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Neutrophil adhesion and chemotaxis depend on substrate mechanics

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Abstract

Neutrophil adhesion to the vasculature and chemotaxis within tissues play critical roles in the inflammatory response to injury and pathogens. Unregulated neutrophil activity has been implicated in the progression of numerous chronic and acute diseases such as rheumatoid arthritis, asthma, and sepsis. Cell migration of anchorage-dependent cells is known to depend on both chemical and mechanical interactions. Although neutrophil responses to chemical cues have been well characterized, little is known about the effect of underlying tissue mechanics on neutrophil adhesion and migration. To address this question, we quantified neutrophil migration and traction stresses on compliant hydrogel substrates with varying elasticity in a micro-machined gradient chamber in which we could apply either a uniform concentration or a precise gradient of the bacterial chemoattractant fMLP. Neutrophils spread more extensively on substrates of greater stiffness. In addition, increasing the stiffness of the substrate leads to a significant increase in the chemotactic index for each fMLP gradient tested. As the substrate becomes stiffer, neutrophils generate higher traction forces without significant changes in cell speed. These forces are often displayed in pairs and focused in the uropod. Increases in the mean fMLP concentration beyond the K_D of the receptor lead to a decrease in chemotactic index on all surfaces. Blocking with an antibody against β_2 -integrins leads to a significant reduction but not an elimination of directed motility on stiff materials, but no change in motility on soft materials, suggesting neutrophils can display both integrin-dependent and integrin-independent motility. These findings are critical for understanding how neutrophil migration may change in different mechanical environments *in vivo* and can be used to guide the design of migration inhibitors that more efficiently target inflammation.

1. Introduction

Neutrophils are subject to numerous spatial and temporal cues in their normal physiological environment, including gradients of signaling molecules, mechanical interactions with surrounding tissues, and direct contacts with other cells (Simon and Green, 2005). It is well appreciated that chemical cues exchanged between neutrophils and their environment play an important role in determining effector functions required for homeostasis and many detailed studies of such interactions have been reported (Tranquillo et al., 1988, Devreotes and Zigmond, 1988, Herzmark et al., 2007). Unstimulated neutrophils are typically non-adherent,

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but their adhesiveness can be upregulated by chemoattractants within minutes (Alon and Ley, 2008).

Sequential interactions between neutrophil receptors and endothelium bound adhesion ligands such as selectins and ICAMs leads to rolling, firm adhesion, migration, and transmigration. β_2 integrin receptors such as LFA-1 and Mac-1 are the most relevant for neutrophil adhesion and migration. Active conformations of integrins on a neutrophil can be induced by chemoattractant binding to G-protein coupled receptors through inside-out signaling or by direct ligand binding through outside-in signaling (Alon and Ley, 2008). Mechanical forces loaded onto integrin receptors during outside-in signaling can accelerate conformational rearrangements leading to stabilization of high affinity conformations and cell arrest (Puklin-Faucher et al., 2006). After migration along a blood vessel wall to an appropriate gap junction, a neutrophil transmigrates through the endothelium into the surrounding extracellular matrix. Neutrophils are capable of infiltrating virtually every organ within the body. Therefore, the ability of a neutrophil to navigate through diverse mechanical environments is critical to tissue homeostasis and host defense.

Previous studies of anchorage-dependent cell motility have elucidated how cells of endothelial, epithelial, or fibroblast origin process mechanical information in their surroundings (Reinhart-King et al., 2003, Paszek et al., 2005, Reinhart-King et al., 2005, Reinhart-King et al., 2008, Smith et al., 2007, Dembo and Wang, 1999, Beningo et al., 2001, Munevar et al., 2001, Balaban et al., 2001). These studies have uncovered a number of important principles, namely that anchorage-dependent cells are capable of sensing and responding to changes in the biophysical properties of their underlying two dimensional matrices through integrin receptors and focal adhesions (Dembo and Wang, 1999, Choquet et al., 1997, Reinhart-King et al., 2005, Balaban et al., 2001, Gupton and Waterman-Storer, 2006). In order to migrate, anchorage-dependent cells must adhere to and generate tension on their underlying substrates through integrin receptors. In the towing model of cell motion, cells must extend their lamellipodia and grab hold of the substrate through integrins, generate cellular contraction which requires exerting tension on the substrate, and then release the uropod which likely requires exertion of mechanical forces or weakened substrate adhesion, or both (Munevar et al., 2001). Another key concept elucidated from prior studies of cell migration is the presence of a biphasic relationship between cell speed and substrate ligand density or substrate mechanics (Palecek et al., 1997, Peyton and Putnam, 2005). It has been shown that maximal migration speeds are typically observed at intermediate levels of adhesion where a balance between generation of propulsive forces within the lamellipod and retraction of the cell body at the rear is achieved.

Given that integrins are required to exert forces on a substrate, substrate compliance should modulate the local concentration of adhesive stresses under a cell; in turn, for a given applied force, substrate compliance could modulate the clustering of adhesion receptors and hence signaling. Pelham and Wang (1977) showed that the compliance of the substrate could affect the size of focal adhesions as well as the rate of cell migration. Specifically, they showed that 3T3 and NRK cells have higher rates of cell motility and lamellipodial ruffling, respectively, when substrate rigidity is decreased (Pelham and Wang, 1997). Recently, Chan and Odde (2008) showed that molecular clutches involving actin and myosin link the cell to the substrate and act as a motor-clutch system that can inherently sense and respond to the elasticity of the substrate. A recent theoretical model from our laboratory (Paszek et al., 2009) illustrates that substrate stiffness can induce receptor clustering by presenting an energy barrier preventing integrins from becoming dispersed. Consistent with the idea that substrate mechanics can alter cell motility, Lo and coworkers presented evidence of durotaxis, in which cells migrate toward stiffer regions of substrate (Lo et al., 2000).

A further question is how tensions and forces within compliant substrates affect adhesion and motility. Forces generated through the substrate can lead to biochemical responses through outside-in signaling, such as phosphorylation, as well as the recruitment of scaffold proteins; then, further downstream signaling pathways are activated leading to changes in cell morphology and motility (Giannone and Sheetz, 2006). However, the role of tension in durotaxis is not well defined. There is conflicting evidence of the role in tension is controlling receptor clustering. Beningo and coworkers indicated that tension preceded the formation of focal adhesions (Beningo et al., 2001), but Balaban and coworkers presented contrary results that suggested tension is generated when or after focal adhesions form (Balaban et al., 2001). The correlation between substrate tension and motility is still unclear and needs to be studied further.

Despite the importance of neutrophils and other amoeboid cells of the immune system, relatively little has been done to probe the mechanochemical control of neutrophil migration. Classic studies performed by Zigmond and coworkers on glass surfaces have demonstrated that neutrophil responses are influenced by both the mean chemoattractant concentration and the slope of the gradient of concentration (Devreotes and Zigmond, 1988, Tranquillo et al., 1988). Recently, we showed that the traction stresses of neutrophils, which migrate rapidly and do not form focal adhesions, could be measured by traction force microscopy (TFM) despite being relatively weak compared to fibroblasts and other endothelial cells (Smith et al., 2007). TFM images showed that during both chemokinesis and chemotaxis toward a chemoattractant exuded from a pipette, neutrophils display a contractile stress that is concentrated in the uropod which appears to drive motion through tail contraction (Smith et al., 2007). Other amoeboid cells such as Dictyostellium also display maps of traction stresses with high concentrations of traction stresses in the uropod (Lombardi et al., 2007), suggesting a shared mechanism of locomotion among amoeboid cells.

