuclei reversibly bent the endothelial actin stress fibers. Surprisingly, formation of both paracellular gaps and transcellular pores by squeezing leukocytes did not require Rho kinase or myosin II mediated endothelial contractility. Electron microscopic analysis suggested that nuclear squeezing displaced without condensing the endothelial actin filaments. Blocking endothelial actin turnover abolished leukocyte nuclear squeezing whereas increasing actin filament density did not. We propose that leukocyte nuclei must disassemble the thin endothelial actin filaments interlaced between endothelial stress fibers in order to complete TEM.
Summary

The endothelial cytoskeleton is a barrier for leukocyte transendothelial migration (TEM). Mononuclear and polymorphonuclear leukocytes generate gaps of similar micron-scale size when squeezing through inflamed endothelial barriers in vitro and in vivo. To elucidate how leukocytes squeeze through these barriers, we co-tracked the endothelial actin filaments and leukocyte nuclei in real time. Nuclear squeezing involved either preexistent or de novo generated lobes inserted into the leukocyte lamellipodia. Leukocyte nuclei reversibly bent the endothelial actin stress fibers. Surprisingly, formation of both paracellular gaps and transcellular pores by squeezing leukocytes did not require Rho kinase or myosin II mediated endothelial contractility. Electron microscopic analysis suggested that nuclear squeezing displaced without condensing the endothelial actin filaments. Blocking endothelial actin turnover abolished leukocyte nuclear squeezing whereas increasing actin filament density did not. We propose that leukocyte nuclei must disassemble the thin endothelial actin filaments interlaced between endothelial stress fibers in order to complete TEM.
Introduction

Leukocyte TEM involves the opening of micron wide gaps through paracellular junctions, and in some settings also though transcellular pores (Carman and Springer, 2008; Isac et al., 2011; Muller, 2011; Nourshargh and Alon, 2014; Vestweber, 2012). Formation of these gaps is initiated by leukocyte protrusions (Shulman et al., 2009), which develop into sub-endothelial pseudopodia, followed by squeezing of the leukocyte nucleus. This step was suggested to be co-regulated by leukocyte-driven actomyosins and endothelial signaling machineries (Cernuda-Morollon and Ridley, 2006; Nourshargh et al., 2010; van Buul et al., 2007), but the mechanisms used by leukocyte nuclei to generate micron scale gaps and pores are still obscure. Gap formation between endothelial cells was traditionally attributed to in situ triggering of endothelial contraction, activated by leukocyte adhesion, clustering of apical and junctional cell adhesion molecules (CAMs), and outside-in signaling to endothelial Rho GTPases and non-muscle myosin II (Garcia et al., 1998; Hixenbaugh et al., 1997; Mehta and Malik, 2006; Saito et al., 1998; Saito et al., 2002; Stroka and Aranda-Espinoza, 2011). However, in these studies the involvement of endothelial contraction in leukocyte TEM was primarily demonstrated with noninflamed endothelial cells, or with leukocyte TEM studied in the absence of physiological shear stresses.

In the present study we addressed how endothelial gaps are formed by squeezing leukocytes in shear flow assays using a model of inflamed microvascular endothelial cells, mimicking flat endothelial cells comprising most peripheral post capillary venules (Carman et al., 2007; Millan et al., 2006). Testing three prototypic leukocytes, namely, neutrophils, peripheral blood T
cells (PB T) and Th1/Tc1 effector T cells, we demonstrate that the TEM of all three types of leukocytes through either paracellular endothelial gaps or transcellular pores is unaltered by inhibition of endothelial actomyosin contractility. Furthermore, inhibition of a key driver of endothelial contractility, the Rho associated kinase, in mouse cremaster muscle post capillary venules did not disrupt neutrophil diapedesis in vivo. These findings collectively suggest that endothelial actomyosin contractility is not obligatory for leukocyte diapedesis across flat endothelial cells. Rather, we show that both T cells and neutrophils extensively displace the dense cytoskeletal networks of the endothelial cells through which they transmigrate and do so without rupturing the endothelial stress fibers. This endothelial remodeling does not involve condensation of displaced actin cytoskeleton, highlighting a checkpoint for leukocyte TEM: the disassembly of endothelial actin filaments by active squeezing of leukocyte nuclei.
Results

Neutrophils and T cells squeeze through continuous VE-cadherin junctions as efficiently as through discontinuous junctions

Discontinuous adherent junctions (AJs) are a subset of endothelial junctions linked by parallel actin stress fibers (McKenzie and Ridley, 2007). To address whether these discontinuous AJs facilitate leukocyte TEM, we exposed human dermal microvascular endothelial cells (HDMVECs), a prototype of a primary flat microvascular barrier, to either short (3 hr) or long (12 hr) stimulation with IL-1β (Figs. 1A, B). As expected, the inflamed endothelial cells contained thicker actin filaments (Fig. 1C). Whereas, AJs in HDMVECs subjected to the short stimulation remained continuous with parallel stress fibers, the AJs in HDMVECs undergoing long IL-1β stimulation acquired a discontinuous pattern, were coupled to perpendicular stress fibers (Fig. 1Ci), and were highly permeable to FITC-dextran (Fig. 1Cii). Surprisingly, despite their low permeability, continuous AJs and lower expression of CAMs and inflammatory chemokines (Barzilai et al., 2015), the briefly stimulated HDMVECs supported identical rates of neutrophil and T cell TEM to HDMVECs stimulated for long periods and enriched with discontinuous AJs (Fig. 1A, B). Thus, the integrity and permeability of AJs and the enrichment of contractile stress fibers perpendicular to these AJs are not necessary for optimal leukocyte TEM across inflamed HDMVECs.

T lymphocytes generate nuclear lobes and insert them into their lamellipodia prior to and during TEM

To delineate the dynamics of leukocyte nuclear squeezing during TEM, Hoechst
labeled neutrophils and T cells were imaged during active migration under shear flow conditions across IL-1β inflamed HDMVECs labeled with a non interfering fluorescent anti VE-cadherin mAb. The TEM of PB T in this system was driven by inclusion of exogenous CXCL12 as was previously reported with HUVECs (Rao et al., 2004; Shulman et al., 2009). This analysis allowed us to determine both the location and duration of nuclear passage. In addition, we used endothelial cells labeled with RFP Lifeact, a widely used F-actin probe (Riedl et al., 2008) to calculate the pore size generated by squeezing leukocytes (Suppl. Fig. 1A). As was found in other in vitro studies (Shaw et al., 2004), neutrophils crossed inflamed HDMVEC monolayers almost exclusively thorough paracellular routes. Consistent with their ability to respond to chemokines stored in intra-endothelial vesicle, primarily CCL2 (Barzilai et al., 2015; Shulman et al., 2012), 50% of effector T cells crossed HDMVECs through either transcellular or perijunctional routes (i.e., 1-3 microns away from paracellular junctions (Fig. 2A). Notably, 20% of transmigrating PB T cells also crossed this barrier via transcellular or perijunctional routes (Fig. 2A). Surprisingly, in spite of the different shape of their nuclei, PB T TEM progressed as rapidly as neutrophil TEM (Fig. 2B) suggesting that the uni-lobular shape of the T cell nuclei is not rate limiting for squeezing. The passage time of neutrophil and PB T nuclei was, however, significantly shorter compared to that of effector T cells (Fig. 2B). This slow kinetics was not due to the preference of effector lymphocytes for transcellular TEM routes, since the kinetics of nuclear squeezing and TEM of individual effector T cells was comparable for paracellular and transcellular routes (Suppl. Fig. 1B). Strikingly, in spite of their different nuclear shapes, different routes of TEM (Fig. 2A), and different kinetics
of nuclear squeezing (Fig. 2C), both the paracellular gaps generated by neutrophils and PB T cells and the transcellular pores generated by effector T cells were similar in size, with an average diameter of approximately 4 microns (Fig. 2D). Furthermore, all three types of leukocytes, once establishing thin subendothelial lamellipodia (< 1 micron high) underneath the endothelial layer (Cinamon et al., 2001), readily pushed their nuclei into these thin structures (Movies S1-3, Fig. 2E-G). Notably, all transmigrating T cells generated small nuclear lobes and inserted these structures into their subendothelial pseudopodia (Figs. 2F, G). While neutrophils inserted their pre-deformed nuclei into their lamellipodia within 15 secs after these pseudopodia were generated (Fig. 2E and Movie S1), transmigrating T cells took 3-fold longer to insert their de novo generated lobes into their subendothelial lamellipodia (Figs. 2F, G, J and Movies S2). Similar nuclear lobes were generated by crawling T cells as well as by T cells migrating underneath the endothelial monolayer (Suppl. Fig. 1C). Furthermore, lymphocytes crawling on surfaces coated with ICAM-1 alone or with chemokine rapidly inserted nuclear lobes into their lamellipodia (Suppl Fig. 1C, D and Movie S4, top). These results suggest that nuclear lobe formation is an inherent property of motile lymphocytes and does not depend on squeezing into the physically constricted space between the endothelial monolayer and the basement membrane.

