

Mechanical Forces Induced by the Transendothelial Migration of Human Neutrophils

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ABSTRACT The mechanisms regulating neutrophil transmigration of vascular endothelium are not fully elucidated, but involve neutrophil firm attachment and passage through endothelial cell–cell junctions. The goal of this study was to characterize the tangential forces exerted by neutrophils during transendothelial migration at cell–cell junctions using an in vitro laminar shear flow model in which confluent activated endothelium is grown on a microfabricated pillar substrate. The tangential forces are deduced from the measurement of pillar deflection beneath the endothelial cell–cell junction as neutrophils transmigrate. The force diagram displays an initial force increase, which coincides with neutrophil penetration into the intercellular space and formation of a gap in VE-cadherin staining. This is followed by a rapid and large increase of traction forces exerted by endothelial cells on the substrate in response to the transmigration process and the disruption of cell–cell contacts. The average maximum force exerted by an actively transmigrating neutrophil is three times higher than the force generated by an adherent neutrophil that does not transmigrate. Furthermore, we show that substrate rigidity can modify the mechanical forces induced by the transmigration of a neutrophil through the endothelium. Our data suggest that the force induced by neutrophil transmigration plays a key role in the disruption of endothelial adherens junctions.

INTRODUCTION

Neutrophil transmigration through the endothelial cell monolayer is a key process in the inflammatory response. It is a multistep cascade consisting of three overlapping steps: neutrophil initial attachment and rolling on the vascular endothelium surface, arrest and crawling (migrating) of the neutrophils to the cell–cell junctions, and transmigration itself (diapedesis) through the junction composed of the VE-cadherin complex and other proteins localized to the junctions, including PECAM-1, CD99, and JAMs (1). In addition, VE-cadherin, which localizes at cell–cell adherens junctions and forms a complex with cytosolic α -, β -, and γ -catenins, is also thought to regulate transmigration (1–4). By live-cell fluorescence imaging of endothelial cell junctions during neutrophil transmigration, previous studies have shown that leukocytes migrate within minutes through the intercellular junctions by transiently disrupting the VE-cadherin complex (2,5). The mechanism underlying rapid diapedesis and gap formation in VE-cadherin remains unclear. A number of hypotheses have been proposed. First, leukocytes induce transient disruption and disassociation of junctional complexes, such as VE-cadherin, to gain passage (1,3,6), or leukocytes actively seek areas of small preexisting gaps in VE-cadherin that expand during diapedesis (3,6,7). Alternatively, the neutrophil itself could exert sufficient force

on the cell–cell junction to rupture the VE-cadherin complex and gain passage (1).

There is a paucity of data on the forces that are generated during leukocyte diapedesis (8), due perhaps to the complexity of the process as well as the lack of appropriate tools (9). In particular, previous studies have evaluated forces during neutrophil attachment to immobilized endothelial adhesion molecules (10,11). The force that drives neutrophil motility comes primarily from two sources: 1), actin polymerization at the leading edge (12); and 2), actin-myosin complexes and RhoA activity during the uropod retraction (13,14). Previous studies analyzed the forces generated by leukocytes crawling on glass, and reported forces reaching 10.7 nN (15). A more recent study that focused on the migration of neutrophils found significantly larger forces, up to 67 nN, generated during chemotaxis on the surface of polyacrylamide gel (16). Both experiments were conducted on artificial surfaces and not on the endothelial cell monolayer. These forces are generated during crawling and not transendothelial migration (TEM). Mechanical forces may also play a role in the disruption of intercellular junctions as a neutrophil penetrates the intercellular junctions, which primarily consists of VE-cadherin dimers, and squeezes between the endothelial cells. However, the force exerted by a neutrophil during diapedesis at cell–cell junctions has not yet been determined. Furthermore, the mechanical properties of leukocytes, and neutrophils in particular, have been studied with various techniques (17,18) and play a key role in determining their function in response to a specific environment (19). For instance, leukocytes have been observed to be activated and modify their mechanical properties when con-

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fined or forced to enter into microchannels (20,21). Additionally, the stiffness of leukocytes could also regulate their adhesion to the endothelium in larger blood vessels by modulating the contact area and thus the mechanical forces between leukocytes and the endothelium (22,23).

Here, we have analyzed the mechanical forces induced by the transmigration of human neutrophils through a human endothelial cell monolayer. Several techniques have been developed to measure the traction forces generated by cells and the biochemical activities associated with them (24–27). To study the relative forces generated in endothelium during neutrophil transmigration, and to characterize their potential role in this process, we used an *in vitro* model consisting of a surface composed of an array of flexible micropillars (Fig. 1). The micropillar system is composed of a high density of flexible poly(dimethylsiloxane) (PDMS) micropillars and has already been used in various experiments to obtain force distributions in isolated or assemblies of cells (28–30). Endothelial cells were cultured at confluence onto the micropillars, and neutrophil transendothelial cell migration was monitored by live-cell imaging under shear flow conditions that mimic blood flow in microvessels (31). By analyzing the deflection of the micropillars, we characterized the tension exerted by the endothelial cells on the substrate, and thus deduced the relative force due to the penetration of adherent neutrophils through the endothelium. In particular, the initiation of the transmigration process corresponds to a sudden and large increase of the traction forces.

Finally, we studied the effect of the substrate rigidity on this process. It has been shown that the compliance of the matrix strongly influences several cellular functions, including migration, adhesion, and even differentiation (32–34). Here, the rigidity of the substrate could play an important role by eventually modifying several key parameters such as cell stiffness, cell spreading, or traction forces during the interactions between the neutrophil and endothelial cells. By changing the spring constant of the micropillars (30,35), we indeed demonstrated that the substrate stiffness could play an important role in the transmigration process.

MATERIALS AND METHODS

Materials

PDMS (Sylgard 184; Dow Corning, Midland, MI) was purchased from Thermo-Fisher Scientific (Medford, MA). Monoclonal antibody (mAb) Hec-1

(murine anti-human VE-cadherin, IgG₁ (36) was conjugated to Alexa 558 (Molecular Probes, Eugene, OR) following the manufacturer's directions. Alexa Fluor 488-tagged goat anti-mouse mAb (Molecular Probes) was used as a nonspecific marker to visualize the top surface of micropillars.