Since motility in neutrophils is associated with tail contraction, membrane interactions with a substrate, which are in turn affected by substrate compliance, might affect neutrophil motility. Recently, Oakes and coworkers showed that on fibronectin-coated gels of different compliances, neutrophils spread and crawl best on stiffer gels, and appear to move more faithfully toward chemoattractant delivered from a pipette (Oakes et al., 2009). Further, based on a different imaging methodology, they substantially confirmed the role of tail contraction in leukocytes by showing that traction stresses were mainly distributed between the uropod and mid-section of the cells as motility progresses. In this paper, we will describe experiments conducted contemporaneously which both complement and extend this work at higher resolution (Jannat, 2009).

In essence, our approach is to use a custom micromachined chamber to make precise gradients of the bacterial chemoattractant fMLP, and to measure the chemotactic response of neutrophils on gels of different elasticity coated with ICAM-1. On each gel we are thereby able to quantify the changes in neutrophil responses as a function of two important chemical cues, the mean concentration of chemoattractant and the steepness of an fMLP gradient. We find that the response of a neutrophil to these cues can vary significantly depending on the mechanical properties of the underlying substrate. To explore how this tactile response depends on integrin-mediated signaling pathways, we blocked integrin molecules and measured the effect on migration parameters. Our results show that integrin engagement is required for motility on stiff hydrogels (12kPa), but not on soft hydrogels (2kPa). Furthermore, high resolution TFM imaging of the traction stresses indicate that during chemotaxis on stiffer gels, centers of force and contraction are punctate, well organized, and highly localized to the uropod. These force centers weaken during motility on softer gels. Thus, the mechanism of directed neutrophil chemotaxis is markedly different depending on the mechanical environment and includes a complex interplay of chemical signaling and interfacial mechanics.

2. Materials and Methods

2.1. Reagents

Adhesive ligands were Protein G (Pierce Biochemicals, Rockford, IL), human ICAM-1 Fc, and E-Selectin Fc (R&D Systems, Minneapolis, MN). TS1/18 β 2 integrin antibody was used at 10 μ g/mL to block cell surface receptors (Pierce Biochemicals, Rockford, IL), Neutrophils were activated with fMLP chemoattractant peptide (Sigma-Aldrich, St. Louis, MO), Cell contractility through RhoA was inhibited using 10 μ M Y-26732 ROCK inhibitor (Calbiochem, San Diego, CA).

2.2. Isolation of neutrophils

Whole blood was obtained from healthy human donors by venipuncture and collected in BD Vacutainer tubes containing citrate-EDTA anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Whole blood was layered onto Polymorphprep density gradient medium (Axis-Shield, Oslo, Norway) and centrifuged at 500 g for 1 hour. After centrifugation, the neutrophil fraction was aspirated and washed once by centrifugation in HBSS without Ca^{2+} and Mg^{2+} . Washed neutrophils were then resuspended in HBSS without Ca^{2+} and Mg^{2+} supplemented with 0.1% human serum (Sigma-Aldrich, St. Louis, MO) and 10mM HEPES (Invitrogen, Carlsbad, CA). Prior to use in flow chamber assays, neutrophil solution was supplemented with Ca^{2+} and Mg^{2+} .

2.3. Preparation of hydrogel substrates

Polyacrylamide hydrogels were prepared as described previously with slight modifications (Pelham and Wang, 1997). Briefly, Corning rectangular glass coverslips were passed through the flame of a Bunsen burner, swabbed with 0.1N NaOH solution, and allowed to air-dry. Coverslips were coated with 3-aminopropyl-trimethoxysilane (APTS, Sigma-Aldrich, St. Louis, MO) and allowed to dry for several minutes inside a chemical fume hood. Excess APTS was removed by rinsing in distilled water. Coverslips were then activated by a 30 minute incubation in 0.5% glutaraldehyde solution at room temperature, rinsed in distilled water, and air-dried.

Acrylamide solutions containing either 5% or 7% w/v of a 40% acrylamide stock solution (Bio-Rad, Hercules, CA), concentrations of N,N'-methylene-bis-acrylamide ranging from 0.1% to 0.76% (Bis, 2% w/v stock solution, Bio-Rad), 35 mM HEPES, pH-8, and distilled water were prepared. The concentration of Bis was varied to control the mechanical properties of the hydrogel. As Bis concentration is increased, the elasticity of the resulting hydrogel increases. 6-((acryloyl)amino)hexanoic acid (N6) crosslinker was synthesized according to the method of Pless et al. (Pless et al., 1983) and co-polymerized with acrylamide and Bis in order to covalently link proteins to the surface of the hydrogel and render it adhesive to cells. Acrylamide solutions were degassed and 0.5% w/v Irgacure 2959 photoinitiator solution (Ciba, Newport, DE) was added to start polymerization.

A drop of gel solution was dispensed onto the activated side of a rectangular glass coverslip. A second Rainex-coated glass coverslip was placed on top of gel droplet to flatten the solution, and the entire assembly was inverted and allowed to sit until beads had settled to the top surface of the gel. Gels were photopolymerized by irradiating with a 365 nm UV lamp (Ultraviolet Products, Upland, CA) for 10-15 minutes until the edges of the gel receded from the top coverslip. The top coverslip was gently peeled away leaving a thin gel immobilized on a rectangular glass coverslip. Gels were rinsed with ice cold distilled water and trimmed to dimensions compatible with the main channel area of the micromachined gradient generator. Gel stiffnesses were confirmed by mechanical testing using a Q800 Dynamic Mechanical Analysis instrument (TA Instruments, New Castle, DE). Samples were dynamically

compressed at a strain rate of $-10\%/minute$ to a final strain of -60% . The elastic modulus of each gel sample was determined from the slope of stress and strain curves.

2.4. Fabrication and assembly of microfluidic flow chambers

Gradient-generating microfluidic networks were designed according to principles described by Dertinger and Whitesides (Dertinger et al., 2001). Microfluidic chambers used in this study were fabricated by bonding PDMS chips (Sylgard 184, Dow Corning, Midland, MI) to glass coverslips as described previously (Li Jeon et al., 2002). PDMS chips were prepared from silicon wafer templates using photolithography as shown in Figure 1. A negative master template was prepared by depositing a thick film of SU8 photoresist (MicroChem, Boston, MA) onto the surface of a clean 3-in silicon wafer. The photoresist film was patterned by exposure to UV light through a high-resolution negative transparency mask in a Karl Suss mask aligner. After exposure, uncrosslinked regions of the wafer were developed away leaving a raised network of channels on the surface of the wafer. PDMS chips with embedded channels were prepared by pouring a 10:1 ratio of PDMS polymer to cross-linking agent onto the silicon template, degassing, and curing in an oven for 1 hour at 80°C . Cured PDMS chambers were cut out and peeled away from the template wafer. Inlet and outlet ports were punched into the chambers using a 20 gauge blunt end needle.

To permanently seal PDMS chambers to glass coverslips containing immobilized hydrogels, PDMS and glass surfaces were briefly exposed to reactive oxygen plasma under vacuum. The main channel of the microfluidic device was aligned with the hydrogel substrate on the glass coverslip, and the surfaces were pressed into contact. Hydrogels within assembled microfluidic devices were rehydrated and incubated with 0.5 mg/mL of Protein G diluted in 50 mM HEPES buffer, pH-6 at 4°C . Unreacted N6 crosslinker on the surface of the hydrogel was capped by incubating with a 1:100 dilution of ethanolamine for 30 minutes at room temperature. Hydrogels were rinsed with 0.1M sodium bicarbonate buffer, incubated with $50\text{ }\mu\text{g/mL}$ of ICAM-1 Fc and $50\text{ }\mu\text{g/mL}$ of E-Selectin Fc at room temperature, and used immediately after incubation was complete. Each assembled microfluidic device was used once.