To elucidate the mechanical regulation of nuclear lobe formation and insertion, we next examined the contribution of myosin II motor activity to lymphocyte lobe formation, insertion and squeezing during TEM. Myosin II based contractility can generate hydrostatic pressure at the back of the nuclei and propel these large organelles (Jacobelli et al., 2013). Myosin II can also pull
the nucleus forward (Wu et al., 2014). Indeed, prolonged (30 min) T cell pretreatment with the myosin II motor inhibitor blebbistatin, with a short (< 10 min) endothelial exposure to the drug significantly delayed their TEM (Fig. 2H). On the other hand, a short co-exposure of T cells and endothelial cells to the myosin II inhibitor blebbistatin (< 10 min) had no effect on TEM kinetics (data not shown), and fully retained the majority of the endothelial stress fibers (Movies S5,6). Interestingly, the prolonged pretreatment of effector T cells with the myosin II inhibitor did not block their ability to form nuclear lobes (Movie S7) and did not affect lymphocyte generation of sub-endothelial lamellipodia (Figs. 2I). However, this myosin II inhibition delayed their nuclear entry to the lamellipodia (Fig. 2J), slowed nuclear squeezing (Fig. 2K and Movie S7) and consequently delayed completion of TEM (Fig. 2H, L). These findings suggest that transmigrating effector T cells use their own myosin II mediated contractility for optimal squeezing of their nuclei across the endothelial barrier.

We next tested how inhibition of myosin II motors in effector T cells affects their motility and nuclear properties during crawling on ICAM-1, which involves nuclear insertion into lamellipodia generated on a non constricted two dimensional (2D) surface (Suppl. Fig. 1Cii,iii and movie S4). As expected, myosin II inhibited T cells crawled much more slowly on the integrin ligand (Fig. 2M). Interestingly, these T cells normally generated lamellipodia (Movie S4) but less frequently inserted their nuclear lobes into these leading edges (Fig. 2N,O). Remarkably, nuclear lobe insertion into the leading edge of T cells crawling on ICAM-1 took place 5 fold faster than nuclear lobe entry into the sub-endothelial leading edge of transmigrating T cells (Fig. 2J,O). Taken together, effector T cells require myosin II motor activity for optimal insertion of their nuclei into their
lamellipodia both during TEM as well as during motility in a non constricted space.

**Inhibition of actomyosin contractility in endothelial cells does not interfere with lymphocyte or neutrophil TEM in vitro or in vivo**

To address whether endothelial actomyosins also contribute to neutrophil and T cell squeezing through inflamed HDMVECs under shear flow, we first verified that inhibition of endothelial actomyosin activity with either the Rho kinase inhibitor, Y-27632, or the myosin II inhibitor, blebbistatin, abolished thrombin induced contractility of these cells (Fig. 3A and Movies S8-10). As expected, both inhibitors also readily disassembled the abundant stress fibers of these ECs and induced occasional actin rich ruffles, but junction integrity remained largely intact (Fig 3B). Surprisingly, however, neutrophils, PB T, and effector T cells all transmigrated normally through either Y-27632 or blebbistatin pretreated IL-1β inflamed HDMVECs (Fig. 3C). Similar results were obtained with TNFα-inflamed HDMVECs and HUVECs (data not shown). Since, leukocytes were only shortly exposed to the inhibitors in the flow chamber (< 10 min), while the endothelial actomyosins were the primary target for these agents, our results suggest that leukocytes can successfully squeeze their nuclei through paracellular gaps and transcellular pores independently of endothelial contractility. Furthermore, when both endothelial and leukocyte myosin II activities were simultaneously blocked by prolonged blebbistatin pretreatment, the gaps generated by these leukocytes were not reduced in size (data not shown) suggesting that leukocyte and endothelial contractility do not collaborate in gap opening.
To validate these intriguing results in vivo, we next tested if introduction of the Rho kinase inhibitor Y-27632 to the extravascular space of post capillary venules (PCV) affects the ability of these venules to support chemotactic neutrophil diapedesis. We used confocal intravital microscopy (IVM) to analyze the diapedesis of endogenous neutrophils towards an extravascular gradient of intramuscularly introduced CXCL1 (Suppl. Fig. 2A). The Rho kinase inhibitor did not reduce the overall accumulation and transmigration of neutrophils, which took place exclusively via paracellular routes (Fig. 3D). Since neutrophil squeezing through these vessels could be readily visualized by PECAM-1 labeling, we next probed the size of PECAM-1 gaps generated by transmigrating neutrophils (Suppl. Fig. 2B). The average diameter of endothelial gaps generated by squeezing neutrophils was similar to that measured by us in vitro (Figs. 2D, 3E). Interestingly, the diameter of these in vivo generated gaps was reduced by 25% in the presence of the Rho kinase inhibitor (Fig. 3E) suggesting a small combined contribution of endothelial and leukocyte contractility to optimal gap opening. As expected, the Rho kinase inhibitor also exerted adverse inhibitory effects on neutrophil motility, an effect that was most notable in the interstitial tissue (Fig. 3F and Suppl. Fig. 2C,D).

**Squeezing leukocytes readily bend but do not rupture endothelial stress fibers**

Ultrastructural cytoskeletal analysis of inflamed HDMVEC monolayers revealed a dense network of filaments interlaced between parallel stress fibers (Fig. 4A). We speculated that leukocyte nuclear squeezing may involve a rupture of the endothelial stress fibers and/or the condensation or disassembly of these
interlaced actin filaments. Tracking in real time the migration of Hoechst labeled neutrophils across inflamed HDMVECs expressing Lifeact-RFP, a widely used fluorescent F-actin probe (Riedl et al., 2008), we found that neutrophil squeezing through paracellular junctions did not induce any noticeable rupture of the endothelial stress fibers (Movies S11 and Fig. 4Bi). Instead, these actin bundles were readily bent by the squeezing leukocytes with a mean net separation of 1.7 +/- 0.8 micron (Fig. 4Bi, ii, Movie S11). Although effector T cells squeezed their nuclei 2-3 fold more slowly than neutrophils, both paracellular, perijunctional or transcellular squeezing of these T cells reversibly bent the endothelial stress fibers, without inducing their rupture (Figs. 4C-E, Movies S12-14 and Suppl. Fig. 3). Occasionally, both gaps and pores were associated with de novo generated actin rings (Fig. 4C,E Movies S12) which were followed by subsequent pore or gap sealing by actin polymerization (Fig. 4E). Interestingly, increased endothelial stress fiber density forced by ectopic expression of constitutively active mDIA1, a nucleator of unbranched actin filaments (Watanabe et al., 1999) (Fig. 4F) only minimally interfered with neutrophil TEM (Fig. 4Gi-iii). The same genetic manipulation, resulted, however, in a small delay in the TEM of effector T cells and slower crossing (Fig. 4Hi-iii), as well as in reduced rate of transcellular TEM (Fig. 4Hiv). Collectively, our data suggests that, leukocytes that squeeze their nuclei through endothelial paracellular gaps and transcellular pores readily bend the endothelial stress fibers and that these elastic filaments exert higher resistance for T cell TEM than for neutrophil TEM.

Squeezing effector T cells displace but do not condense the endothelial actin filaments interlaced between stress fibers
Since nuclear squeezing of all leukocytes tested occurred without rupture of the endothelial stress fibers, we reasoned that the endothelial gaps and pores generated by transmigrating leukocytes involve either a condensation or a disassembly of the interlaced actin filaments between these fibers (Fig. 4A). Since these thin filaments are undetectable by live Lifeact based fluorescence imaging, we developed a correlative videomicroscopy-SEM approach that is based on the membrane stripping approach described in Figure 4 combined with in situ cell fixation and stabilization of all actin filaments at defined time points of the shear flow assay (Figs. 5Ai-iii and Movie S15). This approach allowed us to follow subtle changes both in the density and integrity of the endothelial actin cortex imposed by actively transmigrating effector T cells, the main cell type that used both paracellular and transcellular TEM routes to cross our EC monolayers. Notably, the lymphocyte pseudopodia generated prior to nuclear squeezing, as well as the squeezing nuclei and the T cell uropod were all surrounded by variably sized actin rings, 150-200 nm in width during different steps of TEM (Fig 5B, D-F). In contrast, beads that were co-coated with anti VCAM-1 and anti ICAM-1 mAbs, and settled on the same ECs under low shear and avidly bound to the endothelial surface, were not surrounded by these thin rings (Fig. 5G) nor did they bend the endothelial stress fibers (Fig. 5G). Thus, mere clustering of ICAM-1 and VCAM-1 in the absence of active protrusion and squeezing is insufficient to remodel the EC cytoskeletal barrier. In contrast, and in support of the real time imaging results, the SEM based analysis indicated that effector T cells readily bent the endothelial stress fibers during both paracellular and transcellular squeezing (Fig. 5Aiii, C and Suppl. Fig. 4). Importantly, even during transcellular TEM, no global condensation of the
filamentous actin mesh was apparent within a 1-4 micron distance from the squeezing lymphocyte (Fig. 5 A-F, Suppl Fig. 4). Furthermore, the thin actin rings surrounding squeezing lymphocytes appeared to be de novo generated rather than a product of interlaced actin filaments which were displaced and condensed. Thus, transmigrating T cells do not rupture the endothelial actin stress fibers. Rather, they displace the interlaced actin filaments possibly via the rapid spontaneous disassembly of these short and thin filaments.