Fabrication of the PDMS micropillar substrate

PDMS micropillar substrates were prepared according to du Roure et al. (29). Briefly, using conventional photolithography followed by a deep etching process (the “Bosch process”), silicon wafers were patterned with an array of cylindrical pits and the desired pattern was replicated in positive photoresist by photolithography. Bare parts of the wafers were then etched by the deep Si etching process down to the desired depth to obtain the negative pattern of the array. A drop of liquid PDMS was placed on the silicon template between two coverslips (thickness No. 0, 0.08–0.13 mm) and covered by a third coverslip (thickness No. 2, 0.22 mm) to obtain a thin microchip. This material was cured at 65°C for 12 h and then removed in 70% ethanol to prevent collapse of the micropillars. The ethanol was then gradually replaced by phosphate-buffered saline (PBS). In this study, 2- μ m-diameter pillars of the same area-to-pillar density (22%; ratio of the post surface to the total surface) were used for all experiments, and the pillars were set at a center-to-center distance of 4 μ m. Rigidity was changed by increasing the pillar height from 3.3 μ m to 4.7 μ m.

Fibronectin coating of the PDMS surfaces

The silicone micropillar substrate was coated with fibronectin and an anti-mouse Alexa Fluor 488 fluorescent antibody that binds nonspecifically and allowed visualization of the pillar tops: 40 μ L of 1 mg/mL fibronectin in Dulbecco's phosphate buffered saline and 1 μ L of Alexa488-labeled murine IgG (1 mg/mL) were deposited on the thin PDMS film, gently pressed against the entire surface with a coverslip, and incubated for 1 h at room temperature. The substrate was then rinsed with PBS and pressed against the micropillar surface in the liquid phase and incubated for 20 min. To avoid nonspecific protein adsorption on the sides of the micropillars and thus prevent cell adhesion, micropillar surfaces were saturated by immersion in PBS buffer containing 3% BSA, 0.1% Pluronic F127 (Sigma) for 1 h and then rinsed with PBS as previously described (28,37).

Endothelial and neutrophils cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described (3). The HUVEC (subculture 1) were detached from the culture dish with 3 mM EGTA trypsin-free solution, washed, and placed on the micropillar substrate in three steps at 5-min intervals. This protocol resulted in a confluent monolayer as judged by robust staining of VE-cadherin at cell–cell junctions and differential interference contrast (DIC) microscopy. Confluent HUVEC on micropillar substrates were activated with 25 ng/mL tumor necrosis factor- α (TNF- α) for 3 h to stimulate the endothelium for more effective neutrophil transmigration as previously reported (38). In some

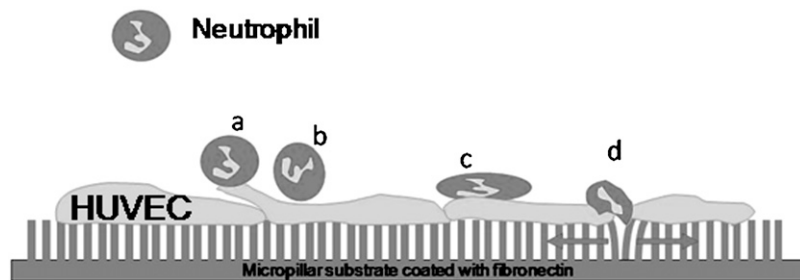


FIGURE 1 Schematic representation of the neutrophil transmigration on a micropillar substrate. HUVEC are plated on the micropillar substrate and inserted into an *in vitro* flow chamber that simulates blood flow. The different steps of interactions between neutrophils and endothelium are shown: (a) attachment, (b) rolling, (c) firm adhesion, and (d) migration toward endothelial junctions and transmigration. The majority of neutrophils attach to the cell surface at cell–cell junctions. Neutrophils that transmigrate induce a change of the force profile on the pillars as they pass between the intercellular junctions and then migrate beneath the monolayer.

experiments HUVEC were treated with conjugated VE-cadherin antibody 30 min before the transmigration experiment to visualize the cell–cell junctions during TEM by live-cell imaging as previously described (39). Human neutrophils (95% pure) were isolated from whole blood obtained from healthy volunteers donors by venous puncture as previously described (6). The human use committee of the Brigham and Women’s Hospital approved all protocols involving the use of human subjects, and signed donor consent was obtained from all blood donors.

Endothelial cell flow transmigration assays

Coverslips containing a confluent monolayer of HUVEC on the micropillar surface were mounted into a parallel plate flow chamber as previously described (31). Freshly isolated polymorphonuclear leukocytes (PMN) were resuspended at 0.5×10^6 cells/mL and drawn across HUVEC monolayers in a parallel plate flow chamber at 0.7 dynes/cm^2 as previously described (3). Neutrophils were allowed to interact with the monolayer, and transmigrated neutrophils were distinguished from those interacting with the apical surface (nontransmigrated) by live-cell imaging, as described below.

Live-cell imaging

Time-lapse, live-cell epifluorescence microscopy experiments were performed on an inverted Nikon TE-2000 microscope (equipped with 40X objective and a heating stage maintaining the temperature of 37°C) coupled to a Hamamatsu digital camera. We used DIC optics to visualize neutrophil transmigration; however, we had to observe cells through a layer of PDMS, which decreased the quality of the images. Images were recorded with MetaMorph software every 5 s for 10 min for every experiment.

Image analysis and calculation of traction forces

To quantify the area of neutrophils after transmigration, we used Image J tools to outline the borders of the neutrophils and calculated the area inside. We measured the local deformation of the pillars by using in-house-made multiparticle tracking software (29). Briefly, by fluorescently labeling only the top of the pillars, we obtained a high contrast between the top of the pillars and the background. We then used the fluorescent signals coming from each pillar to track their center of mass over time. For all experiments the drift (mechanically or thermally induced) of the microscope stage was determined and accounted for automatically. The time resolution, corresponding to the calculation of the overall stress pattern for one image, is $<1 \text{ s}$. The resolution of the pillar displacement is on the order of 25 nm. To calculate the local tangential force, the deflection of the posts is multiplied by the spring constant according to the formula: $F = (3/4)\pi E(r^4/L^3) \times \Delta x$, where E is the Young’s modulus, r is the pillar radius, L is its length, Δx is the displacement from equilibrium, and F is the force. Depending on the spring constant, the force resolution varies. To measure the spring constant, we used dimensionally calibrated macroscopic cylinders of PDMS and measured

their compression under a fixed normal strain to evaluate the Young’s modulus, E . Because E depends on the PDMS cure time, we used a consistent cure time of $12 \text{ h} \pm 2 \text{ h}$ at 65°C corresponding to a Young’s modulus of $2 \pm 0.2 \text{ MPa}$. By performing SEM observations, we measured the dimensions of the pillars (r , L) and calculated their spring constant according to the force-deformation equation.

Confocal microscopy and image analysis

Confocal microscopy was performed on samples fixed by 4% paraformaldehyde for 10 min and then mounted with mounting medium. We used a Zeiss LSM 510 confocal microscope with a 63X oil-immersion lens. Images were acquired in three colors with a $0.2\text{-}\mu\text{m}$ step. After acquisition by Zeiss software (Thornwood, NY), the images were analyzed using Image J.