2.5. Operation of microfluidic flow chambers

Assembled microfluidic devices were vacuum filled according to the method of (Monahan et al., 2001) and connected to syringes filled with chemoattractant gradient solutions. Gradient solutions were pumped through the device using an infusion/withdrawal syringe pump (Harvard Apparatus, South Natick, MA) set up inside a heated microscope stage with temperature controlled at 37°C . Flowrates of each gradient solution were controlled throughout the duration of an experiment resulting in a wall shear stress of less than 1 dyne/cm^2 . The spatial profile of the gradient was tracked during experiments by monitoring the fluorescent intensity of fluorescein dye introduced at different concentrations into each gradient solution. Once fMLP gradients were established, cells were loaded into the microfluidic chamber through a dedicated side inlet port. Neutrophils captured from free stream flow rolled along the surface of the gel and became firmly adherent.

2.6. Cell tracking and quantitation of motility

The positions of firmly adherent cells at different positions within the chamber were captured using a Nikon Inverted Eclipse TE300 microscope with a Nikon $20\times$, numerical aperture 0.40 objective. Time-lapse images were recorded at 30 second intervals for a minimum of 15 minutes. The position of cell centroids at each time interval was determined using ImageJ software. Mean-squared displacements for the centroids of each cell were calculated and used to determine individual cell speed. In the presence of linear gradients within the microfluidic chamber, the chemotactic index was calculated by measuring the distance traveled in the

direction of the gradient divided by the total path length. Data from individual cells was averaged to obtain a single value for each parameter and experimental condition evaluated.

2.7. Traction force microscopy

Traction force microscopy was performed as described previously on firmly adherent neutrophils within the microfluidic chamber using a Nikon Inverted Eclipse TE300 microscope with a Nikon 100 \times , numerical aperture 1.30 oil objective (Reinhart-King et al., 2003, Smith et al., 2007). A phase contrast and corresponding fluorescent bead image were captured simultaneously for each cell at different time intervals. At the end of each experiment, cells were detached from the hydrogel by addition of a 1M EDTA or 0.25% trypsin-EDTA solution, and an image of the unstressed field of view was taken. Traction forces were determined based on deformations of the hydrogel substrate reported by the motion of fluorescent beads embedded within the top surface. Using custom-written LIBTRC 2.0 software, bead displacements within the gel were calculated and the most likely surface traction vectors were obtained as described by Dembo and Wang (Dembo and Wang, 1999). The overall force $|F|$ exerted by the cell on the substrate is an integral of the traction stress over the cell contact

region, $|F| = \int \int \sqrt{T_x^2(x, y) + T_y^2(x, y)} dx dy$, where $T(x, y) = [T_x(x, y), T_y(x, y)]$ is the continuous field of traction vectors defined at any spatial position (x, y) within the contact region.

3. Results

3.1. Neutrophil spreading and adhesion depend on hydrogel mechanics

To investigate the spreading and adhesion of neutrophils on hydrogels in response to spatially and temporally controlled chemical cues, a microfluidic gradient generator was fabricated as described in Figure 1. Cells were perfused into chambers, allowed to adhere to the surface of the hydrogel and exposed to a range of well-defined adhesive, chemical, and mechanical conditions (Figure 2). Stable chemical gradients were generated over hydrogels with shear moduli ranging from 300 Pa (soft) to 12,000 Pa (stiff). The values of hydrogel moduli tested in our studies encompass the range of tissue mechanics that a neutrophil could encounter *in vivo* (Paszek et al., 2005, Engler et al., 2006). To understand the influence of substrate mechanics on cell spreading, neutrophils were activated with a uniform solution of 10 nM fMLP and imaged with time lapse microscopy. As shown in Figure 3a, the time averaged spreading area of neutrophils on hydrogels with constant ligand density increased as the underlying substrate modulus was increased. Neutrophils placed on stiff 12kPa hydrogel surfaces deformed and flattened extensively across the surface generating large spread areas. Spreading on the softer surfaces was much less pronounced. The difference in cell area between the 12kPa and 300Pa hydrogel conditions evaluated was approximately 3-4 fold.

The difference in time-averaged cell spreading area on different hydrogel stiffnesses can be explained by two separate observations. Figure 3b and c show that the initial spread area of a neutrophil just after activation on softer gels is less than that of a cell on a stiff gel. The cell area on the soft gel decreases even further over the span of 10 minutes. After 10 minutes, the neutrophil on the soft hydrogel appears white in phase contrast microscopy indicating its morphology has become more round and the cell body is no longer in the same focal plane as the hydrogel (Figure 3b). Although neutrophils undergo a transition to a rounder morphology on soft gels over time, they remain viable and continue to migrate. Graphs of cell area over time confirm the trend observed by microscopy. Measurements of individual cell areas for a particular gel stiffness plotted as a function of time show that cell adhesion and spreading area is stabilized on stiff 12kPa gels, but decreases as a function of time when the hydrogel stiffness is decreased (Figure 3d). To quantify this observation, the time at which cell area decreased by 50% for a particular gel stiffness was determined (Figure 3e). On soft gels (<7000 Pa),

interactions between the neutrophil and hydrogel substrate lead to a significant decrease in spread area in less than 5 minutes.

3.2. Sensitivity of chemotactic response depends on hydrogel stiffness

We used time-lapse microscopy to observe neutrophil motility in response to varying fMLP cues on hydrogels of varying stiffness. Figure 4 shows the initial and final positions of several cells migrating in response to a uniform stimulus of 10 nM fMLP (a) or a gradient of fMLP of 10nM/10 μ m (b). The data shows that cells on the softest gel in 10 nM uniform fMLP are weakly adherent and migration is biased in the direction of marginal flow in the chamber (left to right). When stimulated with a gradient of fMLP, cell migration on the 0.3kPa gel still appears random, although it is no longer biased in the direction of fluid flow. At an intermediate gel stiffness of 7kPa, cell motility appears random in response to a uniform concentration of 10 nM fMLP and becomes somewhat more directed in the presence of a gradient (10nM/ μ m). Lastly, on the stiff 12kPa gel, cell motility is random in response to uniform 10 nM fMLP stimulation and becomes highly directed in response to stimulation with a gradient. These results show that neutrophils are capable of responding more strongly to a chemical gradient on stiffer hydrogels compared with neutrophils interacting with softer substrates.

To further quantify the differences observed in neutrophil trajectories on hydrogels of varying matrix rigidity, migration parameters such as speed and chemotactic index were measured. The chemotactic index is defined as the distance a neutrophil travels in the direction of increasing gradient divided by the total distance traveled (Lauffenburger and Linderman, 1993). Therefore, the chemotactic index ranges from zero to one and is an index of cell directionality. As shown in Figures 5 a and c, neutrophils are highly motile on hydrogels over a range of stiffnesses during chemokinesis and chemotaxis. As expected, the chemotactic index during chemokinesis (10 nM) is approximately zero for all hydrogel stiffnesses (Figure 5b). In contrast, significant increases in the chemotactic index are observed with stiffer gels in a chemotactic gradient (10 nM/ μ m). On soft hydrogels (0.3 kPa), the chemotactic index in a gradient is only slightly higher than cells in the presence of uniform 10 nM fMLP. The results observed on the stiff gel compare well to previous reports of changes in the chemotactic index in neutrophils in response to different gradients on glass (Herzmark et al., 2007). Both observations of cell trajectories and calculated migration parameters show that changes in hydrogel mechanics influence a neutrophil's response to stimulation with fMLP gradients.

We also determined how changes in substrate stiffness affect the spatial distribution of forces. As shown previously, we find that on stiff hydrogels the highest traction forces are located at the rear of the cell relative to motion (Figure 6a,b) (Smith et al., 2007). Using TFM analysis, we show that on most gel materials, directed motion is driven by punctate, discrete force centers in the uropod. As hydrogel stiffness is decreased, a corresponding decrease in the intensity and spatial area of these regions of high stress within the cell at the rear is observed. At the lowest gel stiffness, the traction stress map displays only a small region of high force that is no longer oriented at the rear of the cell with respect to motion or the external fMLP gradient (Figure 6e). The stress maps illustrate that a steep chemotactic gradient results in an asymmetric distribution of traction forces in neutrophils on stiff gel substrates and that decreased force asymmetry is observed on softer gels.