**Increased density of interlaced actin filaments in inflamed ECs delays but doesn't block leukocyte squeezing**

Since enrichment of stress fibers within inflamed ECs only moderately delayed T cell TEM and minimally inhibited neutrophil TEM (Fig. 4G, H) we next asked if enrichment of endothelial interlaced actin filaments (Fig. 4A) rather than of endothelial stress fibers can block leukocyte nuclear squeezing and TEM. To enrich this type of endothelial actin filament, we exposed IL-1β inflamed HDMVECs to brief thrombin stimulation in the presence of the Rho kinase inhibitor Y-27632, a manipulation that increased actin polymerization with a limited endothelial junction opening (Fig. 6A, B). Although Y-27632 abolished most endothelial stress fibers (Fig. 6A), the mean density of the thinner actin filaments in inflamed thrombin stimulated HDMVECs was elevated by 35% as detected at EM resolution (Fig. 6B). Importantly, the Rho kinase inhibitor exerted negligible effects on leukocyte TEM, even though the endothelial barrier and the leukocytes were exposed to this inhibitor for 30 and 10 min, respectively (Fig. 6C). Notably, the increased endothelial filament density minimally inhibited the TEM of neutrophils, and only moderately slowed down
the TEM of PB T and T effectors (Fig. 6D-F). These results suggest that leukocyte squeezing through the EC cytoskeletal barrier is differentially retarded by an increased density of the cortical endothelial actin cytoskeleton.

Inhibition of endothelial actin turnover totally blocks leukocyte TEM by arresting nuclear passage

Jasplakinolide is a cell permeable cyclo-depsipeptide with potent stabilizing activity on actin filaments due to its ability to inhibit the spontaneous (steady state) disassembly of actin networks by enhancing actin nucleation and inhibiting filament severing (Bubb et al., 2000; Cramer, 1999; Ponti et al., 2004). This drug also increases the thermal stability of individual filaments (Visegrady et al., 2004). Pretreatment of inflamed HDMVECs with this drug alone opened large gaps, precluding our ability to follow its effects on de novo gap and pore formation by squeezing leukocytes. We therefore introduced jasplakinolide in the presence of Y-27632 (Peng et al., 2011), and extensively washed out the jasplakinolide before introducing leukocytes into the flow chamber. Since under these conditions the ECs lost their stress fibers (Fig. 3B, Fig. 6A, B), we were able to follow the net effect of jasplakinolide on the turnover of the interlaced actin filaments (Fig. 6A, middle image) in isolation from its effects on endothelial stress fiber flexibility. Notably, jasplakinolide interchelation into the endothelial actin mesh resulted in total freezing of endothelial lamellipodia dynamics without any apparent increase in actin polymerization within these ruffles (Fig. 7A and Movies S16,17) or reduced endothelial junction integrity (Movie S18). This treatment may therefore have resulted in a total blockade of endothelial actin turnover and filament disassembly. Remarkably, this endothelial treatment
totally inhibited the TEM of all leukocytes tested (Fig. 7B-D, left panels, TEM category), but retained firm neutrophil and T lymphocyte adhesion under shear flow (Fig. 7B-D, left panels). Interestingly, this inhibition of endothelial actin turnover did not impair the ability of neutrophils and T effectors to generate subendothelial lamellipodia (Fig. 7B, D, right panels, grey bars) although it completely eliminated nuclear squeezing of these leukocytes (Fig. 7B, D, right panels, blue bars). Nevertheless, the same endothelial treatment blocked the ability of PB T to generate sub-endothelial lamellipodia even prior to nuclear squeezing (Fig. 7C). Since these lymphocytes were provided with exogenous chemokine, these results could not be attributed to defective secretion and presentation of endogenous endothelial chemokines. As these T cells used primarily paracellular routes of TEM (Fig. 2A), these results reflected their inability to protrude through the paracellular junctions of the endothelial barriers impaired in their actin turnover (Fig. 7C, right panel). The results in Figs. 4, 6 and 7 collectively suggest that interfering with endothelial actin turnover, which likely stabilizes the short interlaced actin filaments, totally abrogates the ability of neutrophils and T cells to squeeze their nuclei through the endothelial barrier. On the other hand, a mere increase in actin filament density does not recapitulate these effects (Fig. 4G, H, Fig. 6). Thus, leukocyte nuclear squeezing seems to proceed by pushing aside the elastic stress fibers (Fig. 4 B-E and Movies S11-14) and by disassembling the shorter the endothelial actin filaments interlaced in between these thick endothelial actin filaments (Figs 5B-G).

Discussion
Leukocyte TEM involves rapid squeezing of their bulky nuclei through relatively tight endothelial barriers (Muller, 2011). Studies both in vitro and in vivo have suggested that this process is initiated by leukocyte protrusions which merge into a large lamellipodia underneath the endothelial monolayer (Shaw et al., 2004; Shulman et al., 2009). Our present work indicates that leukocyte squeezing of the highly deformable leukocyte nucleus though paracellular endothelial gaps and transcellular pores (Suppl. Fig. 5) is initiated by either a preexistent nuclear lobe (in the case of neutrophils) or de novo forming lobes (in PB T and effector T cells). Once a “leader” lobe slides into the thin subendothelial leukocyte lamellipodia, it further lifts the endothelial cell (Suppl. Fig. 5, steps 3,4). This critical step is followed by the rapid squeezing of the bulky leukocyte nucleus either between neighboring endothelial cells or within transcellular pores (Suppl. Fig. 5 step 4).

It was widely believed that squeezing leukocytes drive deformations of the endothelial cytoskeleton during endothelial gap generation which are dependent on endothelial contractile machineries (Muller, 2011). These machineries were proposed to be triggered in situ by clustering of apical and junctional endothelial adhesion molecules, and outside-in signaling to endothelial Rho kinase and other myosin-II activating machineries (Garcia et al., 1998; Saito et al., 1998; Stroka and Aranda-Espinoza, 2011). Testing this hypothesis on a flat microvascular endothelial barrier reminiscent of inflamed peripheral post capillary venules (Carman et al., 2007; Shulman et al., 2012) together with our in vivo study of neutrophil diapedesis across these vessels, we report an alternative mechanism of endothelial actin remodeling controlled by the active squeezing of the leukocyte nucleus. Based on real time analysis of
endothelial stress fibers and correlative light and electron microscopy of the interlaced actin filaments surrounding these thick actin bundles, we propose that at least three endothelial cytoskeletal remodeling steps are involved in leukocyte squeezing and productive TEM in our experimental settings: a reversible bending of endothelial stress fibers; disassembly of the thin actin filaments interlaced in between these endothelial stress fibers; and formation of actin rings around both the protrusions, the nuclei and the uropod of the transmigrating leukocyte.

The actin cytoskeleton comprises the major barrier for leukocyte squeezing, as it is much more dense than the microtubule or the intermediate vimentin cytoskeleton (Fels et al., 2014; Prasain and Stevens, 2009). Surprisingly, in our endothelial cell model, increased density of either endothelial stress fibers or the actin filaments interlaced between these fibers slowed down T cell TEM much more than neutrophil TEM. Thus, the thicker actin stress fibers and the thinner actin filaments in their vicinity seem to comprise a permissive barrier for leukocyte nuclear squeezing. At the slow compression rate involved in leukocyte squeezing, the endothelial actin cytoskeleton deforms in a viscoelastic fashion and can undergo remodeling via its high turnover (Chaudhuri et al., 2007; Fritzsche et al., 2013; Pravincumar et al., 2012). Indeed, steady state actin disassembly rates fall within the range of 30 sec (Ofer et al., 2011; Watanabe and Mitchison, 2002) well within the time frame of leukocyte nuclear squeezing.

Occupancy of ECs by adherent leukocytes also drives extensive actin polymerization around adherent and transmigrating leukocytes (Barreiro et al., 2002; Carman and Springer, 2004; Millan et al., 2006; van Buul et al., 2007;
Wang and Doerschuk, 2000). A recent work utilized a RhoA FRET sensor to probe RhoA activation during neutrophil and monocyte TEM. This study suggested that activation of endothelial RhoA by ICAM-1 occupied by these leukocytes drives the formation of contractile actin rich rings around transmigrating leukocytes (Heemskerk et al., 2016). Prolonged in vitro inhibition of endothelial RhoA activities resulted in larger pores generated by transmigrating neutrophils. Although we did not genetically silence endothelial RhoA, we found that boosting the activities of one of its two main effectors, mDIA1, yielded distinct outcomes on neutrophil and T cell TEM. Future studies should therefore address how individual silencing of this and other Rho family effectors differentially impact the dynamics and routes of TEM of these distinct leukocytes.

In our hands, in vivo inhibition of endothelial and leukocyte Rho kinase activities, although permissive for neutrophil TEM, resulted in somewhat smaller gaps. Endothelial RhoA via its Rho kinase and mDIA1 effectors seems to control the formation of contractile rings that restrain fluid leakage through gaps generated between the transmigrating leukocytes and their surrounding endothelial cells (Heemskerk et al., 2016). In addition to these assemblies, our scanning EM analysis reveals rings 150-200 nm in thickness not only around the squeezed nuclei of transmigrating T cells but also around their invasive pseudopodia, prior to nuclear squeezing. Following maximal gap or pore formation by the squeezed leukocyte nucleus, a much slower filling of the endothelial gap proceeds, possibly via activation of endothelial Rac (Stroka and Aranda-Espinoza, 2011), ventral lamellipodia (Martinelli et al., 2013), and lateral border vesicle recycling (Muller, 2011).
The nucleus is the largest cellular organelle and is mechanically stabilized by a constitutive network of laminar proteins (Friedl et al., 2011). Leukocyte nuclei are 50-100 fold softer than the nuclei of most non hematopoietic cells (Shin et al., 2013) due to their low expression of LaminA/C, a key regulator of nuclear lamina stiffness (Shin et al., 2013). Interestingly, these deformable nuclei are pushed to the lamellipodia of motile leukocytes independently of leukocyte crossing through endothelial barriers by an as yet unknown mechanism. While easily deformed (Shin et al., 2013), the leukocyte nuclei are probably sufficiently stiff to exert (outward) pushing forces on the endothelial cortical cytoskeleton such that it can get displaced and possibly disassembled independently of endothelial myosin II contractility. Similar force-mediated softening has been observed in actin networks growing under compressive load (Chaudhuri et al., 2007). Furthermore, during interstitial migration, neutrophils push their nuclear lobes forward and use their deformable nuclei to squeeze through discontinuities of collagen matrices (Voisin et al., 2010). In sharp contrast to the soft nuclei of neutrophils and T cells, the stiff nucleus of polarized fibroblasts moves rearwards, away from the future leading edge (Gomes et al., 2005). The stiff nuclei of these and other mesenchymal cells are believed to restrict cell squeezing (Friedl et al., 2011; Wolf et al., 2013). Our results suggest, however, that the nuclei of T cells readily deform even when these cells migrate on flat non constrained spaces. The high deformability of T cell and neutrophil nuclei may be similar to that of dendritic cell nuclei (Thiam et al., 2016) and can be beneficial not only for their diapedesis but also for subsequent invasion through low matrix regions within venular basement membrane (Voisin et al., 2010; Wang et al., 2006).
Experimental procedures

Reagents and antibodies

See Supplemental Experimental Procedures.

