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OxLDL and substrate stiffness promote neutrophil transmigration by enhanced endothelial cell contractility and ICAM-1

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Abstract

Elevated levels of oxLDL in the bloodstream and increased vasculature stiffness are both associated with cardiovascular disease in patients. However, it is not known how oxLDL and subendothelial matrix stiffness together regulate an immune response. Here, we used an *in vitro* model of the vascular endothelium to explore the combined effects of oxLDL and subendothelial matrix stiffening on neutrophil transmigration. We prepared fibronectin-coated polyacrylamide gels of varying stiffness and plated human umbilical vein ECs onto the gels. We observed that oxLDL treatment of the endothelium promoted neutrophil transmigration (from <1% to 26% on soft 0.87 kPa substrates), with stiffer substrates further promoting transmigration (54% on 5 kPa and 41% on 280 kPa). OxLDL exposure enhanced intercellular adhesion molecule-1 (ICAM-1) expression on the endothelium, which was likely responsible for the oxLDL-induced transmigration. Importantly, inhibition of MLCK-mediated EC contraction reduced transmigration to ~9% on all substrates and eliminated the effects of subendothelial matrix stiffness. In addition, large holes, thousands of square microns in size, formed in monolayers on stiff substrates following transmigration, indicating that oxLDL treatment and subsequent neutrophil transmigration caused serious damage to the endothelium. Our results reveal that an interplay between ICAM-1 and MLCK-dependent contractile forces mediates neutrophil transmigration through oxLDL-treated endothelium. Thus, microvasculature stiffness, which likely varies depending on tissue location and health, is an important regulator of the transmigration step of the immune response in the presence of oxLDL.

Keywords

myosin light chain kinase; leukocyte; extravasation; cholesterol

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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INTRODUCTION

Oxidation of low-density lipoproteins in the body has been correlated with various pathological conditions, including atherosclerosis, and autoimmune diseases (Levitan *et al.*, 2010). Elevated oxidized low-density lipoproteins (oxLDL) levels in the bloodstream have been identified as a risk factor for development of coronary artery disease and plaque formation (Berliner *et al.*, 2001; Diaz *et al.*, 1997; Holvoet *et al.*, 2001; Holvoet *et al.*, 1998; Toshima *et al.*, 2000). Exposure of the vascular endothelium to oxLDL leads to endothelial cell (EC) damage and dysfunction, including disruption of cell-cell adhesion (Gardner *et al.*, 1999; Liao and Granger, 1995), as well as impairment of nitric oxide release (Blair *et al.*, 1999). *In vivo*, oxLDL affects both large vessels and microvasculature and promotes leukocyte adhesion and transmigration in microvasculature (Lehr *et al.*, 1995; Lehr *et al.*, 1991; Liao and Granger, 1995). Furthermore, EC exposure to oxLDL enhances monocyte invasion through the endothelium *in vitro* (Hashimoto *et al.*, 2007). These changes in EC-leukocyte interactions are likely partially due to upregulation of adhesion molecules necessary for leukocyte adherence and transmigration, including intercellular cell adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (Cominacini *et al.*, 1997; Hashimoto *et al.*, 2007; Takei *et al.*, 2001).

In addition to increased adhesion molecule expression, EC exposure to oxLDL also induces changes in biomechanical properties, including increased contractility (Byfield *et al.*, 2006), stiffness (Byfield *et al.*, 2006; Chouinard *et al.*, 2008), and ability to realign with flow (Kowalsky *et al.*, 2008). It has been proposed that oxLDL acts as a cholesterol acceptor, removing cholesterol from the plasma membrane (Byfield *et al.*, 2006; Kowalsky *et al.*, 2008). Indeed, increases in cell stiffness (Byfield *et al.*, 2004; Byfield *et al.*, 2006) and traction forces (Norman *et al.*, 2010) have also been measured in cholesterol-depleted cells; these results suggest that oxLDL exposure or direct cholesterol removal triggers biomechanical changes through modification of the plasma membrane-cytoskeleton complex (Byfield *et al.*, 2004).