3.3. Effect of hydrogel stiffness on neutrophil chemotaxis

Because the fMLP gradient used in Figure 5 resulted in both a high mean concentration of fMLP and a high gradient steepness ($C_m=10$ nM, slope=10nM/10 μ m), we set out to determine whether the effect of substrate modulus on chemotactic sensitivity of neutrophils would be altered if cells were stimulated with the same gradient but with different mean fMLP concentrations that span the K_d of the receptor, or with a shallower gradient. Such precise

control of the fMLP gradient is made possible with by our micromachined chamber. Previous work has demonstrated that when cells are in a gradient, fMLP concentrations near the receptor K_d produce maximal migration responses (Zigmond, 1977). The K_d of the fMLP receptor is known to be approximately 10 nM (Herzmark et al., 2007). We therefore examined four equivalent gradients of fMLP with different C_m by examining different subregions of a hydrogel within a microfluidic gradient of 0-56.25 nM set over a channel width of 1125 μm . This produced a gradient of 0.5 nM/ μm or 5nM/10 μm . The response of the chemotactic index for each gradient and mean concentration were quantified for five different gel stiffnesses ranging from 300 Pa to 19kPa (Figure 7). The basic trend that the CI increases with increasing gel stiffness is maintained at all values of C_m . As the mean concentration is increased to 35 nM and above for a gradient slope of 0.5 nM/cell diameter, the effect of mechanics on chemotactic sensitivity becomes less pronounced, but only because chemotaxis is itself decreased because of receptor saturation. Because fMLP receptor binding is saturable, receptor occupancy is less sensitive to the gradient of chemoattractant when the mean concentration is high.

In addition to considering the potential effect of the mean concentration of fMLP, we also examined the possibility that the observed effect of substrate modulus on chemotaxis was simply due to a change in cell size that affected the length scale over which gradient sensing could occur. To determine whether the decrease in cell radius on soft gels explained the reduced chemotactic sensitivity, we normalized the measured chemotactic index on each gel stiffness from Figure 5d ($C_m=100\text{nM}$, slope= $10\text{nM}/10\mu\text{m}$) to cell radii obtained from cell spreading data (Figure 3). As shown in Figure 7, accounting for changes in cell radii does not change the trend we report in Figure 5d. Our analysis suggests that simple changes in cell geometry are not sufficient to account for the decrease in chemotaxis that we observe on softer gels.

3.4. Neutrophil integrin requirements during chemotaxis depend on substrate stiffness

Because neutrophil migration on glass and plastic surfaces is known to be mediated by integrin receptors and because integrins are responsive to changes in substrate mechanics, and because we functionalized our surfaces with the β 2-integrin ligand ICAM-1, we examined requirements for integrin receptor usage during neutrophil migration on hydrogels functionalized with ICAM-1 (Woolf et al., 2007, Choquet et al., 1997). Similar to 2D results on glass and plastic surfaces, we find that blocking integrins with a widely used β 2 integrin antibody (TS1/18) reduces cell spreading on both soft (2kPa) and stiff (12kPa) gels (Figure 8). Surprisingly, integrin inhibition does not significantly affect cell speed on either soft or stiff substrates. For neutrophils migrating on stiff gels, a significant decrease in the chemotactic index is observed, while the chemotactic index of cells on soft gels remains the same. Migration parameters of neutrophils on soft (2kPa) gels appear to be unaffected by integrin blocking. This suggests that neutrophils on soft gels may not employ or activate β 2 integrins to the same degree as that observed on the stiff gels.

4. Discussion

In this study, we measured neutrophil chemokinesis and chemotaxis on hydrogel materials under well defined chemoattractant gradients imposed upon the cell in a micromachined gradient chamber. We believe we are the first to combine these two methodologies; most often, chemotaxis on hydrogels has been measured by releasing chemoattractant from a pipette (Smith et al., 2007; Oakes et al., 2009). As a result, we are able to understand the relationship between the magnitude and steepness of the chemoattractant gradient and directed cell motility on hydrogels.

We have observed that neutrophil migration is strongly dependent on shear modulus of the gel. In a chemotactic gradient, varying gel stiffness from 300-12,000 Pa can switch motility from

random to directed, simply due to changes in hydrogel mechanics. This result largely corresponds to that shown previously by Oakes and coworkers (2009). This observation suggests that a neutrophil has a mechanism for tactile sensing that enables it to distinguish between mechanical environments based on stiffness. To identify how such a mechanism might work, we show that a neutrophil first responds to substrates by spreading and exploring the mechanics of the substrate. In the initial stage of spreading, active integrins on the cell surface are required. After initial spreading has occurred, further spreading and the lifetime of adhesion depend on the ability of a neutrophil to generate tension against the substrate through contractility. Apparently because of the requirement for high internal tension, complete spreading and adhesion stabilization is observed only on stiff gels. If the level of tension that is loaded via integrins during the contractile phase of spreading is insufficient, the neutrophil reduces its adhesive area as we observed. The data suggests that a neutrophil possesses tactile machinery that it can use to probe its underlying substrate and to modulate its adhesiveness and tension on a timescale of minutes, consistent with the rapid motility and dynamics of these cells. Anchorage-dependent cells probe their substrates through focal adhesion assemblies that can take hours to form and subsequently generate adhesive responses that occur on the similar time scales (Zhang et al., 2008). Our work suggests that neutrophil spreading involves two different stages, an initial expansion/contact phase and a contraction phase. Similar results have been observed for other cell types that lack focal adhesion machinery (Zhang et al., 2008, Fleire et al., 2006). The contraction phase likely depends on outside-in signaling through integrins and myosin II and is responsible for prolonged, stable spreading.

Changes in neutrophil adhesion that we observe on hydrogels are reflected in changes of migration dynamics. We have shown that the ability of a cell to move directionally up a gradient is strongly dependent on substrate stiffness, as was shown previously (Oakes et al., 2009). We further observed that substantial changes in the chemotactic index are accompanied by avid force generation but without significant changes in speed, and that the magnitude of traction stress were much greater on stiffer gels and concentrated in discrete force centers in the uropod of the cell; these results seem to be in conflict with those presented previously (Oakes et al., 2009). Our current view is that contractile punctate force centers leads to a hydrodynamic squeezing at the tail that propels the cytoplasm and the leading edge forward much in the manner that toothpaste is squeezed from a tube. Because we employed a micromachined gradient chamber, we uniquely were able to correlate directional motion with gradient steepness, mean concentration, and substrate elasticity. We find that our persistence measure is always maximal when the mean chemoattractant concentration is close to the K_d of binding of the fMLP receptor for a given gradient steepness. Further, for a given set of chemotactic parameters, increasing stiffness led to an increasing directional response. However, increasing substrate stiffness was simply a facilitating factor and did not enable a neutrophil to respond to a gradient at very high mean concentrations when all its receptors are saturated. These insights could be uniquely elucidated using our experimental technique.

It is interesting that the ability of a cell to generate speed and to migrate directionally up a linear chemical gradient appear to be separate processes. Because neutrophil morphology and spreading are different on soft and stiff surfaces and because the cells show different requirements for integrins depending on stiffness, it is possible that a neutrophil is capable of switching seamlessly between two different strategies for motility. In the first mode of migration, a high level of cell spreading and tension through integrins is required. In the second mode, migration depends less on integrins and a higher degree of blebbing extensions are observed.