Cells

HDMVECs (C-12211; Promocell, Heidelberg, Germany) were grown in Promocell EC medium (C-22020), according to the supplier's instructions, and were used at passages 2-3. Human neutrophils and T cells and were isolated from citrate-anticoagulated whole blood of healthy donors by dextran sedimentation and density separation over Ficoll-Hypaque, as described (Grabovsky et al., 2000). Neutrophils were assayed up to 3 hours after purification. Peripheral blood T cells (PB T, >90% CD3+ T cells) were cultured for 16–18 h before experiments. For generation of effectors, isolated lymphocytes were seeded on plates coated with anti-CD3 (OKT3; Biolegend) and anti-CD28 (CD28.2; Biolegend) for 48 hrs, and then cultured for 9–12 d with IL-2, as previously described (Shulman and Alon, 2009). A day before the experiment, effector lymphocytes were washed and incubated overnight in fresh IL-2-containing medium. All in vitro experiments using human leukocytes were approved by the Institutional Review Board of the Rambam Medical Center, in accordance with the provisions of the Declaration of Helsinki.

Analysis of leukocyte migration under shear flow

Primary HDMVECs were plated at confluence on plastic- or glass-bottomed dishes spotted with fibronectin, and a day later, were stimulated for 3 h with IL-1β (2 ng/ml). Endothelial cell–coated plates were assembled in a flow chamber
(Shulman and Alon, 2009) and washed extensively. Leukocytes were perfused over the endothelial cell monolayer in binding medium (Hank's balanced-salt solution containing 2 mg/ml of BSA and 10 mM HEPES, pH 7.4, supplemented with CaCl\(_2\) and MgCl\(_2\)) for 40 s at 1.5 dyn/cm\(^2\), and were then subjected to a shear stress of 5 dyn/cm\(^2\) for 10 min. Images were acquired at a rate of four frames per minute using a fluorescence microscope (Olympos IX71 DeltaVision system, Applied Precision or Olympus IX83) equipped with 20x phase-contrast objective and motorized stage. For analysis of migratory phenotype, T cells accumulated in at least three fields of view (~60 cells per field) were individually tracked and categorized as described (Atarashi et al., 2005). For PB T migration, the endothelial monolayer was incubated with 0.5µg/ml CXCL12 for 5 minutes and washed prior to lymphocyte introduction. To determine TEM routes, HDMVECs were prelabeled with 0.5µg/ml anti-VE-cadherin (BV9, Biolegend) 15 min before perfusion of leukocytes. For inhibition of endothelial contractility, cytokine stimulated HDMVECs (alone or overlaid with CXCL12) were pretreated with 20µM Y-27632 or 50µM blebbistatin for 20 minutes, and inhibitors were kept in the binding media during the flow experiment. To arrest endothelial actin dynamics, HDMVECs were preincubated with 20µM Y-27632 for 20 minutes, assembled in a flow chamber and treated with a JY cocktail (1µM jasplakinolide, 20µM Y-27632 in binding medium) introduced into the flow chamber 5 minutes prior to leukocyte introduction. The monolayer was washed extensively with 10ml of binding media containing 20uM of Y-27632. For PB T migration, CXCL12 was included in the JY cocktail. To manipulate actin density, HDMVECs were pre-incubated with 20uM Y-27632 as above, and then stimulated with 5 units/ml of thrombin in the presence of Y-27632 for 3 mins.
Thrombin was washed and leukocytes were introduced in the presence of Y-27632. For inhibition of myosin-II activity T cells were pretreated with 50 μM blebbistatin for 30 min prior to perfusion on IL-1β pre-stimulated HDMVEC monolayer, and the inhibitor was kept in the binding media during the entire experiment. For analysis of leukocyte crawling on purified integrin ligands, Petri dishes were precoated with protein A (20 μg/ml) and ICAM-1–Fc (5-10 μg/ml) as described (Grabovsky et al., 2000). For analysis of nuclear lobe formation and insertion into lamellipodia of crawling T cells, stable leading edge was defined as one that persisted for more than 15 seconds. Recorded movies were analyzed offline with ImageJ (NIH) and Imaris (Bitplane, Oxford Instruments).

**Fluorescence microscopy of squeezing leukocytes and endothelial actin filaments**

Live cell fluorescence imaging of leukocyte TEM under shear flow conditions was performed using DeltaVision RT system (Applied Precision, Issaquah, WA), or Olympos IX83 (Olympus Tokyo, Japan). For live fluorescence imaging of leukocyte nuclei, cells were incubated for 5 minutes with 5μM Hoechst 33342 at 37°C. For actin imaging with the LifeAct probe, HDMVECs were infected with a lentivirus encoding LifeAct-RFP, and imaged by capturing three images at three z planes (0μm, 1.4μm and 2.8μm above the glass bottom of the flow chamber) at 15-20 second intervals. Image stacks were deconvoluted using cellSence software, and projected as maximum intensity over z. Gap and pore size generated by squeezing leukocytes were determined by displacement of endothelial LifeAct-RFP or of the cell permeable actin probe SiR-actin.
(Lukinavicius et al., 2014), introduced 16-20h prior to the experiment at a dose that did not interfere with leukocyte TEM dynamics.

**Statistical analysis**

All data are reported as mean values ± SD or SEM and were analyzed by Student’s t test. Group comparisons were deemed significant for two-tailed unpaired p values below 0.05.
Author Contributions
S.B. designed and performed most of the in vitro experiments, analyzed the data and contributed to the writing of the manuscript; S.Y. performed and analyzed major parts of the in vitro experiments; S.M. conducted mouse cremaster muscle experiments. F. R. performed the mDIA1 experiments. O.K performed SEM processing and analysis; L.S.B, performed in vitro experiments with HUVECs; O. G. assisted with image analysis. S.F. supervised parts of the experiments; A.Z. wrote parts of the discussion; S.N. supervised and contributed to the design and interpretation of the in vivo data. R.A. supervised all the experiments and wrote the manuscript.

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References


**Figure legends**

**Figure 1. Discontinuous adherence junctions are not required for leukocyte TEM**

(A, B) Migratory phenotype of neutrophils (A), or T effectors (B) interacting with HDMVECs at 0, 3 and 12 hours after IL-1β stimulation. The numbers of neutrophils and T cells which completed transmigration across HDMVECs pre-stimulated for either 3 or 12 hrs with IL-1β at the indicated time points are shown in Aii and Bii, respectively. Values represent the mean ± SD of three fields in each experimental group. The experiment shown is representative of three. (Ci) Immunostaining of VE-cadherin (green) and F-actin (red) in HDMVECs pre-stimulated with IL-1β for either 3 or 12 hrs. Bottom panels depict enlarged images of the areas indicated in the top panels. Scale bars= 15μm. (Cii) Relative permeability of HDMVECs pre-stimulated with IL-1β for either 0, 3 or 12 hrs to FITC-dextran (70 kDa). A representative experiment of 2. A detailed description of the migratory phenotypes analyzed is included in the Suppl. Material.