There is also evidence that the subendothelial layers of blood vessels stiffen in cardiovascular disease. Clinically, pulse wave velocity measurements have demonstrated increased vasculature stiffness in patients with atherosclerosis or hypertension (Blacher *et al.*, 1999; Boutouyrie *et al.*, 2002), and because these waves also reflect deep into microvasculature, it is believed that there is also a change in microvasculature stiffness. However, these measurements are unable to specify which parts of the blood vessel change biomechanically. Several *in vitro* studies have recently addressed this issue by scraping away the endothelium, or by cross-sectioning the artery to measure the media layer. Using these methods, the reported stiffness of healthy subendothelial matrices ranges from 2.5 kPa to 8 kPa (Engler *et al.*, 2004; Kothapalli *et al.*, 2010; Peloquin *et al.*, 2011), while arteries in ApoE-null mice, a model for atherosclerosis, are much stiffer, around 28 kPa (Kothapalli *et al.*, 2010). These experiments indicate that the mechanical properties of the subendothelial matrix in vasculature vary significantly in cardiovascular disease. Moreover, we hypothesize that the stiffness of microvasculature in a particular tissue varies with the stiffness of that tissue; for example, brain tissue is very soft (300–500 Pa), while collagenous bone tissue is much stiffer (~100 kPa) (Engler *et al.*, 2006; Norman and Aranda-Espinoza, 2010).

Leukocyte transmigration requires an interplay between signaling events and mechanical forces (Stroka and Aranda-Espinoza, 2010; Stroka and Aranda-Espinoza, 2011b). We have previously shown that stiffer EC substrates promote neutrophil transmigration through tumor necrosis factor- α (TNF- α)-activated endothelium (Stroka and Aranda-Espinoza, 2011b). This substrate stiffness-dependent transmigration is due to biophysical changes that occur in the endothelium; stiffer substrates support larger myosin light chain kinase

(MLCK)-mediated contractile forces upon neutrophil adherence to the endothelium (Stroka and Aranda-Espinoza, 2011b). Larger contractile forces lead to formation of intercellular gaps that promote transmigration. In addition, we showed that the fraction of transmigrated cells depends on the amount of ICAM-1 available for binding on the surface of the ECs, independent of substrate stiffness (Stroka and Aranda-Espinoza, 2011b). Because oxLDL exposure also leads to upregulation of ICAM-1 and increased contractility in ECs, we hypothesized that treatment of the endothelium with a physiological concentration of oxLDL also affects transmigration in a subendothelial matrix stiffness-dependent way. In particular, we explored transmigration of neutrophils, which primarily transmigrate in microvasculature, where oxLDL exposure has been shown to affect leukocyte-EC interactions, and where vessel stiffening is relevant in cardiovascular disease.

MATERIALS AND METHODS

Preparation of substrates

Thin (~80 μm) polyacrylamide gel substrates were prepared on activated coverslips as described by Wang and Pelham (Wang and Pelham, 1998) and as used previously in our publications (Norman and Aranda-Espinoza, 2010; Stroka, Aranda-Espinoza, 2009; Stroka and Aranda-Espinoza, 2011b). Concentrations in this work included 15% acrylamide + 1.2% bis (280 kPa), 8% acrylamide + 0.07% bis (5 kPa), and 3% acrylamide + 0.1% bis (0.87 kPa). After sulfo-SANPAH treatment and UV activation, gels were coated with 0.1 mg/mL fibronectin. Gel mechanical properties and surface-bound fibronectin were characterized as previously described (Norman and Aranda-Espinoza, 2010; Stroka and Aranda-Espinoza, 2009; Stroka and Aranda-Espinoza, 2011b).

Cell culture and treatments

HUVECs were purchased from Lifeline Cell Technology (Walkersville, MD) and cultured as previously described (Stroka and Aranda-Espinoza, 2011a; Stroka and Aranda-Espinoza, 2011b). Cells (passages 2–5, 4×10^5 total) were plated onto fibronectin-coated polyacrylamide gels and formed monolayers within 2 days (Stroka and Aranda-Espinoza, 2011b). After 2 days, cells were treated with 25 $\mu\text{g/mL}$ oxLDL for 24 hours prior to transmigration experiments. In some assays, cells were treated with oxLDL for only one hour prior to experiments. To inhibit MLCK, HUVECs were pre-treated with 10 μM ML-7 (Sigma, St. Louis, MO) for 8 minutes prior to transmigration assays. For removal of cholesterol, HUVECs were pre-treated with 5 mM methyl- β -cyclodextrin (M β CD; Fisher Scientific, Pittsburgh, PA) for one hour prior to transmigration assays. To block ICAM-1, cells were pre-treated with 10 $\mu\text{g/mL}$ monoclonal anti-human ICAM-1 (R&D Systems BBA3, Minneapolis, MN) for one hour prior to transmigration assays.