RESULTS

Characterization of the traction forces induced by the endothelial cell monolayer

HUVEC were cultured on substrates composed of micropillars as detailed in Materials and Methods. We used a specific coating of the micropillars to restrict endothelial cell adhesion to the tips of the posts (see Materials and Methods section). Using microcontact printing, we delivered fibronectin from a stamp onto the tips of the posts, and adsorbed Pluronic onto the remaining unstamped regions of the array to block nonspecific protein adsorption and cell adhesion as previously described (37). This procedure results in confluent monolayers that exhibit robust staining by anti-VE-cadherin mAb (Fig. 2). VE-cadherin is a key component of adherens junctions, and its robust staining at cell–cell junctions indicated that the cells had established functional intercellular complexes. We analyzed forces generated by endothelial cells plated at different densities to obtain the range of forces and thus the mechanical background induced by those cells in the absence of neutrophils. All experiments and controls were conducted on 3-h TNF- α -activated endothelial cells. TNF activation is necessary for the transmigration to occur. First, endothelial cells were grown to confluence and the deflections of the underlying micropillars were measured. The force per pillar, averaged over 14,000 micropillars, generated by a confluent HUVEC monolayer is $1.5 \pm 0.3 \text{ nN}$ on micropillars with a $45 \text{ nN}/\mu\text{m}$ -spring constant (Fig. 3). We calculated the force exerted by the endothelial cells on a per-

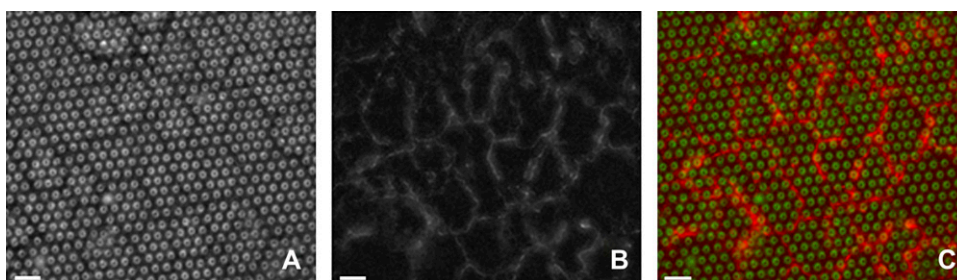


FIGURE 2 HUVEC form a confluent monolayer with VE-cadherin present at cell–cell junctions. (A) DIC image of a confluent HUVEC monolayer cultured on a micropillar substrate. (B) Live-cell image of VE-cadherin staining of the endothelial junctions. (C) Merge of VE-cadherin (red channel) and DIC (green channel) images that identify intercellular junctions (red). The lack of clear focus in the red signal is due to the scattering of light by the PDMS body of the micropillars. Scale bar is $10 \mu\text{m}$.

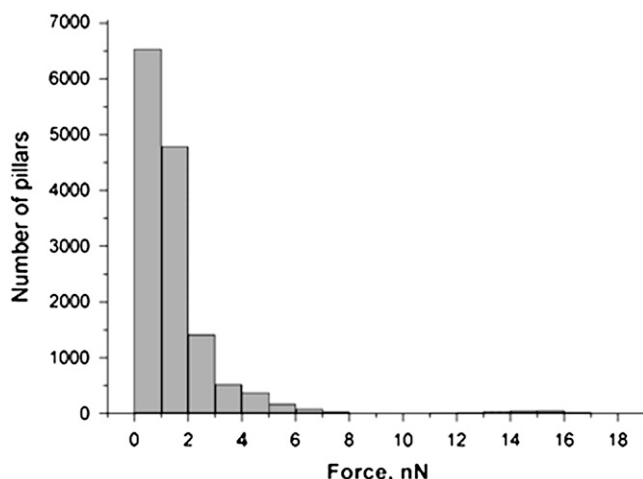


FIGURE 3 Distribution of forces generated by the HUVEC monolayer on the micropillar substrate. We analyzed 14,000 pillars in three different experiments beneath 30 endothelial cells and plotted a distribution diagram.

pillar basis instead of an averaged per-cell force because the pillar density was unchanged. Then, the average force we calculated for individual human endothelial cells was 3.2 ± 0.6 nN ($n = 15$ cells with 7000 pillars analyzed), which is significantly larger than that for a monolayer. These results are in good agreement with our previous studies on epithelial cells and, in particular, the range of forces is similar (29,30). It is interesting that in the case of a monolayer, we obtained an average force in the endothelial junction area that was higher (5 ± 1 nN, $n = 20$ studies with 3000 pillars analyzed) than the mean force calculated for all the underlying pillars (1.5 ± 0.3 nN). Forces in the area of cell–cell junctions were calculated by averaging over pillars selected on each side of the intercellular junction. Basically, a two-pillar-wide band was selected around VE-cadherin positive cell–cell edges. The main part of the mechanical activity of an endothelial monolayer transmitted to the substrate was located at the cell–cell junctions. Fig. 3 shows that the histogram of the force distribution for a confluent monolayer exhibits an exponential decay. Forces below 6 nN correspond to 96% of events (Fig. 3). Few events in the area of high forces correspond to the cells generating an abnormal pattern of force. Because these cells could be abnormally adherent cells, we did not analyze any transmigration events on them. The force pattern within confluent monolayers has an important implication for transmigration experiments. In particular, the force generated by HUVECs in the area of cell–cell contacts (5 ± 1 nN) represents the force baseline against which the neutrophil transmigration force will be compared.

Neutrophil transmigration triggers VE-cadherin disruption and micropillar displacement

First, we compared the kinetics of the transmigration process of neutrophils through endothelial cells on micropillar sub-

strates versus flat substrates using the same fibronectin coating. We verified that cellular interactions, including neutrophil attachment, kinetics, and number of transmigration events, were not affected by the array of closely spaced pillars (Figs. 4 and 5). Indeed, neutrophils bound at or close to endothelial cell–cell junctions with $78 \pm 8\%$ initially attached within one neutrophil cell diameter from the VE-cadherin-labeled junctions, in a manner similar to that on glass (2). About 40% of the neutrophils that attached to the surface of the endothelial cell ultimately transmigrated. The length of time required for a transmigration event was 85 ± 20 s based on 35 cells examined in detail, which was similar to the time frame obtained with TNF- α HUVEC cultured on fibronectin-coated glass coverslips (Fig. 4; (2)). Finally, most of the transmigration events occurred in the area of contact among three cells (tricellular junctions), which was consistent with previous reports using endothelium plated on glass coverslips and laminar shear flow conditions (2,7,40). Thus it appears that neutrophil transmigration processes on a flat surface and on a micropillar surface are similar and go through the same sequence of steps.