**Figure 2. T cell nuclei deform and generate de novo lobes while crossing endothelial barriers**

(A) Leukocyte migration across HDMVECs stimulated for 3hrs with IL-1β was imaged under shear flow as in Figure 1, and routes of TEM of individual leukocytes were determined in three independent experiments using inflamed monolayers prelabeled with a non blocking anti VE-cadherin 5 min before each experiment. (B) Neutrophils, PB T, or effector T cells were labeled with the Hoechst nuclear dye. Overall TEM kinetics of individual leukocytes crossing
HDMVECs stimulated for 3hrs with IL-1β as in Figure 1 was measured from first identification of leukocyte subendothelial leading edge to final uropod detachment from the apical endothelial aspect. (C) Nuclear passage kinetics from earliest identification of nuclear insertion underneath the endothelial monolayer. (D) Sizes of endothelial holes generated by the crossing leukocytes determined as described in the Experimental Procedures. As most holes were oval and not round, the average hole diameter is presented. (E) Serial images from a movie (Movie S1) depicting a representative Hoechst labeled neutrophil squeezing through a paracellular EC junction. The white arrow denotes a stable basolateral leading edge generated by the neutrophil underneath the EC monolayer. The white arrowhead denotes the time point of insertion of the first nuclear lobe. The white numbers in the right panel indicate individual nuclear lobes imaged underneath the plane of the EC monolayer. Time intervals are depicted in each image. Scale bars= 5µm. (F,G) Serial images of a representative effector T cell (F), or of a PB T cell (G), each labeled with Hoechst. Scale bars= 5µm. The contours of the leukocyte leading edges and their nuclei are outlined in each image in green and red respectively. Surface rendering of nuclear periphery kymographs of either the effector T or PB T cells are shown in the right corner of each respective series of images. (H-L) Effects of myosin-II blocking within effector T cells on nuclear insertion, squeezing and TEM under shear flow. T cells were pretreated with the myosin-II inhibitor blebbistatin for 30 min prior to perfusion on an IL-1β pre-stimulated HDMVEC monolayer. Values represent the mean ±SD of three fields in each experimental group. The results shown are from one experiment representative of 4. (H) Time course of TEM. (I) Percentages of adherent T cells that generated a stable
subendothelial leading edge (white bars) and successfully squeezed their nuclei and crossed the EC monolayer (blue bars). (J) The delay in nuclear entry to the subendothelial leading edge of individual T cells. (K) Duration of nuclear passage of individual T cells. (L) Duration of the entire transmigration of individual T cells. (M-O). Effects of myosin-II blocking in effector T cells interacting under shear flow with a substrate coated with high density ICAM-1. (M) Effects on T cell crawling velocity. (N) Effects on nuclear entry to the leading edge of individual crawling T cells. (O) Effects on the delay in nuclear insertion into the leading edge of individual crawling lymphocytes. The experiments shown in H-O are each representative of three. **P < 0.005 ***P < 0.001 by Student’s t-test.

**Figure 3. Endothelial cell contractility is not required for lymphocyte or neutrophil TEM**

(A) Effects of Y-27632 (20µM) and blebbistatin (50µM) on thrombin stimulated endothelial cell contractility. HDMVECs (prestimulated with 2ng/ml IL-1β for 3 hours) were incubated with each inhibitor for 20 min before addition of thrombin (5 units/ml). Representative ECs within a confluent monolayer were monitored for an additional 20 min by live videomicroscopy. The panel depicts the outline of individual cells (indicated by numbers) determined at 5 min intervals. See also the corresponding movies (Movies S8-10). Scale bar= 50 µm. (B) Effects of Y-27632 and blebbistatin pretreatments on the actin cytoskeleton of IL-1β stimulated HDMVECs. Actin filaments in the treated monolayers were visualized by phalloidin staining (red) together with VE-cadherin immunostaining (yellow). Scale bar= 20 µm. (C) Effects of Y-27632 or blebbistatin (Blebb.) on adhesive
and migratory phenotypes of neutrophils (left), PB T cells (middle) and T effectors (right). IL-1β stimulated HDMVECs were pretreated with each of the inhibitors as in A and B, and inhibitors were left in the medium during the TEM assay. These endothelial pretreatments did not affect the number of leukocytes initially accumulated on the monolayers under shear flow. Values represent the mean ± S.D. of three fields in each experimental group. Results shown are from a representative experiment of 3 (left), 4 (middle), or 6 (right). (D) Extravasation of EGFP neutrophils across cremasteric post capillary venules stained with anti-PECAM-1 mAb as induced by local administration of CXCL1 alone or in the presence of Y27632. Values are means from 3-5 different 3D fields of view, represented as cell numbers/mm³ regions ± SEM measured in 3-6 independent experiments. (E) Endothelial cell pore size generated by the transmigrating neutrophils studied in (D) was assessed by RFR-Lifeact imaging. Number of events quantified were n= 42 for neutrophil diapedesis induced by CXCL1 alone, and 30 in tissues treated with Y-27632 and CXCL1. P< 0.0001. (F) Representative confocal images of cremasteric venules (stained with anti-PECAM-1 mAb, red) and extravasating neutrophils (green) analyzed in D and E. Bar= 30 µm. For more details, please refer to Suppl. Methods.

**Figure 4. Leukocyte nuclei squeezed between and through endothelial cells bend endothelial stress fibers without rupturing these bundles**

(A) SEM image of a representative HDMVEC (pre-stimulated with 2ng/ml IL-1β for 3 hours) stripped of its plasma membrane. Note the parallel stress fibers (yellow arrowheads) and the interlaced filaments between them. Scale Bar= 1µm. (B-E) Real time tracking of leukocyte nuclei (labeled with Hoechst) and of
endothelial stress fibers and filaments labeled by Lifeact RFP. Serial images of movies of leukocytes transmigrating across IL-1β stimulated HDMVECs with indicated time points (seconds) elapsed from initial leukocyte protrusion into the subendothelial space. (Bi) Paracellular neutrophil squeezing. (Bii) Minimal and maximal separation of endothelial stress fibers (SF) prior to and during neutrophil squeezing. n=12 (C) Paracellular squeezing of a representative T effector. See Movie S12. (D) Perijunctional squeezing of an effector T cell. See Movie S13. (E) Transcellular squeezing of an effector T cell. See Movies S14. See an additional tracking of neutrophil squeezing in Movie S11. Scale Bars in B-E = 10µm. (F) Phalloidin staining of stress fibers in inflamed HDMVECs transfected with a GFP labeled constitutively activated mDIA1 (Green, cell 2) and a sham transfected EC (Green negative, cell 1). VE-cadherin staining is shown in purple. (G, H) Migratory phenotypes, TEM kinetics and crossing times of neutrophils (Gi-iii) or T effectors (Hi-iv) interacting under shear flow with IL-1β stimulated HDMVECs which were pretransfected either with the constitutively activated mDIA1 mutant (mDIA1 ΔN3), or control. The fractions of TEM routes taken by T cells directly interacting with mDIA1 vs. control cells are shown in Hiv. Values in G-H represent the mean ± SEM of 3-6 fields in each experimental group. In Hi * P < 0.03 and in Hiii * P < 0.02.

Figure 5. Effector T cell squeezing involves reversible disassembly of interlaced endothelial F-actin rather than filament condensation

Correlative video and SEM microscopy. Ai depicts successive images 15 seconds apart of a representative effector T cell (marked with an asterisk) during paracellular TEM across IL-1β stimulated HDMVECs under shear flow,
taken from Movie S15. At t= 4 min, the lipid extraction and cytoskeleton-stabilizing solution perfused into the flow chamber reached the video recorded sample. The fixed sample (which corresponds to the last successive image at the right) was further processed for SEM and individual images were correlated to individual video recorded fields of view. Aii depicts a SEM image of the field shown in Ai. The stripped effector T cell (marked by an asterisk and shaded in brown for clarity) corresponds to the lymphocyte highlighted in Ai. Aiii depicts an enlargement of the square in Aii. A bent endothelial actin bundle is indicated by the yellow arrowheads. (B-F) A similar correlative microscopy SEM approach was used to follow actin remodeling during distinct steps of video recorded transmigrating T cell. (B) Initial protrusion of an effector T cell sent into an endothelial cell. The three white arrows mark a thin ring surrounding the T cell pseudopodium. (C) An effector T cell sends a basolateral leading edge in between endothelial cells and bends a nearby actin stress fiber. The bent endothelial stress fiber is marked by the yellow arrowheads. (D) An effector T cell is squeezed (or squeezes) through an endothelial cell. Note the thick bundle, possibly an endothelial stress fiber, marked by the yellow arrowheads and the de novo generated ring marked by the two white arrows. (E) An effector T cell during its squeezing through an endothelial cell. (F) The uropod of a transmigrating T cell at the final stage of transcellular TEM. The arrows mark a de novo generated ring. (G) Lack of endothelial actin remodeling around a bead coated with anti ICAM-1 and VCAM-1 mAbs adhered under shear to inflamed HDMVECs for 5 min. Bars in Aiii and in B-G= 1 μm.
Figure 6. Increased density of endothelial actin networks delays leukocyte squeezing but does not block TEM

(A-F) HDMVECs were stimulated with IL-1β for 3 hours and then incubated with Y-27632 (20 min) followed by stimulation with thrombin (3 min). (A) SEM of the differentially stimulated inflamed endothelial samples subjected to membrane stripping as in Fig 4A 2 min post thrombin stimulation. Scale bar= 1µm. Note the thicker stress fibers and the thinner filaments interlaced in between these fibers in the control panel. (B) Densitometry analysis of 4 fields of view from the SEM analyzed endothelial samples shown in A. The fields analyzed in 8k magnification were 1000 µm² in area. (C) TEM kinetics of neutrophils (left), PB T (middle) and effector T cells (right) migrating over inflamed HDMVECs pretreated with Y-27632 or control. Endothelial cells were pretreated with 20µM Y-27632 for 20 minutes and the inhibitor was present during the migration assay. Values represent the mean ± SD of three fields in each experimental group. (D-F) Migratory phenotypes, TEM kinetics and crossing times of neutrophils (D), PB T cells (E), and effector T cells (F) interacting with inflamed HDMVECs treated with either Y-27632 alone or exposed to short thrombin stimulation as in A. Plots and crossing times of individual leukocytes crossing the treated ECs in the absence and the presence of thrombin are depicted in green and blue plots and dots, respectively. The numbers of leukocytes initially accumulated on the monolayers were not affected by thrombin. Results shown are representative of 4 experiments.
Figure 7. Inhibition of endothelial F-actin disassembly blocks leukocyte nuclear squeezing