Isolation and oxidization of LDL

Normal human plasma, prepared in acid citrate dextrose, was purchased from a local blood bank (Life Source, Chicago, IL). LDL was isolated by sequential centrifugation in KBr between the density ranges 1.019 to 1.063 g mL^{-1} . The preparation was dialyzed against three changes of 10mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA at 4°C to remove KBr. To proceed to the copper oxidization, further dialysis against PBS (pH 7.4) was performed to reduce the EDTA presence. Copper sulfate was then added to LDL, with a final concentration of 25 μM . The oxidation was set in 37°C for 16 hours and stopped by adding 1 mM EDTA. Oxidation level of oxLDL was assayed by measuring the content of thiobarbituric acid-reactive substances (TBARS) in LDL and oxLDL by using TBARS Assay Kit (ZeptoMetrix, Buffalo, NY). The average TBARS values were 21.8 ± 5.1 nanomoles per MDA per milligram of LDL protein for oxLDL.

Transmigration assays

Neutrophils were isolated from human blood as previously described (Stroka and Aranda-Espinoza, 2009). Protocols were approved by the University of Maryland Institutional Review Board, and informed consent was obtained from all volunteers. Transmigration experiments are described elsewhere (Stroka and Aranda-Espinoza, 2011b). Following oxLDL treatment, HUVECs were washed thoroughly with PBS, and fresh regular HUVEC media was added. Neutrophils ($\sim 1 \times 10^6$ total) were added to the HUVEC monolayer and allowed to gravitate to the EC apical surface for about 30–60 seconds. Phase contrast timelapse microscopy was completed as previously described (Stroka and Aranda-Espinoza, 2011b). Fraction of neutrophil transmigration was quantified by dividing the number of neutrophils that transmigrated any time during the 30 minute timelapse by the total number of neutrophils in the first frame of that sequence. Neutrophils that entered or exited the field of view after the first frame of the sequence were not counted. The fraction of non-activated neutrophils was calculated by dividing the number of non-activated neutrophils in each timelapse sequence by the total number of neutrophils. Neutrophils that were activated had small protrusions (white arrows, Fig. 1A), while non-activated neutrophils remained spherical. All experiments were repeated at least 3 times. Hole area and density were quantified as previously described (Stroka and Aranda-Espinoza, 2011b). Briefly, in each of 4 independent experiments, 10–20 images were taken at each timepoint after addition of neutrophils. In each of those images, both the number of holes per area and size of holes were quantified in ImageJ. The final reported values are the means of the pooled data from 4 experiments. Statistical significance was determined using ANOVA and the pooled data.

Cell staining

HUVECs were fixed, blocked for non-specific binding, and immunostained for ICAM-1 as previously described (Stroka and Aranda-Espinoza, 2011b). Alternatively, HUVECs were fixed, permeabilized, blocked for non-specific binding, and immunostained with antibodies to β -catenin to label cell borders, or stained with phalloidin-TRITC to label F-actin (Stroka and Aranda-Espinoza, 2011a). Fluorescence microscopy was completed at room temperature on immunostained HUVECs using an inverted microscope (Olympus IX81). Images were captured with a Hamamatsu ORCA-ER CCD digital camera (Leeds Precision Instruments, Minneapolis, MD) using Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO). To quantify ICAM-1 expression, ImageJ was used to find the average intensity value (in AU) for each image. At least 10 images from 3 independent coverslips were captured under identical microscope conditions. From each image an average fluorescence intensity of all pixels was obtained, and those values were averaged from each of the three independent experiments to obtain the final result. ANOVA was used to determine statistical significance, by comparing all intensity values from the three experiments between the different stiffnesses.