We performed an analysis of neutrophil transmigration by acquiring images of the HUVEC and neutrophils with DIC microscopy and simultaneously captured sequential, two-color fluorescence of micropillars (Fig. 5, *green*) and VE-cadherin (Fig. 5, *red*) (essentially three-color imaging; see also [Movie S1a](#), [Movie S1b](#), and [Data S1](#) in Supplementary Material). We first established the baseline position of micropillars (Fig. 5, *a* and *c*, control) before neutrophil attachment. DIC microscopy (Fig. 5 *a*) depicts the behavior of a neutrophil that adheres at a tricellular junction at time = 0 s (Fig. 5 *a*, panel *B*), transmigrates (panels *C* and *D*), and accumulates beneath the endothelial monolayer (Fig. 5 *a*, panel *E*; [Movie S2](#) and [Data S1](#)). A blue circle outlines the neutrophil transmigration area. We also observed neutrophils that stably adhered but did not transmigrate, as shown in Fig. 5 *a* (*red arrow*). This can be deduced by the lack of disruption of the junctional staining of VE-cadherin as shown on Fig. 5 *b*. Only neutrophils that successfully transmigrate trigger transient disruption, followed by resealing of VE-cadherin staining (Fig. 5 *b*, panels *F–J* in the circle). Strikingly, during this same time period, we detected a sudden transient and reversible displacement of micropillars located directly beneath the endothelium at the site of transmigration (Fig. 5, *c* and *d*, panels *N* and *S*). The yellow arrows in panel *S* illustrate the vector force applied to corresponding pillars. The initial increase in pillar displacement correlated with loss of VE-cadherin staining and neutrophil migration into intercellular space (Fig. 5, panels *H*, *M*, and *R*).

The process of neutrophil penetration in between the junction was previously detailed in electron microscopy observations by Burns et al. (7) and our own DIC microscopy results (2) (Fig. 5). The maximum increase in pillar displacement (Fig. 5, panels *I*, *N*, and *S*) corresponded to a large increase of the disruption of cell–cell contacts. Based on the

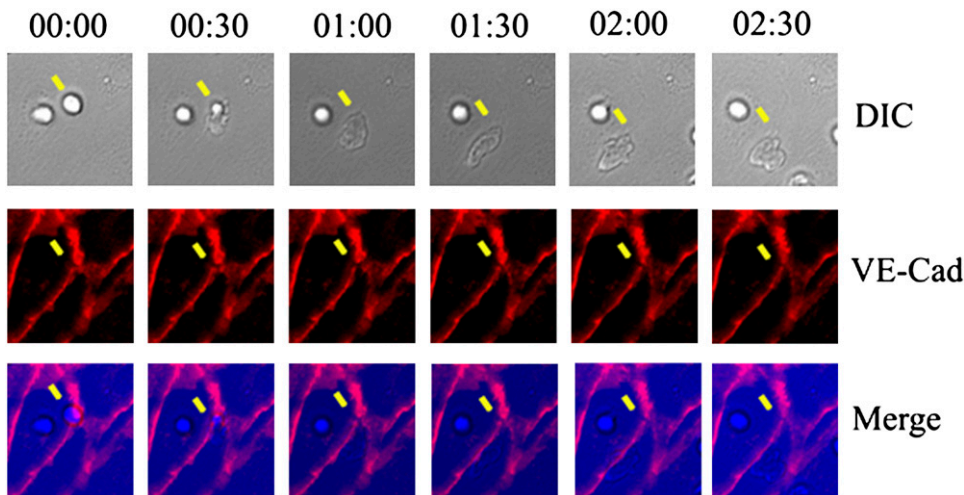


FIGURE 4 Neutrophil transmigration across TNF-activated HUVEC cultured on glass coverslips. The yellow arrow indicates a transmigrating neutrophil near a tricellular junction. The DIC panel shows that the neutrophil penetrates at cell–cell junctions and spreads underneath the endothelial cell body. The VE-Cad panel shows that VE-cadherin junctional staining is transiently disrupted during transmigration and then reseals after transmigration is complete. The merge of the DIC and VE-cad (merge panel) shows the relative position of a transmigrating neutrophil and the intercellular junctions rupture area, and clearly indicates that junction rupture occurs exactly in the place of transmigration.

DIC microscopy live-cell imaging (Fig. 5), we also observed that the position of the neutrophil, relative to the monolayer and the top of the pillars, varied with time. The modification of the force pattern induced by endothelial cells on the substrates thus correlates with the bulk of the neutrophil passing through the cell–cell junctions. After completing transmigration, the neutrophil, located in the top left area of the blue circle in Fig. 5, causes significantly less change in micropillar displacement at the original area of transmigration. Finally, transmigrated neutrophils moved randomly beneath the endothelial monolayer. After transmigration, neutrophils continued to migrate away and induced forces on the substrate

([Movie S3](#) and [Data S1](#)); however, the force measurements were difficult to interpret because they were due to the superposition of forces exerted by endothelial cells and neutrophils located beneath the monolayer.

Using standard optical microscopy, we could not verify the precise location of neutrophils after the transmigration. To verify the precise location of the neutrophils after transmigration, we used confocal microscopy. We analyzed 30 independent transmigration events and observed neutrophils that penetrated the endothelial monolayer and reached the bottom of the micropillar substrate (Fig. 6 *A*). In Fig. 6 *B*, the labeling of intercellular connections with a conjugated anti-