(A) Effect of HDMVEC pretreatment with a cocktail of jasplakinolide and Y-27632 (JY cocktail) or with Y27632 alone on endothelial cytoskeletal remodeling. A single representative endothelial cell was monitored and the cell contours were determined at the indicated time points. Top and bottom panels depict phase contrast images and dynamic changes of EC contours before and after JY treatment, respectively. Scale Bars= 10µm. (B-D) Left panels depict adhesive and migratory phenotype of neutrophils (B), PB T (C), and T effectors (D) interacting with IL-1β stimulated HDMVECs pretreated with Y-27632 for 20 min followed by incubation with jasplakinolide for 5 minutes in the presence of Y27632. Jasplakinolide was removed by extensive washing prior to leukocyte introduction in the continued presence of Y27632. Right panels depict percentage of the corresponding adherent leukocytes that generated a stable basolateral leading edge (white bars) and successfully squeezed their nuclei and crossed the EC monolayer (blue bars) during the assay. Y-27632 was included throughout the assay to prevent EC collapse. Values represent the mean ± SD of at least three fields in each experimental group. Results shown are from one experiment representative of 3. The numbers of leukocytes initially accumulated on the differently treated monolayers were not affected by the jasplakinolide pretreatment.
Figure 1

Ai

Migratory phenotype (%) Resting 3h IL-1β 12h IL-1β

Ali

TEM (%) 3h IL-1β 12h IL-1β

Bi

Migratory phenotype (%) Resting 3h IL-1β 12h IL-1β

Bii

TEM (%) 3h IL-1β 12h IL-1β

Ci

F-actin/VE-cadherin/Nucleus

Cii

Fluorescence units

Resting 3h IL-1β 12h IL-1β

***

NS
Figure 3

A

Thrombin: - + + +
Inhibitor: - Y-27632 Blebbistatin

0 min 5 min 10 min 15 min 20 min

B

Carrier Y-27632 Blebbistatin

C

Neutrophils

Migratory phenotype (%)

Carrier Y-27632 Blebb.

PB T

Migratory phenotype (%)

Carrier Y-27362 Blebb.

Effectors

Migratory phenotype (%)

Carrier Y-27632 Blebb.

D

Average neutrophil extravasation (μm²)

Y-27632 - + + +
CXCL1 - - + +

E

Average PECAM pore size (μm)

Y-27632 - + + +
CXCL1 - - + +

F

Carrier + CXCL1 Y-27632 + CXCL1

Figure 3
Figure 4

A

Bi: 00:00 00:08 00:32 00:48 01:36 02:40

Bii: Minimum Maximum
0 1 2 3 4 5

Distance between the SFs

B

C

00:00 01:40 02:20 02:40 03:00 03:20 04:00 04:40

D

00:00 00:15 00:30 01:00 01:30 02:00 02:15

E

00:00 00:40 01:20 02:00 02:20 02:40 03:00

F

1 1 2 2

Gi: Migratory phenotype (%)
0 2 4 6 8

Gii: Control mDIA1 ΔN3

Giii: Entire TEM duration (sec)

Hi: Detach Arrest Crawl TEM

Hii: Migratory phenotype (%)

Hiii: Entire TEM duration (sec)

Hiv: Paracellular Transcellular

Figure 4
Figure 6

A. Control | Y-27632 | Y-27632 + Thrombin

B. Normalized actin density

C. Neutrophils

D. Neutrophils

E. PB T

F. Effectors
Supplemental Information

Supplemental Figures

A

-20sec
Maximal
+20sec

Transcellular
Paracellular

B

Delay in nuclear entry (sec)

Effectors

Nuclear passage (sec)

Effectors

Entire TIM duration (sec)

Effectors

Paracellular
Transcellular

Ci

Cii

Ciii

D

PB T

Effectors

Lobes per leading edge

Apical Basolateral

Lobes per leading edge

Apical Basolateral
**Figure S1** Related to Figure 2

**Kinetics of distinct steps of paracellular and transcellular TEM of effector T cells**

(A) Examples of transcellular and paracellular gaps generated during leukocyte transendothelial migration across IL-1β stimulated HDMVECs under shear flow, as detected by the displacement of the ectopically expressed lifeact-RFP probe. Images were taken 20 sec before and after the maximal displacement by the given squeezing T cell, shown in the middle panels of each row. Scale bars= 5µm

(B) Kinetics of distinct steps of TEM of effector T cells through either paracellular or transcellular routes. Green and red dots denote the kinetics of the indicated steps of paracellular (green) and transcellular (red) TEM completed by individual T effector cells. **Left panel:** Delay in nuclear entry to the basolateral leading edge of individual T cells. **Middle panel:** Duration of nuclear passage of individual T cells. **Right panel:** Duration of the entire transmigration of individual T cells. (C) T cell nuclei deform and generate de novo lobes while crawling on endothelial cells or on purified ICAM-1. (Ci) Effector T cells were labeled with the nuclear dye Hoechst and allowed to crawl over HDMVEC, stimulated for 3hrs with IL-1β. Serial images from a movie depicting a representative Hoechst labeled effector T cell crawling under shear stress of 5 dyn/cm². Time intervals in seconds are indicated on top of each image. (Cii) Serial images of a representative Hoechst labeled effector T cell crawling on immobilized ICAM-1 at a shear stress of 5 dyn/cm². (Ciii) Serial images of a representative Hoechst labeled PB T cell crawling on ICAM-1 co-immobilized with CXCL12. T cells were allowed to interact with the substrate under shear free conditions and then subjected to a shear stress of 2 dyn/cm². Scale bars= 10µm. D-F Effector T cells and PB T cells generate nuclear lobes at high frequency on adhesive surfaces independently of TEM. (D) Frequency of nuclear lobe formation per leading edge generated by effector T cells or by PB T cells crawling under shear flow on (apical) or below (basolateral) IL-1β inflamed HDMVEC monolayers. Each value represents a single Hoechst labeled cell tracked by videomicroscopy, as in C. (E) Frequency of nuclear lobe formation per leading edge generated by individual effector T cells crawling under shear flow on ICAM-1. (F) Frequency of nuclear lobe formation per leading edge generated by individual PB T cells crawling under shear flow on ICAM-1/CXCL12 substrates. The experiments analyzed in E and F were conducted as described in (C).
**Figure S2. Related to Figure 3**

Intravital microscopy protocol for testing the in vivo effects of Rho kinase inhibition on neutrophil diapedesis and interstitial motility in cremaster muscles

(A) Top line: the time course of the intra-scrotal injection of the anti-PECAM-1 mAb, chemokine and Rho kinase blocker (Y-27632). Images depict the viewing platform and time lapse images recorded by confocal microscopy. For more details, see Suppl. Experimental Procedures. (B) LysM-EGFP-Ki mice were anaesthetized with i.p. Ketamine (125mg/kg) and Xylazine (12.5mg/kg) and maintained with Ketamine/Xylazine via intra-muscular dosing during IVM. KC (300ng) +/- Y-27632 (50uM final concentration) + Alexa-fluor-555-labelled anti-PECAM-1 mAb were injected via the intrascrotal route.
(in a total volume of 400ul). After 30 minutes, the cremaster muscle was surgically exteriorized for analysis by IVM as previously detailed (Woodfin et al., 2011). (C,D) Effects of intra-muscular injection of the Rho kinase inhibitor on interstitial motility of extravasating neutrophils. Neutrophil motility was analyzed by intravital microscopy of cremaster muscles perfused continuously throughout the entire course of the intravital microscopy experiment with either Y-27632 or a carrier solution, as described in Fig. S2C. (C) Total distance (in microns) traveled by endogenous EGFP labeled neutrophils outside of blood vessels. *** P< 0.0001. (D) Net displacement of extravasating neutrophils in microns. *** P< 0.003. Results in C and D are the mean values +SEM of 16 neutrophils (without Rho inhibitor), and 24 neutrophils analyzed in the presence of the Rho kinase inhibitor.

Figure S3. Related to Figure 4
Endothelial cell actin stress fibers are not disrupted during tranendothelial migration
An image panel of movie S12 showing lifting of endothelial cell stress fibers by an effector T cell that has successfully crossed the endothelial monolayer and continues to crawl below the endothelial monolayer. The top row shows reconstituted images (extended focal imaging, EFI) obtained from the three series of RFP images depicted in the bottom 3 rows. Each row corresponds to images taken at the indicated focal planes at the corresponding time points depicted in the top row. The locations of each row in the Z direction i.e., Z=0µm, Z=1.5µm and Z=3.0µm are depicted. Note the dramatic displacement of actin stress fibers by the squeezing nucleus of the T cell (starting at t=0:02:47).
Figure S4. Related to Figure 5
SEM images of membrane stripped T cells and endothelial cells fixed during TEM
Positions of the transmigrating T cells were determined by correlative SEM videomicroscopy, as described in Figure 5. Each image depicts a representative effector T cell squeezed in between endothelial cells (left) or through cells (right) and bending and bending endothelial bundles (actin stress fibers). The bent endothelial bundles are indicated by the white arrows. Scale bars= 1µm.
Figure S5. Related to Figures 4-7
Leukocyte TEM across flat endothelial cells consists of six main steps
A proposed model for paracellular T cell TEM based on our findings. 1. Initial actin-filled leukocyte protrusions through ECs and adherens junctions. 2. A 200-500 nm thick sub-endothelial leading edge (lamellipodium) develops underneath the EC. 3. Nuclear deformation and insertion of an anterior lobe into the sub-endothelial leading edge. This step is associated with lifting of the EC over the inserted nuclear lobe. 4. A nucleus-guided widening of the paracellular gap or transcellular pore to 4 microns. Thin endothelial actin filaments interlaced between the endothelial stress fibers undergo disassembly (not shown). 5. Sealing of the EC gap or pore by actin polymerization steps within the endothelial cells. 6. Retraction of the leukocyte uropod from the apical endothelial compartment. The TEM step most sensitive to inhibition by endothelial pretreatment with jasplakinolide (jasplak.) is shown in pink. All steps are shared by neutrophils (not shown), though they use their preexistent nuclear lobes for squeezing. Transcellular TEM of T cells follow a similar series of steps, but both initial protrusion and nuclear squeezing take place via a transcellular pore rather than through a paracellular inter-endothelial gap. The enlarged squares in step 3 depict schematic actin assemblies from imaginary top views of an endothelial cell engaged by a transmigrating leukocyte based on our Lifeact and SEM images in Figs. 4 and 5. The right square depicts endothelial stress fibers parallel to the endothelial junctions and the short actin filaments interlaced in between these fibers. The left square depicts the same stress fibers bent but not ruptured by the squeezing leukocyte nucleus. In contrast, the interlaced actin filaments are disassembled (rather than pushed aside and condensed) as a consequence of the forces exerted by the leukocyte nucleus.
Supplemental Movie Legends

All in vitro experiments of leukocyte TEM were conducted under shear flow. Unless otherwise indicated, a constant shear stress value of 5 dyn/cm² was used throughout the following experiments.