RESULTS

Stiff substrates promote neutrophil transmigration through oxLDL-treated HUVECs

Using our *in vitro* model, we observed activation of neutrophils (Fig. 1A; white arrowheads) and subsequent transmigration through oxLDL-treated HUVECs using phase contrast microscopy (Fig. 1A, bottom). Meanwhile, few neutrophils on untreated HUVECs were activated (Fig. 1A, top). Neutrophils on top of the endothelium appeared bright white, while transmigration events could be identified by the phase-darkened portions of the neutrophils between the endothelium and the gel (Fig. 1A, bottom; white arrows). Twenty four-hour treatment of HUVECs with oxLDL promoted neutrophil transmigration on all substrates, in comparison with untreated HUVECs ($P < 0.001$ for all substrates) (Fig. 1B). Furthermore, transmigration increased with subendothelial matrix stiffness (Fig. 1B), from 26%

transmigration through ECs on 0.87 kPa, to 54% on 5 kPa ($P<0.01$). Transmigration on even stiffer substrates (280 kPa) was about 41% and was not different from 5 kPa or 0.87 kPa ($P>0.05$). Some neutrophils (~15%) were not activated after being plated onto an oxLDL-treated endothelium, but the fraction not activated was independent of substrate stiffness ($P>0.05$ across groups) (Fig. 1C), indicating that the differences in transmigration with substrate stiffness were due to the ability to transmigrate, not due to the ability to become activated.

ICAM-1 expression on oxLDL-treated HUVEC monolayer apical surface is independent of substrate stiffness

To evaluate the amount of ICAM-1 on the apical surface of the oxLDL-treated endothelium, we immunostained non-permeabilized HUVECs and quantified fluorescence intensity as a function of substrate stiffness (Figs. 2A and 2B). For both untreated and 24-hour oxLDL-treated HUVECs, ICAM-1 intensity was independent of substrate stiffness ($P>0.05$), though on all substrates there was increased ICAM-1 expression after 24 hours of oxLDL treatment ($P<0.05$) (Figs. 2A and 2B). Further evidence of similarities in ICAM-1 expression with substrate stiffness is that the fraction of non-activated neutrophils did not depend on substrate stiffness (Fig. 1C), since binding to ICAM-1 activates neutrophils via adhesion of neutrophil $\beta 2$ integrins.

Further, to evaluate the role of oxLDL-induced ICAM-1 expression in neutrophil transmigration, ICAM-1 on the apical surface of the oxLDL-treated endothelium was blocked using an ICAM-1 antibody. In this blockade assay, neutrophil transmigration was significantly reduced to the level of the untreated controls (Fig. 2C). Further, blocking ICAM-1 in oxLDL-treated HUVECs eliminated the effects of substrate stiffness on transmigration, as there was no difference in transmigration between the substrates ($P>0.05$).

Inhibition of MLCK-mediated EC contraction eliminates effects of substrate stiffness

We evaluated the possible mechanism by which substrate stiffness promotes neutrophil transmigration through oxLDL-treated endothelium. We hypothesized that MLCK played a role, and therefore we suppressed contraction in the 24-hour oxLDL treated HUVECs by incubating the monolayers with 10 μ M ML-7 to inhibit MLCK. Neutrophil transmigration was significantly reduced on all substrates ($P<0.05$), and the dependence of transmigration on substrate stiffness was eliminated ($P>0.05$ across substrates) (Fig. 3A). Importantly, the percent decrease in transmigration was significantly greater on 0.87 kPa in comparison with the stiffer substrates (Fig. 3B; $P<0.05$). Together, these results signify that contractility was higher in the oxLDL-only treated ECs on stiffer substrates. We also tested the role of contractility versus ICAM-1 expression using a 1-hour treatment of oxLDL or M β CD, where there is increased EC contractility (Byfield *et al.*, 2006) but still close to basal levels of ICAM-1. Neither of these treatments led to neutrophil transmigration (~0%), indicating that contractility does not promote transmigration without sufficient ICAM-1 expression.