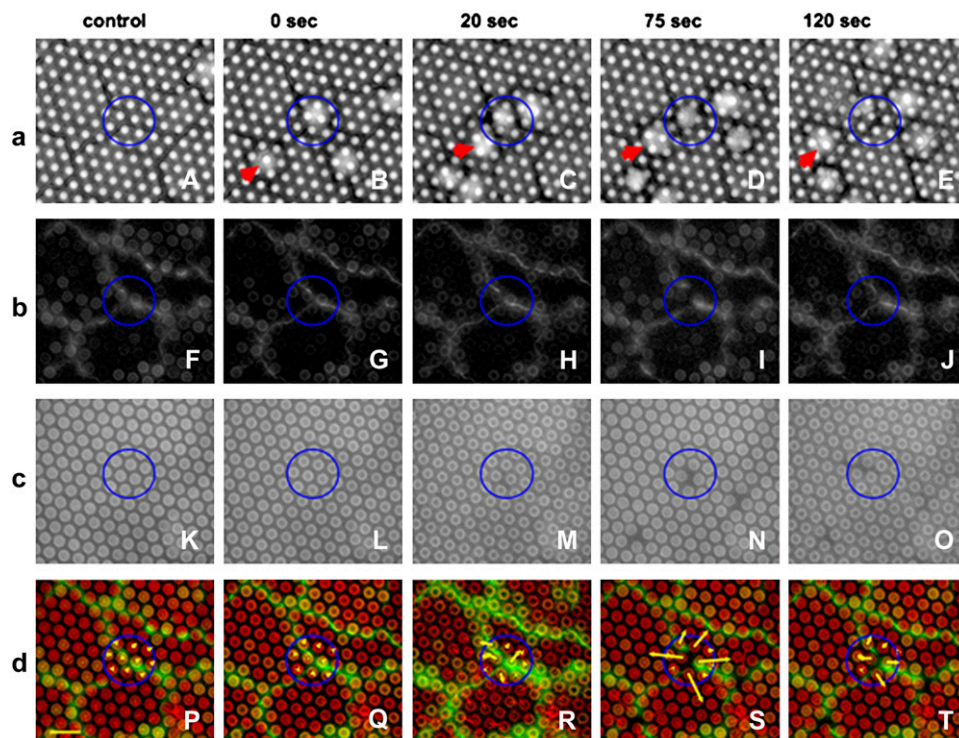


FIGURE 5 DIC and two-color fluorescent imaging of the endothelial monolayer during transmigration. (*a*) Micrographs of images. *A–E* are DIC images of the process of neutrophil transmigration: baseline, no neutrophil (*A*), a neutrophil arrests (*B*), extending lamellipodium (*C*), transmigration (*D*), and crawling beneath the monolayer (*E*). (*b*) Micrographs of images *F–J* depict the VE-cadherin junction labeling of an intact junction (*F*, *G*), the initial rupture by neutrophil (*H*), maximal gap in VE-cadherin (*I*), and resealing of VE-cadherin (*J*). (*c*) (*K–O*) micropillar surface with no displacement (*K* and *L*), low-force stage (*M*), radial displacement (*N*), and relaxation (*O*). (*d*) Merged images *P–T* represent overlays of the VE-cadherin junctional staining (green) and micropillars image (red). The forces exerted on the pillars are represented by red lines originating from the centers of the pillars. A nonmigrating neutrophil to the left of the circle is indicated by a red arrow in panel *a*. Scale: diameter of pillar is 2 μm , yellow bar is 10 nN.

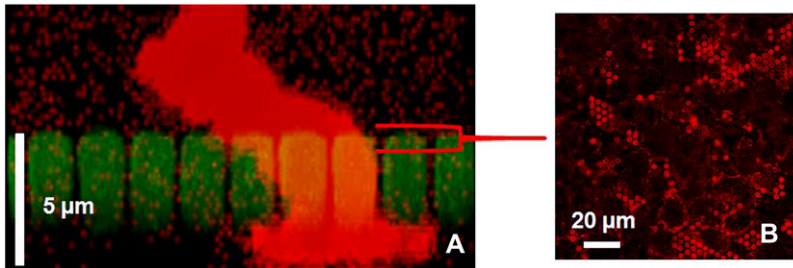


FIGURE 6 Confocal images of neutrophil transmigration. (A) Z-projection of a neutrophil transmigration site (neutrophil is shown in red, pillars in green). (B) VE-cadherin staining with a conjugated anti-VE-cadherin antibody averaged over 10 slices, shown in red. The length of the pillar was $\sim 5 \mu\text{m}$ for this experiment.

VE-cadherin antibody shows that cell–cell junctions are visible above the micropillar tops. The fluorescent signal observed on some micropillars was due to nonspecific binding of the antibody on PDMS substrate.

Neutrophil transmigration force profile

We analyzed 30 complete transmigration events using the imaging protocol described above and found that in most cases, six pillars consistently showed a distinct radial displacement pattern on both sides of the endothelial junction and had the largest absolute change in displacement. This number corresponded to an area of $10 \times 6 \mu\text{m}^2$ (Fig. 7), which was in between the area occupied by a nonspread neutrophil $\sim 40 \mu\text{m}^2$ and that of a spread neutrophil $\sim 120 \mu\text{m}^2$ on glass (41). We also estimated the average area occupied by neutrophils after transmigration on the pillars and obtained $96 \pm 12 \mu\text{m}^2$ for 30 neutrophils analyzed (see Materials and Methods). At the same time, the actual size of the gap ob-

served in VE-cadherin staining was on average $\sim 4 \mu\text{m}$. Thus, it appeared that six pillars were significantly displaced during neutrophil transmigration through a narrow gap in intercellular junctions and then when a neutrophil spread underneath the monolayer on a similar area as on flat surfaces. Therefore, in all of the experiments, only the six pillars directly beneath the transmigration site were monitored and analyzed for consistency of calculation of an average force.

Fig. 7, A–C show the change in the displacement of the six pillars directly underneath a transmigrating neutrophil for one representative transmigration event, and Fig. 7 D depicts the plot of the elapsed time versus the average force generated on these six pillars (the triangles identify the transmigrating neutrophil). Notably, this timescale of the complete process was similar to the one obtained for neutrophil transmigration of endothelium cultured on fibronectin-coated glass coverslips (2). The time point labeled A identifies the average force per pillar (4 nN) in a resting monolayer before the neutrophil arrests (i.e., baseline force level). This force at the cell–cell