We considered the possibility that changes in chemotactic sensitivity across gel mechanics could simply be due to decreases in cell radius that in turn lead to reduced gradient sensing capability. This type of mechanism has been reported for anchorage-dependent cells (Meyers

et al., 2006, Neves et al., 2008). However, normalizing the chemotactic index obtained across a range of hydrogel stiffnesses to the corresponding cell radius does not affect the trend of the data. Further, lowering the mean fMLP concentration to values approximately equal to the receptor K_d , where gradient sensing should be most sensitive, produces the same strong dependence of motility on gel stiffness. Although we observe that neutrophil migration on soft gels is less directed in two dimensions, it is possible that the formation of membrane extensions directed towards a gradient coupled with low integrin adhesiveness in constrained 3D tissue environments could lead to different results.

Lastly, numerous papers have reported the requirements for integrin receptors during cell motility (Palecek et al., 1997, Zaman et al., 2006). This notion has recently been challenged by Lammerman and coworkers who show that pan-integrin deficient neutrophils are also capable of motility *in vivo* (Lammermann et al., 2008). In addition, Woolf and Alon show that T-cells which share the same integrin receptors as neutrophils are capable of undergoing motility responses in the absence of integrin adhesiveness using the same blocking antibody we employ (Woolf et al., 2007). Two explanations are proposed for this behavior in prior work. The first is that neutrophils only require integrins for migration in two dimensions, and that integrins are not required for motion in three dimensions. Second, depending on how integrin adhesiveness is regulated for a specific cell type, chemical signals in certain environments may silence integrin activation. Our work on 2D hydrogel substrates shows there are integrin-dependent and an integrin-independent modes of motility. Based on our work, we hypothesize that simple changes in mechanics that occur between 2D glass and plastic surfaces as compared to soft 3D hydrogels that are commonly used may help to explain some of the differences observed in immune cell motility behavior between these two different *in vitro* systems.

An interesting speculation of this work is that neutrophils should crawl more avidly toward a target when the substrate stiffens. Since neutrophils must move very quickly from the endothelial apical surface to the extracellular matrix, a gradient in elasticity might accelerate extravasation. If some or all integrins are not required for neutrophil migration in softer mechanical compartments *in vivo*, such as the interstitial space where chronic inflammation and accumulation of inflammatory cells typically occurs, then inhibition of integrins would not represent an optimal targeting strategy for inflammatory disease. Indeed, many of the integrin inhibitors targeting inflammation tested in humans have met with limited or little success (Staunton et al., 2006, Yonekawa and Harlan, 2005). Moreover, due to the plasticity in integrin use by neutrophils and other immune cells, a more effective strategy for targeting migration in inflammation would be to apply inhibitors of integrin and integrin-independent motility in parallel. We provide here results of the first quantitative study of neutrophil responses to substrate mechanics in well-defined chemotactic gradients.

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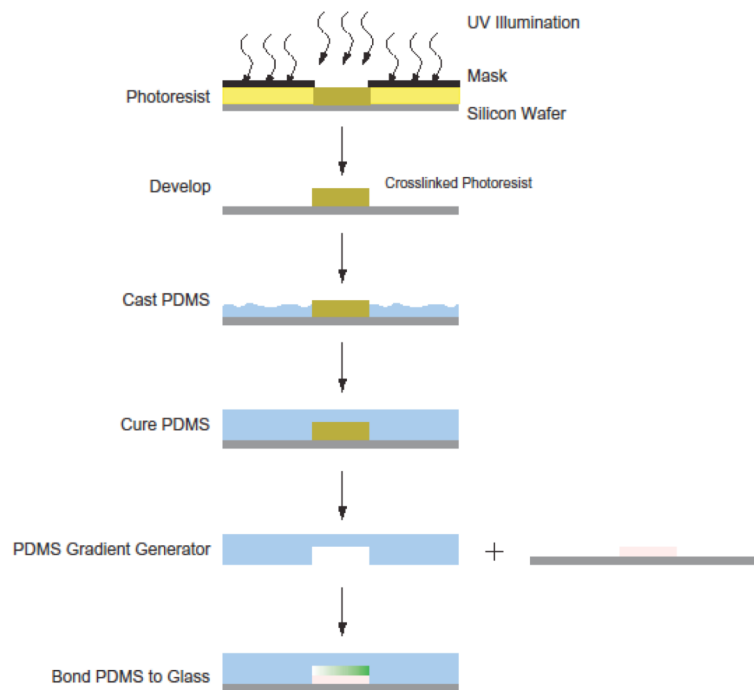


Figure 1. Fabrication of a microfluidic system to study neutrophil responses to gradients on hydrogel substrates
 A schematic of the photolithography process used to prepare microfluidic devices that generate gradients over the surface of polyacrylamide hydrogels as described in Materials and Methods.

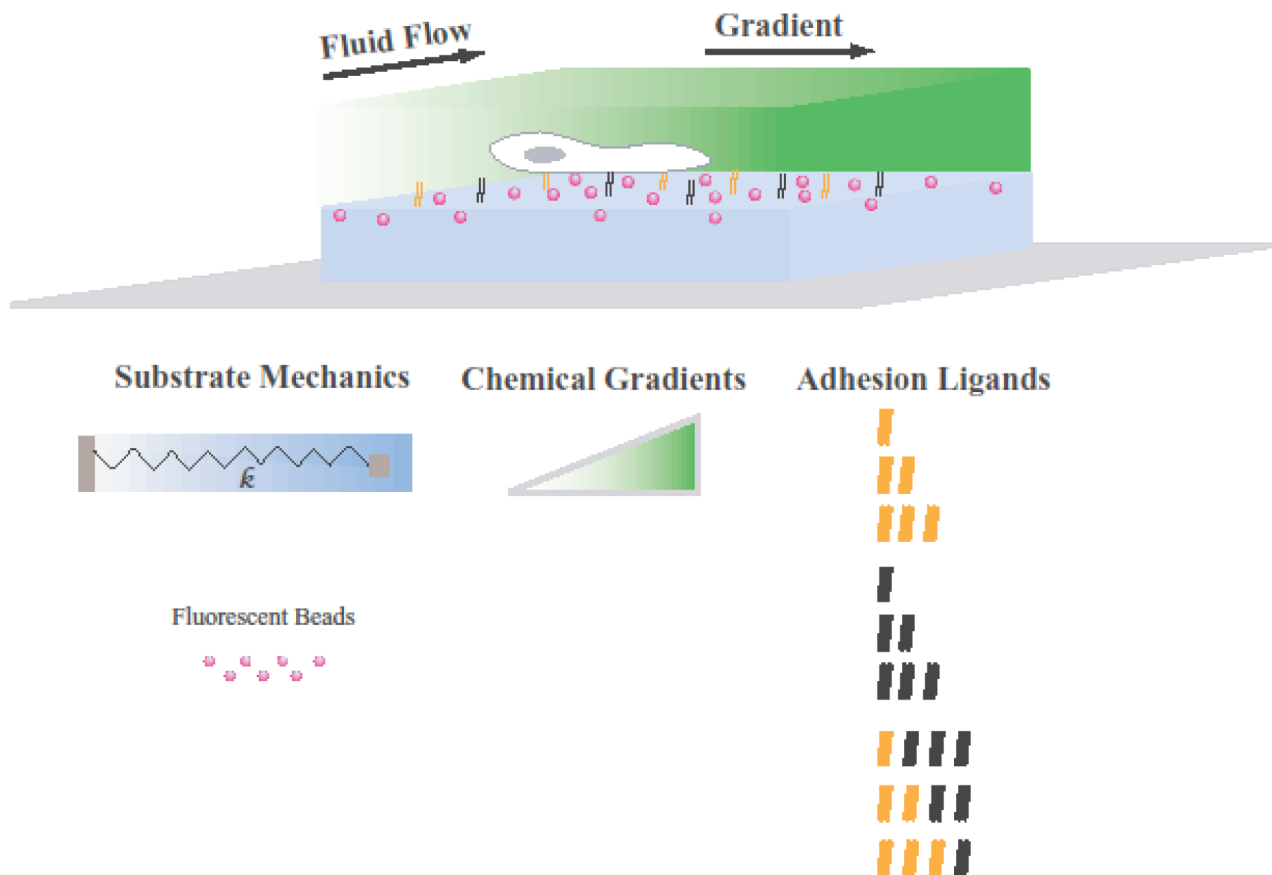


Figure 2. Schematic representation of an adherent neutrophil on a hydrogel within a microfluidic gradient chamber

A single cell is shown interacting with a ligand-coated surface oriented in the direction of increasing chemical gradient. Ligands are immobilized on the surface of the hydrogel via covalent bonds as described in Materials and Methods. The direction of fluid flow as well as chemical, and mechanical parameters within the system that can be varied independently of each other are also illustrated.