Neutrophil transmigration injures the ox-LDL-treated endothelium on stiff substrates

OxLDL is known to cause EC dysfunction, and here we investigated how subsequent leukocyte transmigration through the endothelium might further damage the ECs. In untreated or 1 hour oxLDL-treated ECs (no transmigration), we observed intact monolayers up to 2 hours after addition of neutrophils. Remarkably, we observed formation of large holes, on the order of thousands of square microns, after neutrophil transmigration through 24-hour oxLDL-treated endothelium (Fig. 4). Cells at the perimeter of each hole detached from each other at one side, leaving the other sides still adhered to neighboring cells and to the substrate, indicating that holes formed due to cell retraction and not cell death. Both the size of the holes ($P<0.001$) and number per area ($P<0.001$) increased with increasing EC

substrate stiffness (Fig. 4A–B). By 2 hours after plating neutrophils, holes did not reseal; in fact, holes on 280 kPa were significantly larger than at 1 hour ($P < 0.001$; Fig. 4B). Histograms of hole area at 1 hour and 2 hours (Figs. 4C and 4D) indicate that at 2 hours, there were about half as many small holes ($\sim 1000 \mu\text{m}^2$) in comparison with 1 hour; meanwhile, there was a greater amount of larger holes at 2 hours. These results suggest one of two possibilities at 2 hours: either about half of the smaller holes became larger, or that the smaller holes healed, while more larger holes formed. Monolayers on soft substrates (0.87 kPa) were usually still intact 1 hour after plating neutrophils (Fig. 4E), while monolayers on stiffer substrates (5 kPa and 280 kPa) were severely disrupted (Fig. 4F). Further, inhibition of MLCK prevented hole formation in the endothelium after neutrophil transmigration (Figs. 4A and 4B). Thus, we conclude that the substrate stiffness-dependent neutrophil transmigration and EC injury were due to MLCK-mediated contractile forces, as we have previously found for the case of the TNF- α -activated endothelium (Stroka and Aranda-Espinoza, 2011b).

OxLDL treatment induces EC stress fiber formation on all substrates

To rule out that EC morphology, density, or F-actin cytoskeletal arrangement could not account for the dependence of transmigration on subendothelial matrix stiffness, we evaluated these parameters as a function of substrate stiffness. We immunostained for β -catenin as a marker of cell borders to observe oxLDL-treated HUVEC morphology and found no visible changes as a function of substrate stiffness (Fig. 5A), indicating constant monolayer cell density.

Because leukocytes may interact with F-actin as they cross this cytoskeletal barrier during transmigration, we observed the arrangement of the actin cytoskeleton as a function of subendothelial matrix stiffness. In F-actin-labeled untreated cells, less stress fibers formed on softer substrates (Fig. 5B). In comparison, in oxLDL-treated HUVECs, mature stress fibers formed on all substrates, with no apparent differences between substrates (Fig. 5B), suggesting that F-actin arrangement cannot account for the substrate stiffness-dependent transmigration behavior.

DISCUSSION

In this manuscript we present two important results: (1) oxLDL treatment of the endothelium and (2) stiffer subendothelial matrices both promote neutrophil transmigration. The ICAM-1 blockade experiment (Fig. 2C) suggests that enhanced ICAM-1 expression following oxLDL treatment accounts for the increase in neutrophil transmigration in comparison with untreated cells (Fig. 1B). However, an alternative, and equally plausible, explanation is that transmigration is promoted by some other mechanism that requires ICAM-1 binding, or that ICAM-1 adhesion and downstream signaling together act in concert to promote transmigration. Meanwhile, the MLCK inhibition experiment (Fig. 3) explored the mechanism by which subendothelial matrix stiffness influences neutrophil transmigration through the oxLDL-treated endothelium. Recently, direct measurements of traction forces in islands of ~ 15 ECs demonstrated larger contractile forces in EC monolayers on stiffer substrates (Krishnan *et al.*, 2011). In monolayers of cells exerting enhanced traction forces, there are larger intercellular gaps that can promote leakage of molecules, or, in our case, transmigration of leukocytes. Presumably, in a more contractile endothelium, it takes less energy for the leukocytes to break the endothelial cell-cell adhesions. Here, increased MLCK-mediated EC contraction on stiffer substrates led to more transmigration (Fig. 3) in the presence of the same amount of ICAM-1 (Figs 2A–2B). Notably, the percentage decrease in transmigration due to MLCK inhibition was larger on stiff substrates (5 kPa and 280 kPa) than soft substrates (0.87 kPa). These results indicate

higher contractility in ECs on stiff substrates after oxLDL treatment and possibly after neutrophil binding to ICAM-1, and this contractility promoted neutrophil transmigration.