**Movie S1 Related to Figure 2**
**Neutrophils rapidly insert their multilobular nuclei immediately after establishing a stable basolateral lamellipodium**

Time lapse video of a representative Hoechst labeled neutrophil crossing IL-1β-stimulated HDMVEC under shear flow. Phase contrast and fluorescence images were taken 4 sec apart. Elapsed time is designated as mm:ss. Bar, 5 µm.

**Movie S2 Related to Figure 2**
**Effector T cells inserting multiple nuclear lobes into their multiple basolateral lamellipodia**

Time lapse movie of a representative Hoechst labeled T cell crawling under shear flow on IL-1β-stimulated HDMVEC. DIC and fluorescence images were taken every 6.5 sec. Elapsed time is designated as mm:ss. Bar, 5 µm. Note that the first lobe sent out by the T cell retracted immediately upon contacting the endothelial nucleus (also labeled in Hoechst, top left).

**Movie S3 Related to Figure 2**
**Peripheral blood T cells push forward their nuclei during crawling and TEM through endothelial cells**

Time lapse video of a representative Hoechst labeled PB T cell crawling and crossing IL-1β-stimulated HDMVEC under shear flow. Transendothelial migration took place between 0:14 and 1:03 of the movie. Note the de novo lobes generated by the crawling and transmigrating lymphocyte. Phase contrast and fluorescence images were taken every 7 sec. Elapsed time is designated as mm:ss. Bar, 5 µm.

**Movie S4 Related to Figure 2**
**Effector T cells push forward their nuclei and insert nuclear lobes into their lamellipodia during crawling under shear flow on glass coated immobilized ICAM-1 even after blocking myosin II activity**

Phase contrast and fluorescence images of representative T cells allowed to adhere to the ICAM-1 coated substrate and then subjected to continuous shear flow (2 dyn/cm²). Cells were pretreated for 30 min with the DMSO carrier (top) or with blebbistatin (bottom). Elapsed time is designated as h:mm:ss. Bar, 5 µm.

**Movie S5 Related to Figure 3**
**Endothelial actin stress fibers of intact IL-1β-stimulated HDMVEC are stable throughout the imaging period**

A time lapse movie of a representative IL-1β stimulated endothelial cell expressing lifeact-RFP showing steady state turnover of actin stress fiber. The DMSO carrier was added to the media at time 0:00:00 as a control. Images were taken every 1 minute. Elapsed time is designated as h:mm:ss. Bar, 20 µm.

**Movie S6 Related to Figure 3**
**Endothelial cells actin stress fibers diminish during prolonged exposure to blebbistatin**

A time lapse movie of a representative IL-1β stimulated endothelial cell expressing lifeact-RFP showing the collapse of actin stress fibers as a function of exposure to blebbistatin. Blebbistatin was added to the media at time 0:00:00. Images were taken every 1 minute. Elapsed time is designated as h:mm:ss. Bar, 20 µm.

**Movie S7 Related to Figure 2**
**Blocking myosin II activity in effector T cells does not inhibit nuclear deformation and lobe formation**

A time lapse movie of a representative T cells prelabeled with Hoechst pretreated for 30 min with blebbistatin as in Figure 2 H-L, and allowed to adhere, crawl, and transmigrate through IL-1β-stimulated HDMVEC under shear flow. Elapsed time is designated as mm:ss. Bar, 5 µm.
**Movie S8 Related to Figure 3**
Time lapse movie of IL-1β stimulated HDMVEC treated with thrombin and recorded by phase contrast microscopy

Cells were stimulated with thrombin and their contraction was imaged by phase contrast microscopy. The contours of the individual endothelial cells are outlined in different colors every 5 minutes of the experiment. Elapsed time is designated as mm:ss. Bar, 20 µm.

**Movie S9 Related to Figure 3**
Time lapse movie of IL-1β stimulated HDMVEC pretreated with the Rho kinase inhibitor Y-27632 and stimulated with thrombin
The pretreated cells were stimulated with thrombin and their contraction was imaged by phase contrast microscopy. The contours of the individual endothelial cells are outlined in different colors every 5 minutes of the experiment. Elapsed time is designated as mm:ss. Bar, 20 µm.

**Movie S10 Related to Figure 3**
Time lapse movie of IL-1β stimulated HDMVEC pretreated with the myosin-II inhibitor blebbistatin and stimulated with thrombin
The pretreated cells were stimulated with thrombin and their contraction was imaged by phase contrast microscopy. The contours of the individual endothelial cells are outlined in different colors every 5 minutes of the experiment. Elapsed time is designated as mm:ss. Bar, 20 µm.

**Movie S11 Related to Figure 4**
**Transmigrating neutrophils do not rupture endothelial stress fibers during paracellular TEM**
A time lapse movie depicting Hoechst labeled neutrophils crossing under shear flow through paracellular gaps of IL-1β-stimulated HDMVEC expressing RFP-Lifeact. Note the strong Lifeact signal within the endothelial stress fibers and the actin ring formed around the squeezing neutrophils. Fluorescence images were taken 15 sec apart. Elapsed time is designated as mm:ss. Bar, 5 µm.

**Movie S12 Related to Figure 4**
**Transmigrating effector T cells do not rupture the endothelial stress fibers during paracellular TEM**
A time lapse movie depicting Hoechst labeled effector T cell crossing under shear flow through paracellular gaps of IL-1β-stimulated HDMVEC expressing RFP-Lifeact, a probe for filamentous actin. Note the strong Lifeact signal within the endothelial stress fibers. Phase contrast and fluorescence images were taken 20 sec apart. Elapsed time is designated as h:mm:ss. Bar, 10 µm. For more details see also Figure S7.

**Movie S13 Related to Figure 4**
**Transmigrating effector T cells cross inflamed endothelial cells at perijunctional sites without disrupting adjacent endothelial stress fibers**
A time lapse movie depicting a Hoechst labeled effector T cell crossing through an endothelial cell near a paracellular junction of IL-1β-stimulated HDMVEC expressing RFP-Lifeact. Note the strong Lifeact signal within the endothelial stress fibers of the left endothelial cell and their bending by the squeezing T cell. The right endothelial cell was not transfected in order to enable probing in real time the location of the edge of the two endothelial cells at their paracellular junction. DIC (green channel) and fluorescence images (T cell nucleus in blue; endothelial F-actin in red) of a time lapse movie. Images were taken 15 sec apart. Elapsed time is designated as mm:ss. Bar, 5 µm.

**Movie S14 Related to Figure 4**
**Stress fiber bending by the nucleus of a transmigrating effector T cell crossing transcellularly through inflamed HDMVECs**
A time lapse movie depicting a Hoechst labeled effector T cell crossing through a transcellular route of IL-1β-stimulated HDMVECs expressing RFP-Lifeact. Images were taken 20 sec apart. Elapsed time is designated as h:mm:ss. Bar, 5 µm.

**Movie S15 Related to Figure 5**
**Correlative videomicroscopy SEM**
A time lapse movie of effector T cells crawling and crossing IL-1β-stimulated HDMVECs under shear flow, through a paracellular route, recorded by phase contrast microscopy. Images were taken 15 sec apart. Elapsed time is designated as mm:ss. Bar, 5 µm. At the end of this movie the sample was
exposed to the lipid extraction and cytoskeleton-stabilizing solution perfused into the flow chamber as explained in the legend of Figure 5.

**Movie S16 Related to Figure 7**

**Effect of jasplakinolide perfusion over inflamed HDMVECs on steady state actin turnover and dynamics of spontaneous endothelial ruffles**

Time lapse movie showing phase contrast microscopy of an individual HDMVEC recorded shortly before and after introduction of Jasplakinolide containing medium at t=12:30. For more details, refer to the legend of Figure 7. Elapsed time is designated as mm:ss. Bar, 10 μm.

**Movie S17 Related to Figure 7**

**Effect of jasplakinolide perfusion over inflamed HDMVECs on actin density within endothelial ruffles**

Time lapse movie showing relative actin density imaged by RFP-Lifeact. Intensity is presented in a rainbow pattern (black to red). The peripheral segment of an inflamed HDMVEC was recorded shortly before and after introduction of jasplakinolide. The time at which jasplakinolide reached the flow chamber is indicated by the appearance in the time lapse movie of the red label “Jasp”. For more details, refer to the legend of Figure 7. Elapsed time is designated as mm:ss. Bar, 10 μm.