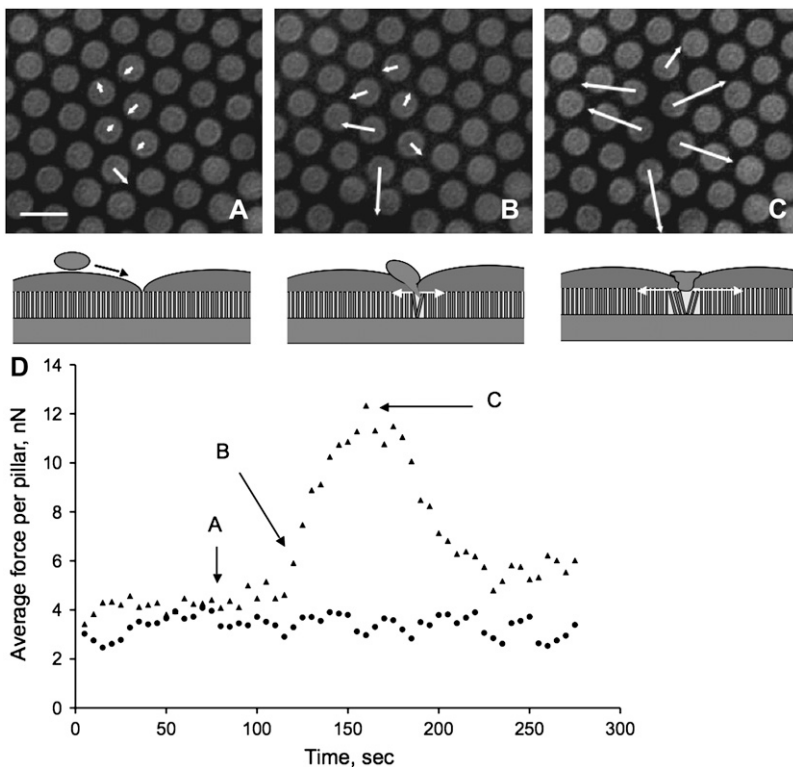


FIGURE 7 Force profile and leukocyte transmigration. (A) Cells before neutrophil transmigration. (B) Initiation of transmigration. (C) Peak force during transmigration. The length of the yellow line represents the force in the corresponding direction, with white bar = 10 nN. (D) The force profile during neutrophil transmigration is shown with (\blacktriangle), and the force profile induced by a neutrophil that does not transmigrate on Fig. 5 is shown (\bullet).

contact area is randomly oriented and is comparable to the average force generated by the monolayer as noted above, reaching 5 ± 1 nN. Point *B* represents the time point when the dissociation of the junctions begins as a result of the neutrophil penetrating the junction, as detected by VE-cadherin staining and DIC observation. The increase of the force (Fig. 7, panel *B*) is correlated with an increase in the radial displacement of the pillars. We suggest that this event is caused by an initial increase of the forces exerted by endothelial cells due to their interaction with the neutrophil. Point *C* indicates the time of maximal strain and coincides with the neutrophil actively passing between adjacent endothelial cells. For this experiment done on a micropillar substrate with a 45 nN/ μm spring constant, we observed an increase in the average force from 4 to 13 nN per pillar, and from 24 to 78 nN per neutrophil. Based on 30 experiments, the average maximum force increased from 4.8 ± 1 to 14 ± 4 nN per pillar.

Careful analysis of the DIC and fluorescent images in combination with a force diagram enabled us to understand the mechanics of neutrophil penetration of the endothelial cell–cell junctions during transmigration. The initial intercellular junction disruption, as detected by the gap in VE-cadherin staining, was a relatively low-force process (Fig. 7 *D*, phase *B*). However, frame-by-frame analysis of the DIC and two-color fluorescence images revealed that the force increased at the same time as the neutrophil started to penetrate into the intercellular space and further VE-cadherin junction dissolution was observed (Fig. 5 *b–d*, panels *H* and *I*, *M* and *N*, *R* and *S*). Only when the bulk of the neutrophil penetrated the cell junction of the HUVEC were large traction forces detected (Fig. 7 *D*, phase *C*). Subsequently, it appeared that the neutrophil literally pushed the endothelial cells apart for further penetration. This phenomenon was confirmed by the radial distribution of forces pointing away from the center of penetration (Fig. 5 *d*). Indeed, the direction of the forces confirms that the transmigration of the neutrophil induces an increase of the forces exerted by endothelial cells on the substrate in correlation with the disruption of cell–cell junctions.

After the neutrophil completely penetrated the junction, we observed its spreading underneath the endothelial cells and a continuous local change of the force profile as it migrated under the monolayer (see [Movie S3](#)). Moreover, as the neutrophil left the area of penetration at the end of the transmigration process, we observed a local resealing of the junction and the force profile recovered its original baseline value (Figs. 5 *c* and 7 *D*).

Fate of nonmigrating neutrophils proves that neutrophils have an active role in force generation

We analyzed the force generated by neutrophils that attached to the endothelial cell surface, migrated on the apical surface to the junctions, and attempted to penetrate intercellular junctions but never did. The fate of one such neutrophil is shown in Fig. 5 *A–E* and identified by the red arrow. Although this neutro-

phil remained attached under flow conditions, there was no visible disruption of the VE-cadherin junction staining or pillar displacement in the corresponding area (Fig. 5 *b*, panels *F–J*, and Fig. 5 *c* for VE-cadherin staining, and panels *K–O* for pillar displacement). The force diagram analysis of the interactions of such neutrophils with the endothelial monolayer revealed a fluctuating pattern (Fig. 7 *D*, circles) in contrast to a clear-cut elevation in the force diagram for transmigration events (Fig. 7 *D*, triangles). Fluctuations of the force diagram may be caused by the neutrophil's attempts to project lamellipodia in the intercellular space, but we could not confirm this phenomenon because of the limitation of our optical resolution. Thus, we observed a net increase of the traction forces only when a neutrophil migrated between the cells and disrupted VE-cadherin staining.

A separate subgroup of events was represented by neutrophils that did rupture the VE-cadherin junctions but failed to transmigrate (Fig. 8). In these cases, significant forces were observed, reaching values of 6 ± 1 nN per pillar on average. This was slightly above the noise level, but could be clearly detected by the change in the distribution of force vectors from random to radial. The force exerted was still two times lower than the forces induced during a full transmigration event. It is interesting that these force values are comparable to that observed during the initial phase of a complete transmigration event (Fig. 7 *D*, point *A*). However, such neutrophils failed to complete the transmigration.

Neutrophil transmigration on substrates with different stiffness values

Finally, we addressed the possibility that the rigidity of the substrate can affect the transmigration process. By changing the length of the micropillars, one can vary their spring constant. Therefore, we studied the interaction between neutrophils and endothelial cells on substrates with the same density of micropillars but with two different rigidities: 45 nN/ μm pillars and 131 nN/ μm pillars (Fig. 9). In each experiment we analyzed the six pillars directly underneath the location where a neutrophil transmigrated (i.e., the penetration site). We analyzed 10 independent events for each substrate. We observed that the transmigration process exhibits a rigidity-dependent force generation mechanism because the force induced by the transmigration is higher on the more-rigid substrate (39 ± 6 nN) than on the soft one (14 ± 4 nN). The force exerted on the pillars at the peak phase of the transmigration (i.e., maximum force) is threefold greater than the baseline for each substrate rigidity and reaches 250 ± 42 nN per neutrophil for the rigid pillars compared to 84 ± 24 nN for endothelial cells alone.