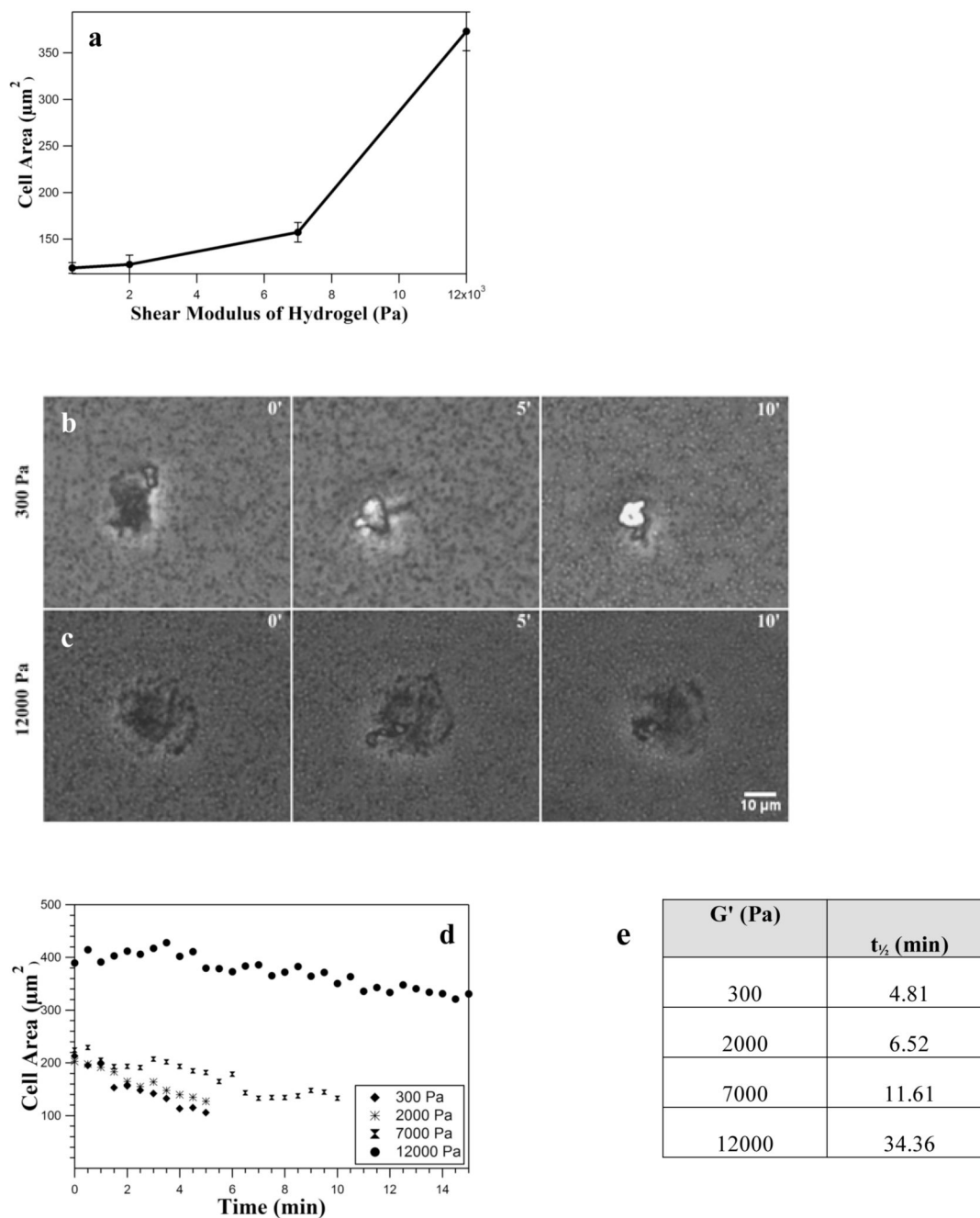


Figure 3. Neutrophil adhesion and spreading on hydrogels depends on substrate mechanical properties

(a) Neutrophils were activated with 10 nM fMLP and observed during adhesion to hydrogel substrates. The area of individual cells attached to a hydrogel during a 15 minute time interval was averaged and plotted against hydrogel shear modulus to show the influence of substrate mechanics on cell spreading. (b,c) Phase contrast images of neutrophils adhering to hydrogel surfaces in uniform solutions of 10 nM fMLP on soft (300 Pa) and stiff (12 kPa) gels as a function of time. (d) Cell area of neutrophils activated with uniform solutions of 10 nM fMLP on hydrogels at the indicated stiffnesses were measured and plotted as a function of time. (e) The time at which cell spreading area for a given hydrogel stiffness had decreased by 50%.

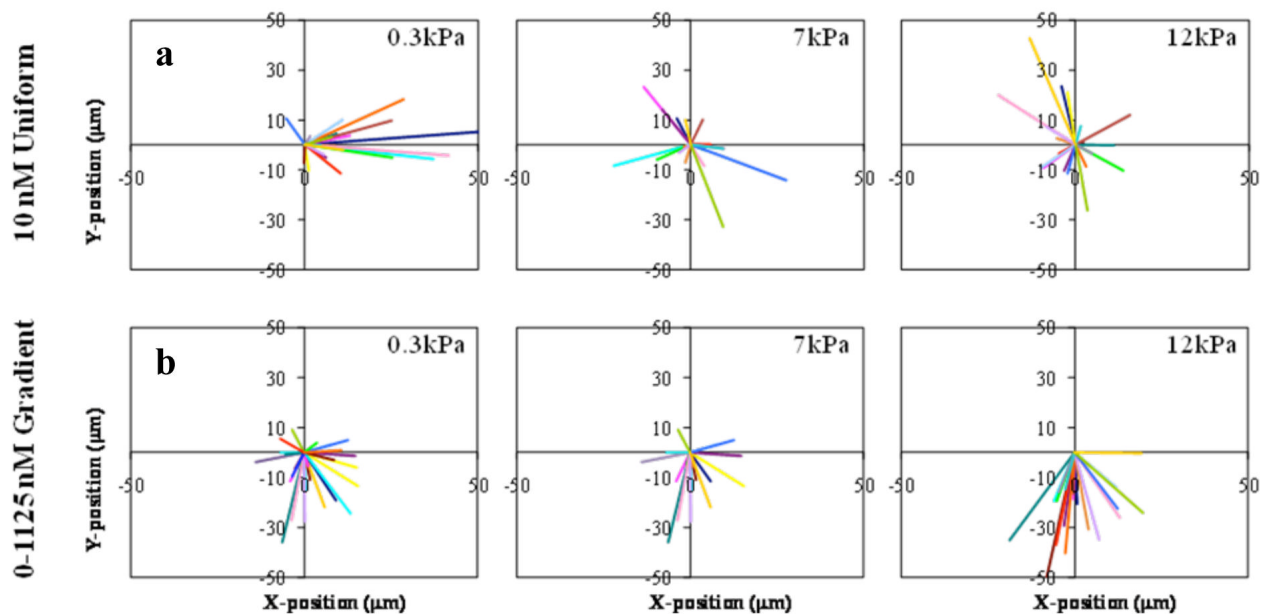


Figure 4. Neutrophil Trajectories During Chemokinesis or Chemotaxis on Hydrogels with Varying Mechanical Properties

Representative 2D Wind-Rose plots showing individual cell trajectories for a range of gel stiffnesses in both uniform (a) and gradient (b) solutions of fMLP. After cells are imaged using time-lapse microscopy, centroids are computed and individual cell tracks are generated. Trajectories were obtained by tracking cell centroids for a total time of 15 minutes per condition. Lines begin at the zero-centered initial position of each cell and end at the final position to display net cell dispersion. For each graph with a gradient, the spatial gradient of fMLP increases from top to bottom.