**Movie S18 Related to Figure 7**

**Effect of jasplakinolide perfusion over inflamed HDMVECs on integrity of paracellular junctions.**

Time lapse movie showing phase contrast images of a confluent monolayer of inflamed HDMVEC recorded shortly before and after introduction of jasplakinolide containing medium. Elapsed time is designated as mm:ss. Bar, 10 μm.
Supplemental Experimental Procedures

Reagents and antibodies
Recombinant human CXCL12, IL-1β and IL-2 were from PeproTech (Rocky Hill, NJ) and recombinant mouse CXCL1 was from Tocris Bioscience (Bristol, UK). Blebbistatin, bovine serum albumin (BSA), fibronectin, Hoechst 33342, polyethylene glycol, thrombin and TRITC-phalloidin, were from Sigma-Aldrich (St. Louis, MO). Jaspaklinolide and Y-27632 were purchased from Tocris Bioscience. The Sir-actin probe was purchased from Spirochrome (Stein am Rhein, Switzerland). Ca²⁺, Mg²⁺ free Hank’s balanced salt solution (HBSS) was from Gibco. Anti-CD3 (OKT3), anti-CD28 (CD28.2) and anti VE-cadherin (BV9) were purchased from Biolegend (San Diego, CA).

FITC-Dextran permeability assay
Permeability across the endothelial cell (HDMVEC) monolayer was measured using 70kDa FITC-Dextran. 10⁵ HDMVECs were seeded on fibronectin-coated 24-well transwells (Costar, Corning, 3.0µm, Polyester membrane) and grown for 60h before the assay. Cells were stimulated with IL-1β (2 ng/ml) for 3 and 12 hrs. 100µl of 1 mg/ml FITC-Dextran in HBSS containing 0.2% BSA was added to the upper chamber of the transwell and 600µl of FITC-Dextran free HBSS was added to the lower chamber. FITC-Dextran was allowed to diffuse from upper to the lower chamber for 60 minutes at 37°C. After 60 minutes, FITC-Dextran concentration in the lower chamber was measured using a fluorescence multi-well plate reader (TECAN) with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Categories of leukocyte adhesion, crawling and transmigration analyzed in vitro
Neutrophils or T cells were perfused over the EC monolayer at 0.75 dyn/cm² for 1 min (accumulation phase) and then left under constant shear flow (5 dyn/cm²) for 10 mins. Images were video recorded through a 20x phase contrast objective at 2 frame/s. For migratory phenotype analysis, accumulated leukocytes were individually tracked throughout the assay and categorized as described in (Cinamon et al., 2001). Any leukocyte that initially accumulated but detached during the 10 min exposure to constant flow was grouped in the detachment category. Any leukocyte which arrested and remained adherent at its original site of arrest without any lateral locomotion was grouped in the arrest category. Crawling leukocytes which moved at least 1 cell diameter (~10 microns) away from their initial site of arrest but failed to cross the endothelial monolayer during the entire assay period was grouped in the crawling category. All transmigrating leukocytes (with a TEM phenotype) either crawled before crossing the endothelium or crossed the endothelium at their original point of arrest. All different groups (termed migratory phenotypes) were calculated as fractions of leukocytes which originally accumulated during the first 1 min phase.

Intravital confocal microscopy (IVM)
All experiments were carried out under UK legislation, and protocols employed were approved by the Ethical Review Committee of Queen Mary University of London. Lys-EGFP-ki mice expressing GFP-labelled myeloid cells were anaesthetized with i.p. Ketamine (125mg/kg) and Xylazine (12.5mg/kg) and maintained with Ketamine/Xylazine via i.m. dosing as previously detailed (Woodfin et al., 2011). To induce an inflammatory response, the mice were injected via the intrascrotal route (i.s.) with recombinant mouse CXCL1 (300ng in 400µl of PBS). Control mice received i.s. PBS. In some groups of mice, PBS and CXCL1 were co-injected with Y-27632 (50µM solution). All mice were additionally injected i.s. with an Alexa-555-labelled non-blocking anti-PECAM-1 mAb to stain endothelial cell junctions of the cremaster microvasculature, as previously detailed (Woodfin et al., 2011). After 30 mins, the cremaster muscle was surgically exteriorised and imaged using an upright confocal microscope (Woodfin et al., 2011). The exteriorised tissue was continuously superfused during the 1 hr observation period with warm Tyrode solution or Tyrode containing Y-27632 (50µM). In each mouse, 4-6 post-capillary venules were imaged in 4D, and subsequently analyzed by IMARIS Bitplane software. Neutrophil transmigration into tissues, dynamics, and the size of endothelial cell pores in the PECAM-1 channel was quantified as previously described (Colom et al., 2015; Woodfin et al., 2011).

Transient transfections in endothelial cells
HDMVEC were infected with a lentivirus (pLenti6/v5, Invitrogen Life Technologies) encoding LifeAct-RFP. Cells were harvested 1 day after infection, seeded and confluent monolayers were taken for flow experiments a day later. HDMVECs were also transfected with pEGFP-N3 encoding GFP fused to the constitutively active mDia1 mutant ΔN3 (Watanabe et al., 1999). Transfection was
performed using Amixa Basic Nucleofector™ Kit for Primary Mammalian Endothelial Cells according to manufacturer’s protocol. A day later cells were harvested and reseeded to form confluent monolayers consisting of ECs with variable levels of the GFP mDIA1 fusion protein. Leukocytes adhered and squeezed through or immediately near cells expressing high levels of the GFP fused mutant were distinguished from leukocytes interacting with identically electroporated endothelial cells lacking a detectable GFP signal.

**Fluorescence microscopy**
For immunostaining, samples were fixed with PBS containing 4% (wt/vol) paraformaldehyde and 2% (wt/vol) sucrose. Fixed cells were blocked with 10% (vol/vol) goat serum and incubated with either primary fluorescence-labeled mAb or unlabeled mAb, followed by secondary antibody, or with the Hoechst 33342 nuclear dye or the F-actin probe, TRITC (tetramethylrhodamine isothiocyanate)-phalloidin. Images were obtained with a PlanApo phase contrast 60× oil-immersion objective on an Olympus IX83 microscope (Olympus, Tokyo, Japan). Sections were acquired as serial z stacks and were subjected to digital deconvolution (CellSence, Olympus).

**Densitometric analysis of filaments**
The area of the actin fibers was determined using Fiji by a simple fixed threshold analysis and was normalized to the area of the whole image (Schindelin et al., 2012). Mean actin density was determined in at least 4 different fields of view using either 8k (1000µm²) or 25k (100µm²) magnifications.

**Image analysis of nuclear shape changes**
To visually illustrate the nucleus movement we prepared a 3D-stack of time lapse images of Hoechest labeled leukocytes, and created a surface rendering of the “3D-nuclei” using Imaris software. Z-spacing was 4 times the x/y spacing.

**Correlative electron microscopy**
Scanning electron microscopy of cellular actin networks was performed according to Hoelzle and Svitkina (Hoelzle and Svitkina, 2012). Briefly, endothelial cells and T cells were extracted in situ at 37°C for 5 min with a cytoskeleton-stabilizing solution perfused into the flow chamber (50 mM imidazole, 50 mM KCl, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, and 0.5 µM TRITC-phalloidin supplemented with 1% Triton X-100 and 4% polyethylene glycol, Mr 35,000). Extracted cells were briefly washed with the same solution without Triton X-100 and polyethylene glycol, and fixed for 20 minutes with 2% glutaraldehyde in 0.1M cacodylate solution (pH=7.4) supplemented with 5mM CaCl₂ and 1% sucrose. Next, samples were fixed in aqueous solution of 0.1% Tannic acid for 20 minutes, washed twice in DDW and incubated with 0.1% uranyl acetate for an additional 20 minutes. Dehydrations were performed by washing three times with increasing ethanol concentrations (25% Vol/Vol, 50%, 75%, 100%). Prior to CPD (critical point drying) samples were incubated in 0.1% uranyl acetate in 100% ethanol for 20 minutes and washed four times in pure ethanol. After CPD the specimens were made conductive by coating with a 5 nm layer of Chromium, deposited by sputtering (Emitech K575X).

**Endothelial remodeling around beads coated with anti CAM mAbs**
Protein G Dynabeads (Life technologies, Carlsbad, CA) were washed 3 times in PBS and blocked with PBS containing 2% BSA (sigma) for 1h. Beads were incubated for 16 hrs at 4°C in blocking solution containing a 1:1 mixture of 0.2µg/µl anti ICAM-1 mAb (eBioscience HA58 , San Diego, CA) and anti VCAM-1 mAb (Clone 4B9, a gift from Dr. T.K. Kishimoto, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT). Coated beads were overlaid for 30 sec on HDMVECs monolayers prestimulated for 3 hour with IL-1β as described in the main text and let settle for 30sec. Samples were agitated for 5 minutes at 37°C and washed twice in HBSS before being exposed to the lipid extraction and cytoskeleton-stabilizing solution as described in the main text. All anti ICAM-1 and VCAM-1 antibody coated beads resisted detachment from the IL-1β stimulated HDMVEC up to a shear stress of 10 dyn/cm² whereas IgG coated Protein G beads were readily detached from the endothelial monolayer at a shear stress of 5 dyn/cm².
Supplemental References