DISCUSSION

In this study we investigated the role of the mechanical interactions that occur between neutrophils and endothelial cells during the transmigration process. By combining the

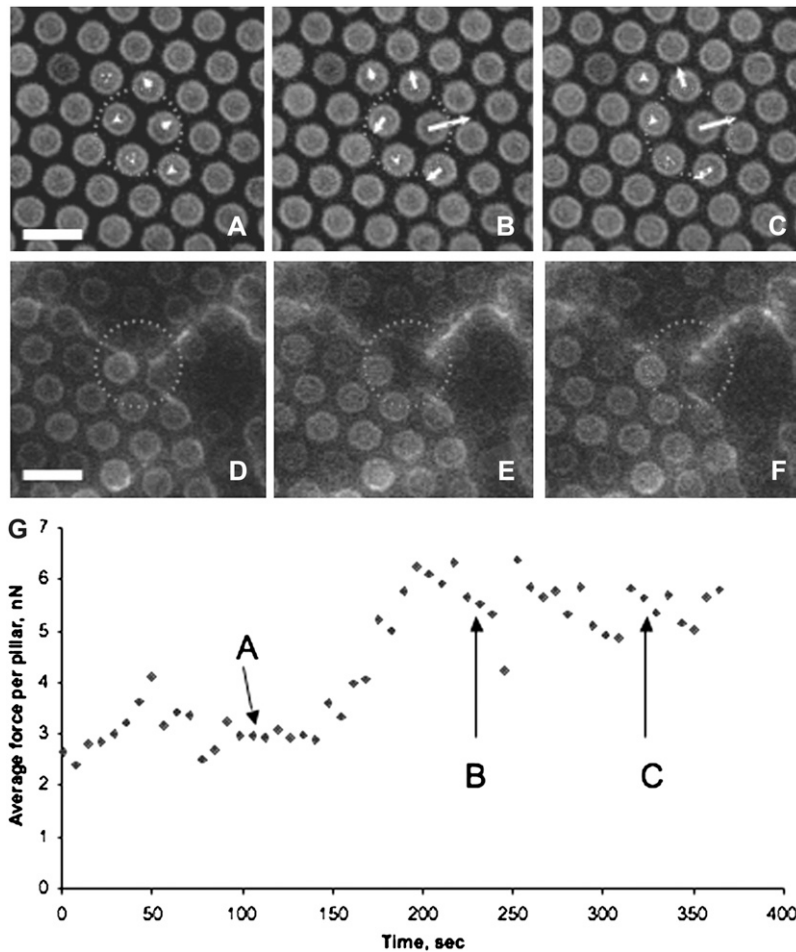


FIGURE 8 Nonmigrating neutrophil force diagram. Some of the neutrophils project lamellipodium that rupture the intercellular junctions but then fail to transmigrate. *A* shows a neutrophil before an attempt to transmigrate, and *B* and *C* are force distributions during the transmigration attempt. *D–F* show corresponding VE-cadherin junctional staining with clear rupture on *E* and *F*. *G* represents the average force per pillar corresponding to *A–C*. Maximal average force reaches 6.3 nN per pillar and is significantly less than that observed in the events of full transmigration.

use of microfabricated substrates and an *in vitro* human cell culture assay, we analyzed the different steps of the TEM in terms of mechanical traction forces. We performed experiments in a well-characterized *in vitro* flow adhesion model that mimics physiological laminar shear flow conditions

(2,3,38), and cultured cells on a substrate made of a closely spaced array of flexible micropillars. To our knowledge, this is the first study to provide quantitative results regarding the forces transmitted by neutrophils to endothelial cells during transmigration, and to address their implication in the disruption of cell–cell junctions.

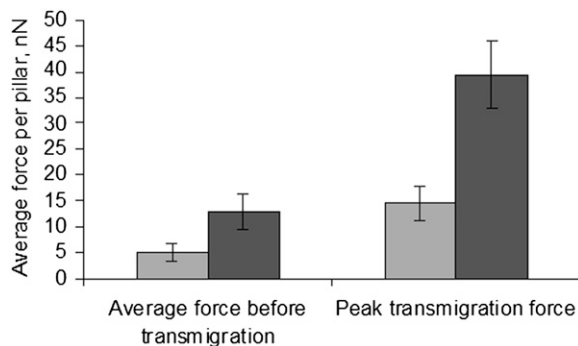


FIGURE 9 Force profile of transmigration on micropillars substrates with different rigidities. Average force per pillar recorded underneath the neutrophil transmigration site. (Light gray) 45 nN/μm pillars; (dark gray) 131 nN/μm pillars. Six pillars were analyzed in each experiment, and 10 experiments were conducted for each rigidity. $p < 0.05$ for before transmigration versus peak force for both stiffnesses.

First, the analysis of the traction forces generated by endothelial cells in the absence of neutrophils was necessary to measure the force deviation induced by the transmigration. It is interesting that the results revealed that the mechanical activity within a confluent monolayer was localized mainly at cell–cell junctions. The average force exerted on the substrate and located under cell–cell junctions was 5 nN, which was more than three times larger than the force averaged over the entire monolayer (~ 1.5 nN). These data were consistent with our previous observations on epithelial cells (29). This indicates that cell–cell junctions should play an important role in the mechanical integrity of an endothelial monolayer, in particular by regulating the cell–substrate interactions. Hence, the low basal value of the forces that were generated by the endothelial cells allowed us to detect changes in the force profile at the intercellular junctions during transmigration.

Neutrophils transmigrated normally through our hybrid HUVEC-micropillar flow system model and followed the well-described multistep adhesion cascade paradigm (1,2). The analysis of the neutrophil diapedesis force profile revealed a precise sequence of events. The first events corresponded to the adhesion of neutrophils onto the endothelial cells, followed by neutrophil penetration at the intercellular space. It is interesting that a low force was measured during this event despite the fact that this step correlated with the disruption or the formation of opening gaps at intercellular junctions as detected by VE-cadherin staining. Further force increase correlated with the size of the junction disruption (Fig. 5 *b*) as the neutrophil migrated between the endothelial cells. We observed a clear radial force distribution induced by the transmigration on the underlying pillars that correlated with the location of the neutrophil body. The maximal force, which corresponded to the displacements within the estimated area of interaction between the neutrophil and the monolayer, could reach up to 250 nN per neutrophil. The overall time frame for this process was ~ 60 s as measured from the force increase initiation to the peak force. After that, the neutrophil spread underneath the endothelial cell surface and the force profile became randomly distributed at the original area of diapedesis. The last step correlated with the resealing of VE-cadherin within the junction (see Fig. 5 and [Movie S1a](#), [Movie S1b](#), and [Data S1](#) for the detailed process).

Thus, the force profile in the area of transmigration appears to be a good indicator of the state of the diapedesis process. Forces were initially distributed randomly in the zone of neutrophil penetration as the endothelial cells in the monolayer acted in synergy and did not exert large differential forces in the cell junction area. When the bulk of the migrating neutrophil reached the intercellular space, the force profile changed dramatically: the magnitude of forces increased and their direction exhibited a radial pattern (Fig. 7). This radial orientation clearly indicated that the forces were due to endothelial cells and the rupture of cell–cell junctions in response to the neutrophil transmigration.