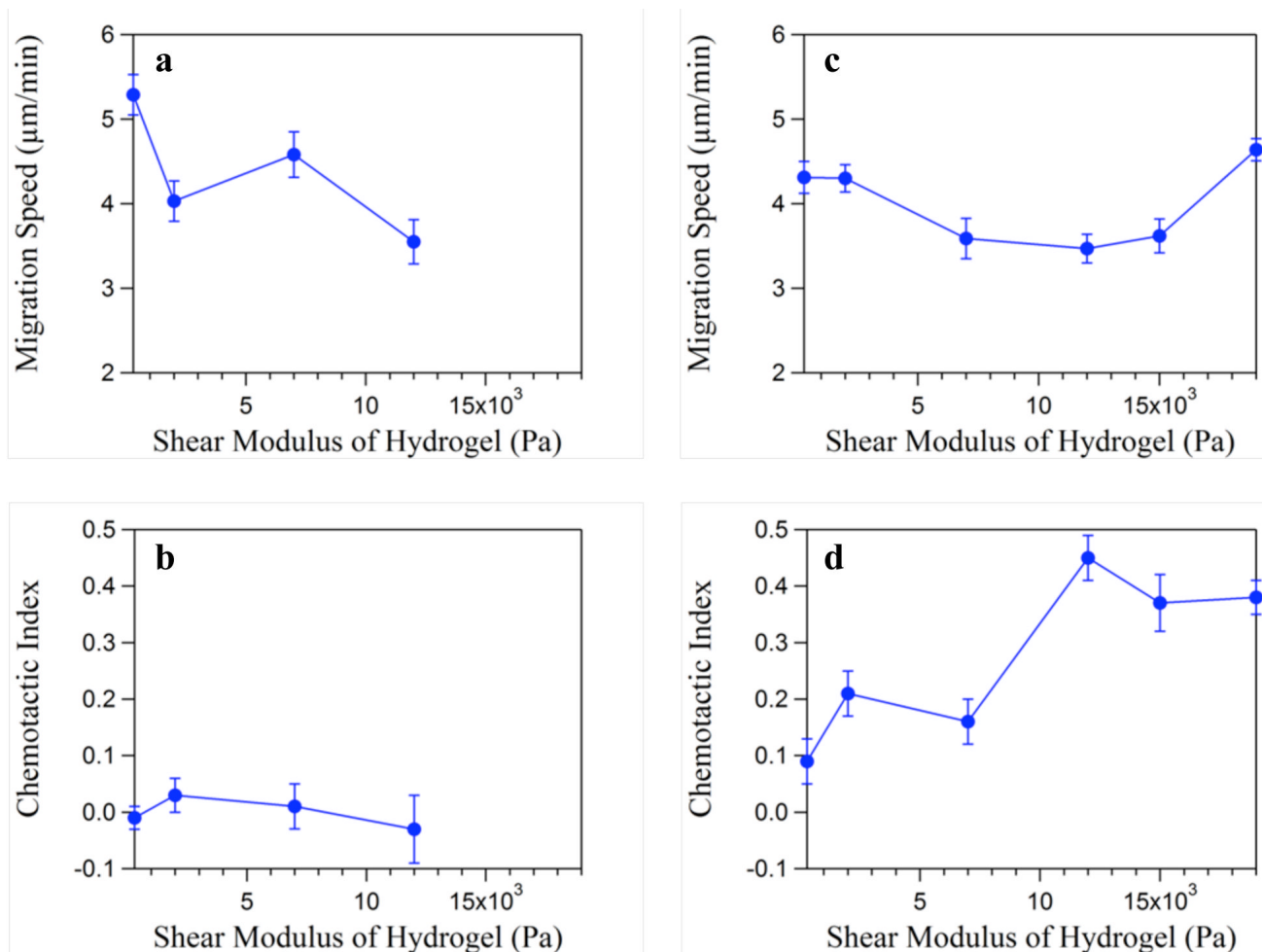


Figure 5. Relationship between cell speed and directionality as a function of hydrogel stiffness
 Migration parameters for neutrophils obtained on hydrogels functionalized with ICAM-1/E-Selectin in the presence of uniform or gradient solutions of fMLP. (a) Migration speed, (b) chemotactic index in a uniform concentration of 10 nM fMLP. (c) Migration speed, (d) chemotactic index in a gradient of 0-1125nM fMLP or 10nM/cell diameter (10nM/10μm).

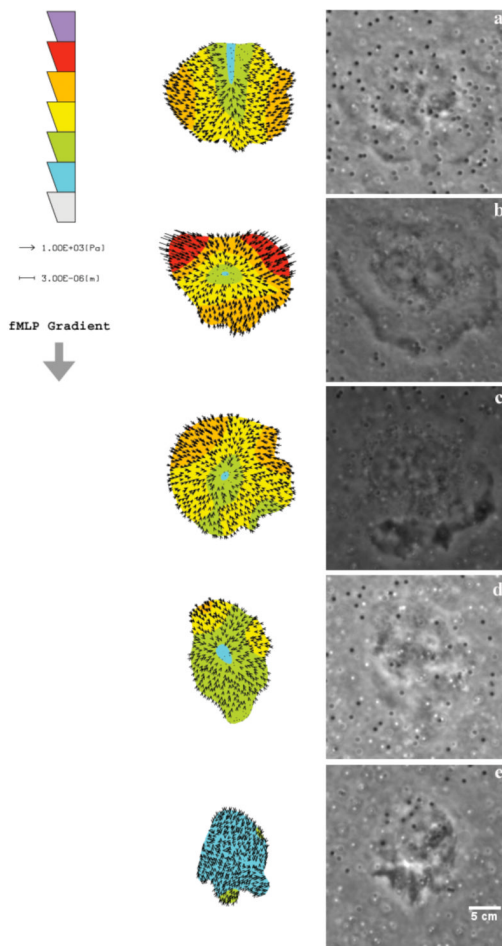


Figure 6. Traction Stress Maps of Neutrophils Migrating on Hydrogels of Varying Stiffness in Response to a Gradient of fMLP

Neutrophil traction stress maps and corresponding phase contrast images during migration on stiff hydrogels (12,000 Pa) in response to a gradient of fMLP (10 nM/cell diameter). Arrow pointing downwards indicates the direction of the fMLP gradient from low to high concentration within the microfluidic chamber. Color map indicates the magnitude of stress in different regions of the cell. Horizontal arrow indicates the magnitude of the force vectors drawn within each cell.