We have previously characterized the kinetics of hole formation in relation to neutrophil transmigration through TNF- α -activated ECs, showing that hole formation initiates a few minutes after a maximum fraction of neutrophils has transmigrated (Stroka and Aranda-Espinoza, 2011b). However, for the case of TNF- α -treated ECs, the endothelium was mostly able to reseal the holes by 2 hours after plating neutrophils (Stroka and Aranda-Espinoza, 2011b). Here, with oxLDL treatment, the endothelium did not reseal by 2 hours (Figs. 4A and 4B), despite reduced transmigration in comparison with TNF- α . For both oxLDL and TNF- α treatments, holes are likely due to proteases released by neutrophils either during or after transmigration, as they migrate between the endothelium and the gel. These proteases could cleave endothelial cell-cell and/or cell-substrate adhesions, and larger EC contractile forces on stiffer substrates could promote retraction as proteases act on them, leading to large holes. Also, neutrophil transmigration and subsequent migration beneath the monolayer could weaken endothelial cell-cell and/or cell-substrate adhesions. It would be useful to repeat our experiments using a system where neutrophils could continue migrating through the matrix below the endothelium; this could be done using a material neutrophils can degrade, such as collagen. If this type of injury also occurs *in vivo* due to the presence of oxLDL, transmigration through the endothelium in stiffened vasculature could add another layer of complication to the molecular and cellular events in cardiovascular disease.

Because neutrophil transmigration occurs primarily in the microvasculature, where shear stress is very low, we chose to investigate the static condition. In the future we could examine whether shear stress plays a role in EC substrate stiffness-dependent neutrophil transmigration. Shear stress is also known to affect EC biomechanical properties (Davies *et al.*, 1994; Sato *et al.*, 1987; Shiu *et al.*, 2004). Thus, fluid flow might also affect MLCK-dependent cell contraction along with subendothelial matrix stiffness.

In summary, we showed that 24-hour oxLDL treatment of the vascular endothelium promoted neutrophil transmigration, with stiff EC substrates further promoting transmigration. ICAM-1 expression was enhanced on all substrates after 24-hour oxLDL treatment, an effect that might account for the promotion of transmigration in comparison with the untreated controls. Furthermore, large holes formed in the monolayers on stiff substrates following neutrophil transmigration and were further indicators of enhanced contractile forces in ECs on stiff substrates. Using an inhibitor of MLCK in oxLDL-treated ECs, we showed that MLCK-mediated EC contractility was responsible for the dependence of subendothelial matrix stiffness on transmigration and hole formation in the monolayers. These results indicate that microvasculature stiffness, which likely varies depending on tissue location and health, is an important regulator of the immune response in the presence of oxLDL. These results also suggest that the effects of EC exposure to oxLDL during the onset of atherosclerosis depend on the biomechanical changes that take place in vasculature as the disease develops.

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Figure 1.

Stiffer substrates promote neutrophil transmigration through 24-hour oxLDL-treated human umbilical vein endothelial cells (HUVECs). (A, top) Phase contrast time sequence of two neutrophils on untreated HUVECs. Cell labeled with “1” does not activate, while cell labeled with “2” does activate, as indicated by protrusive morphology (white arrowheads). (A, bottom) Also shown is a phase contrast time sequence of two neutrophils on 24-hour oxLDL-treated HUVECs. Cell labeled with “3” transmigrates through the endothelium (portion beneath the endothelium indicated by white arrow), while cells labeled with “4” is activated (white arrowheads) but does not transmigrate. Transmigration is indicated by a change in phase from bright white (on top of the endothelium) to darkened (between the endothelium and the gel; white arrows). T=0 corresponds to the time when transmigration begins in cell 3. Image sequence with cells 1 and 2 are representative of activity on untreated HUVECs at all timepoints. Scale bar is 10 μ m and applies to all images. Both untreated (top) and oxLDL-treated (bottom) are on 5 kPa substrate. (B) Fraction of transmigrated neutrophils through untreated or oxLDL-treated HUVECs, as a function of substrate stiffness. ** indicates $P < 0.01$ with 5 kPa by one-way ANOVA followed by Tukey’s honestly significant difference criterion. Bars are averages of at least 8 experiments, while error bars indicate standard error. (C) Fraction of neutrophils not activated as a function of substrate stiffness. Bars are mean $\pm$ standard error of 4 experiments. $P > 0.05$ across groups by ANOVA.

Figure 2.