To exclude the possibility that the force we observed was due to the induction of endothelial contraction in response to neutrophil adhesion, and not to the neutrophil diapedesis itself, we studied the fate of neutrophils that formed strong adhesions to the intercellular space area but did not penetrate the junctions. These neutrophils did not rupture the junctions, as deduced by the lack of change in VE-cadherin staining, and did not induce a force increase. These data support our conclusion that the increase in tensile forces measured under the endothelial cells is most likely caused by an extension of a neutrophil lamellipodia into the junction, as shown in confocal analysis (Fig. 6) and by previous reports (2). In addition, the analysis of the force diagram after the transmigration revealed a continuous deformation of the substrate as neutrophils migrated beneath the monolayer and interacted with both the basolateral surface of endothelium and the pillars. Another important piece of evidence in support of an active

role of neutrophils in force generation was shown by the force diagram obtained from the adhesion of neutrophils on the endothelial monolayer that ruptured VE-cadherin junctions between the cells but did not transmigrate. A small dissociation of VE-cadherin junctions was observed, and was comparable to that observed in the first steps of a complete transmigration event. Consequently, the resulting force is just 1–2 nN per pillar above the force generated by endothelial cells without neutrophils. Here, we established that the forces generated during diapedesis proceeded in at least two distinct mechanical phases: first, a low-force dependent mechanism of around 6 ± 1 nN that involves an initial increase in the opening of the junction, and second, a high-force phase (~ 13 nN) in which the bulk of the neutrophil's body penetrates, causing a large dissociation of cell–cell junctions concomitant with a transient loss of VE-cadherin staining. These forces respectively correspond to ~ 1 –2 and ~ 7 nN per pillar above the force baseline defined by the forces exerted by endothelial cells in the absence of neutrophils. This sequential process involves complex events that regulate interactions between the mechanical and adhesive properties of both neutrophils and endothelial cells. In particular, previous studies have shown that transendothelial neutrophil migration is regulated by endothelial cell-dependent cytoskeletal mechanisms, and a variety of intracellular signaling steps contribute to this process (39,43,44). Our findings illustrate that transmigration may be governed by a combination of different force-generation mechanisms, and thus the increase of the forces probably requires a remodeling of the actin cytoskeleton and actin-myosin contractile machinery in both neutrophils and endothelial cells. In particular, one would expect that transmigration induces a reinforcement of the adhesion of endothelial cells onto the substrate and the actin cytoskeleton to help the neutrophils migrate across the monolayer. Such a cytoskeleton reorganization of endothelial cells could also play a role at the end of the process to allow the penetration of the neutrophil body through the junctions. The increase of the traction forces exerted by endothelial cells during transmigration clearly demonstrates that an active process is involved in locally remodeling the adhesive contacts on fibronectin-coated pillars, as previously shown in other cellular systems (45). Furthermore, transient disruption of VE-cadherin-mediated cell–cell adhesion is also an important event in the process of neutrophil transmigration (2,3,46). In agreement with previous studies (47), our results suggest that a “tug of war” may occur between cell–cell and cell–substrate adhesions, and the structure that is able to develop the most tension on its ligand in response to neutrophil adhesion “wins.” However, further experiments need to be done to directly correlate the remodeling of the different adhesions and the corresponding mechanical forces.

Furthermore, our interpretation is supported by the fact that neutrophils can generate higher forces during transmigration of the endothelium cultured on stiffer micropillar substrates. The average maximal force, calculated as an average of the

sum of six moduli of force vectors induced on pillars under the transmigration site at peak force, increases from 78 to ~ 250 nN as the substrate rigidity varies from 45 up to 131 nN/ μm . This change corresponds to a change from 0.026 pJ to 0.08 pJ in terms of energy spent, calculated as a sum of energies stored in deflected pillars, with a total increase of 0.054 pJ per neutrophil on rigid substrate compared to soft substrate. The increase of the energy barrier with the stiffness of the substrate could reflect that the neutrophil transmigration affects the mechanical interactions of endothelial cells. Indeed, many cell types respond to an increase of substrate rigidity by forming well-defined and stable focal adhesions and thus remodeling their cytoskeleton (48). Again, our findings point out that the observed neutrophil migration could be associated with endothelial actin polymerization and myosin II reorganization, as suggested by previous studies (43,49). The force increase with the substrate stiffness could be attributed to a higher resistance of endothelial cells to the cell transmigration on rigid substrates due to an increase of cytoskeleton contractility and stronger adhesion complexes between endothelial cell and pillars. Along the same line, it is of interest to compare our results with the calculated force necessary to break the bonds of VE-cadherin at junctions. From our observations, the average size of the VE-cadherin gap is $\sim 4 \mu\text{m}$ at maximal force (2), and we estimate from the literature that the VE-cadherin concentration in the intercellular space is $\sim 5 \times 10^3$ dimers/ μm^2 (50), assuming that the height of the intercellular connection is 1 μm and a 50 pN for the unbinding force (51). This leads to an estimated force of around $\sim 1 \mu\text{N}$ for simultaneous unbinding of the cadherin-cadherin bonds. Our measurements on substrates with different rigidities yielded lower values but in the same range of amplitudes, from 0.08 up to 0.25 μN . These differences could be explained by the fact that the adhesions at the junctions are not broken at the same time, but certainly in a sequential process. The source of the process that induces a dissociation of cell–cell junctions remains unclear. It could be due to actin polymerization at the front edge of the neutrophil in conjunction with biochemical interactions between the neutrophil and endothelial cells that trigger phosphorylation of the cytoplasmic tail of VE-cadherin (46). This process could affect signaling pathways that govern cell–cell adhesive interactions.

In summary, we measured the tangential force exerted by the interaction of neutrophils with a confluent TNF- α -activated endothelial cell monolayer. We demonstrated that neutrophil transmigration through the intercellular junctions is a multistep process with a clear mechanical signature. Our assay, which combines an in vitro flow adhesion model that mimics physiological shear flow conditions and a micro-force sensor array, enabled us to characterize and quantify the mechanical interactions between neutrophils and endothelial cells that occur during diapedesis. Finally, by changing the substrate rigidity, we obtained indirect evidence that the modulation of cytoskeleton contractility changes the transmigration force profile. This suggests that competition be-

tween cell–substrate adhesions and cell–cell junctions plays a key role in determining whether neutrophils successfully transmigrate through an endothelial cell monolayer.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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