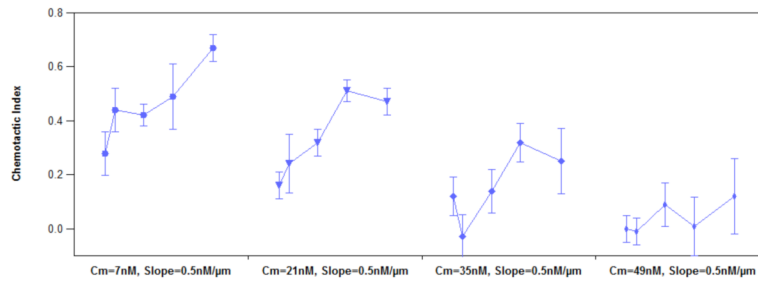


Figure 7. Chemotaxis in linear fMLP gradients with varying mean concentrations as a function of hydrogel stiffness

Neutrophil chemotaxis in linear fMLP gradients with mean concentrations ranging between 7-49nM and constant slope was measured on hydrogels with shear moduli of 300 Pa, 2000 Pa, 7000 Pa, 12000 Pa, and 19000 Pa. For each line, shear modulus increases from left to right.

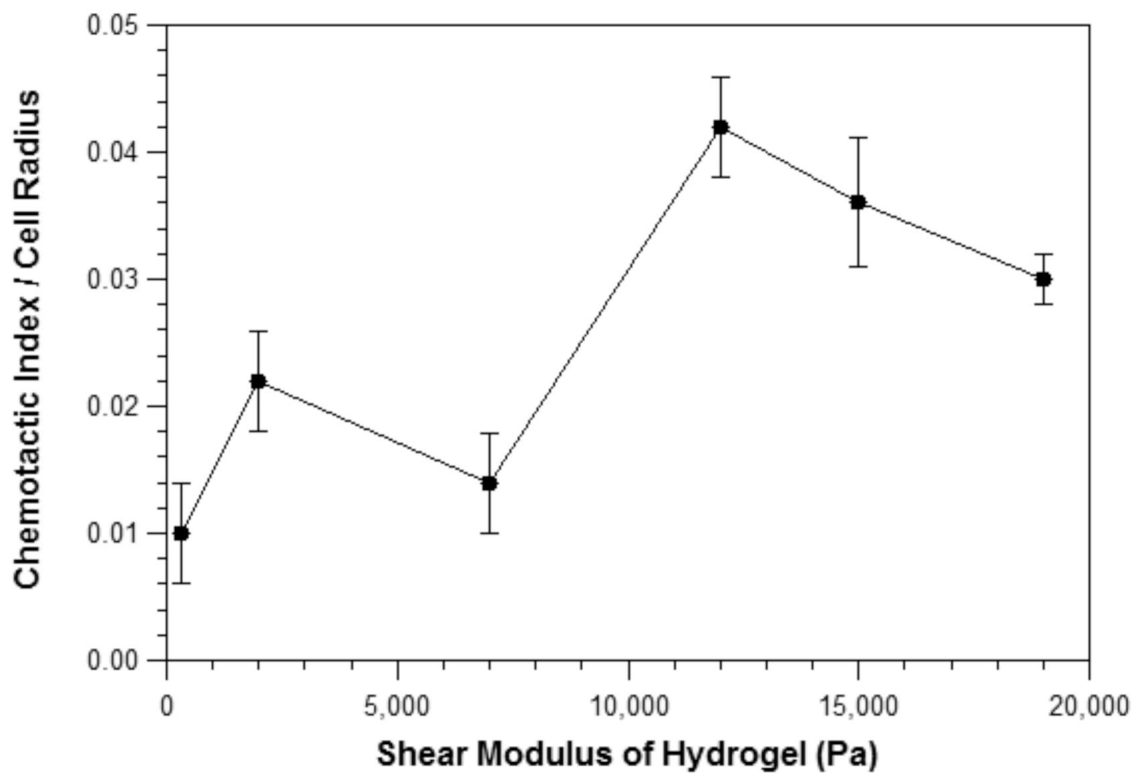


Figure 8. Chemotactic index values for neutrophils on hydrogels of varying stiffness normalized to observed cell radius

To account for differences in spatial gradient sensing that would result from changes in cell spreading, chemotactic index values were replotted after normalization to average cell radii observed on different gel stiffnesses. The cell radii was used as the characteristic length for normalization because spatial sensing occurs across a cell's diameter.

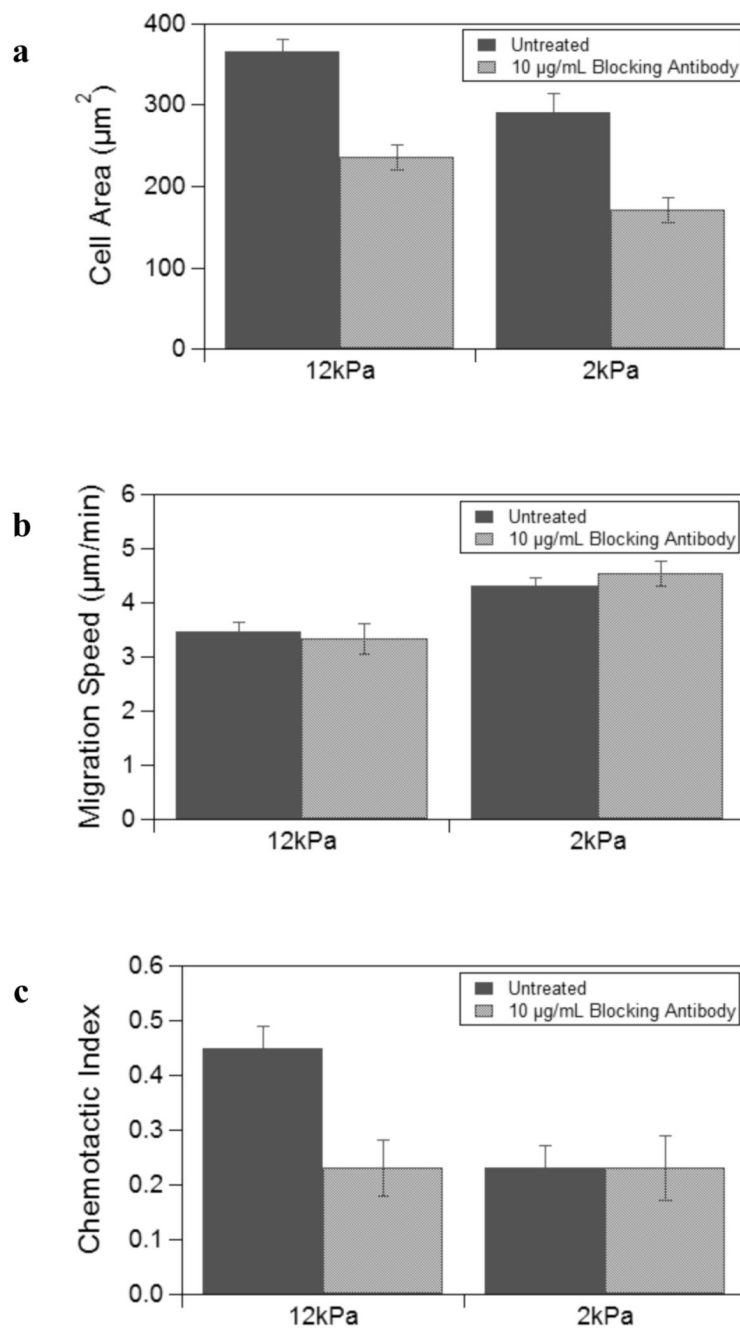


Figure 9. Effect of $\beta 2$ integrin blocking antibody

Neutrophils were incubated with 10 $\mu\text{g/mL}$ TS1/18 antibody and allowed to attach and migrate on either soft (300 Pa) or stiff (12,000 Pa) hydrogels in the presence of a 0-1125 nM gradient (10nM/ μm). (a) Cell spreading, (b) migration, (c) chemotactic index were measured for individual cells tracked for a total of 15 minutes.