ICAM-1 expression is enhanced in 24-hour oxLDL-treated HUVECs, and blocking ICAM-1 significantly reduces transmigration on all substrates. (A) HUVECs were immunostained for ICAM-1 and fluorescence images were taken at many different locations across each of at least 3 different monolayers for each substrate. Bars are mean $\pm$ standard error of 3 experiments. $P > 0.05$ by ANOVA across substrates for untreated and oxLDL-treated HUVECs. For each substrate, * indicates that $P < 0.05$ between control and oxLDL using a t-test. (B) Representative fluorescence images of HUVEC monolayers immunostained for ICAM-1. Scale bar is 20 μm and applies to all images in panel B. (C) HUVECs were left untreated, or treated with oxLDL followed by an ICAM-1 antibody and neutrophil transmigration was quantified as a function of substrate stiffness. Bars are mean $\pm$ standard error of at least 3 experiments. No statistical difference was found between the untreated and oxLDL+ICAM-1 antibody-treated HUVECs, nor was there any dependence on substrate stiffness for either case ($P > 0.05$).

Figure 3.

Inhibition of MLCK-mediated EC contraction reduces transmigration on all substrates and eliminates the effects of substrate stiffness. (A) 24-hour oxLDL-treated HUVECs were incubated with ML-7 to inhibit MLCK and neutrophil transmigration was quantified for each substrate. Bars are mean $\pm$ standard error of 3 experiments. Statistical significance between oxLDL-treated HUVECs and oxLDL+ML-7-treated HUVECs on the same substrate stiffness is indicated by * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$). (B) The percentage decrease in transmigration was quantified by dividing the difference between the transmigration fractions for oxLDL and oxLDL+ML-7 by the transmigration fraction for oxLDL, and multiplying by 100%. * indicates $P < 0.05$ with 5 kPa and 280 kPa by ANOVA, followed by Tukey's honestly significant difference criterion.

Figure 4.

Stiff substrates promote hole formation in the 24-hour oxLDL-treated endothelium following neutrophil transmigration. (A) Quantification of hole density as a function of substrate stiffness at 1 hour and 2 hours after plating neutrophils. *** or ^^ indicates $P < 0.001$ with 5 kPa and 280 kPa, while & indicates $P < 0.05$ with 280 kPa by ANOVA followed by Tukey's honestly significant difference criterion, in comparison with data from the same timepoint. ### indicates $P < 0.001$ with same stiffness 1-hour timepoint without ML-7, using a t-test. Bars are mean $\pm$ standard error of $N = 42\text{--}70$ images from at least 4 experiments. (B) Quantification of hole area as a function of substrate stiffness at 1 hour and 2 hours after plating neutrophils. *** indicates $P < 0.001$ with 5 kPa at 1 hour, while ^^ indicates $P < 0.001$ with 5 kPa at 2 hours, both by ANOVA followed by Tukey's honestly significant difference criterion. In addition, $P < 0.001$ between hole areas at 1 hour and 2 hours for 280 kPa using a t-test. # or ### indicates $P < 0.05$ or $P < 0.001$, respectively, with same stiffness 1-hour timepoint without ML-7, using a t-test. Bars are mean $\pm$ standard error of $N = 8\text{--}204$ holes from at least 4 experiments. (C) Histogram of hole areas for HUVECs on 280 kPa at 1 hour after plating neutrophils. (D) Histogram of hole areas for HUVECs on 280 kPa at 2 hours after plating neutrophils. (E) Phase contrast image of mostly intact HUVEC monolayer on a soft (0.87 kPa) substrate, 1 hour after plating neutrophils. (F) Phase contrast image of HUVEC monolayer on a stiff (280 kPa) substrate, 1 hour after plating neutrophils. Large holes have formed in the monolayer. Scale bar is 50 μm and also applies to panel E.

Figure 5.

HUVEC morphology in the monolayer is independent of substrate stiffness. (A) 24-hour oxLDL-treated HUVEC monolayers on all substrates were immunostained for β -catenin as a marker of cell borders. (B) Untreated and oxLDL-treated HUVEC monolayers were stained for F-actin. Scale bar for panel A, β -catenin images, is 50 μ m, while scale bar for panel B, F-actin images, is 20 μ m. Substrate stiffness is indicated at the top of each column.