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Motile Dendritic Cells Sense and Respond to Substrate Geometry

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Abstract

Dendritic cell (DC) migration is required for efficient presentation of antigen to T cells and the initiation of an adaptive immune response. In spite of its importance, many aspects of DC migration have not been characterized. DCs encounter a variety of environments with different stiffness and geometry, but the effect of these parameters on DC migration has not yet been determined. We addressed this question by comparing DC motility on standard migration surfaces (polydimethylsiloxane (PDMS)-coated coverslips) and micropost array detectors (mPADs). These two surfaces differ in both stiffness and geometry. We found that DC migration was affected by substrate type, with significant increases in speed and significant decreases in persistence time on mPADs made of PDMS as compared to spin-coated PDMS coverslips. To determine whether the geometry or compliance of the post arrays was responsible for these changes in DC migration, we quantified DC motility on mPADs of identical geometry but different stiffness. Migration was indistinguishable on these mPADs, suggesting that DCs are responsive to geometry of ligand presentation and not stiffness. Further, by micropatterning ligands on flat PDMS surfaces in similar geometries to the mPAD arrays, we determined that DCs respond to the geometry of printed ligand. Finally, we used a variety of small molecule inhibitors to identify pathways involved in geometry sensing. We saw a significant role for myosin contractility and $\alpha_5\beta_1$ integrin engagement. We also noted significant reorganization of the actin cytoskeleton into dynamic actin rings when DCs were motile on posts. From these experiments, we conclude that DCs are insensitive to substrate compliance in the range tested but respond to changes in geometry via a mechanism that involves integrin function, myosin contractility, and remodeling of the actin cytoskeleton. As a possible explanation, we postulate a consistent role for filopodial extension and contraction as the driver of DC motility.

Key Terms

mPAD; geometry; stiffness; actin; dendritic cells; migration; mechanosensing

Introduction

DCs are antigen presenting cells, capable of activating naive T cells and launching potent adaptive immune responses²⁶. As immature cells, DCs are distributed throughout peripheral tissues, where they continuously capture and degrade material from the environment, displaying peptide fragments on surface-bound MHC complexes. When DCs detect molecular signs of infection and inflammation²⁴, they undergo a series of phenotypic and functional changes called maturation¹. Mature DCs become highly migratory and travel to T cell-rich areas of lymphoid tissues where they present surface bound antigens to T cells¹⁹. DC migration is therefore critical for the ability of the body to launch a proper immune response.

One remarkable aspect of DC biology is their ability to migrate robustly through a wide range of body tissues²⁰. Like all cells, migrating DCs must adjust to the mechanical and geometric characteristics of the microenvironment³. Geometry, whether physical structure or spatial patterning of extracellular matrix ligands, has been shown to control cell adhesion, guide cytoskeletal organization and regulate migration^{30,4,12}. Likewise, stiffness strongly influences the physiology of many cell types, including their adhesion, force generation and migration^{16,23,9,18}. In particular, most immune cells respond to differences in the mechanics of their substrate. For example, neutrophil adhesion, directed migration and force generation increase with substrate stiffness, and macrophage force magnitude is dependent on substrate stiffness^{14,13}. To our knowledge, it is unknown how DCs respond to the biophysical properties of their surroundings.

Micropost array detectors (mPADs) were developed as tools for probing cellular traction forces²⁸. We have previously used mPADs to measure the forces that DCs exert during chemotaxis and chemokinesis^{21,2}. We found that DCs exert traction stresses from the front, consistent with the elongation, adhesion, and contraction of filopodial that were oriented in the front of the cell by an imposed chemotactic gradient²¹. While mPADs are useful tools for measuring forces of migration, we have not considered how switching the substrate from a PDMS-coated coverslip to an mPADs might affect the ability of the DCs to migrate. It stands to reason that the geometry of the contact points, as presented by the tips of the posts, might influence the ability of the DC to organize its migration machinery. Also, the compliance of those contacts might affect the ability of DCs to pull on the substrate.

In this paper, we use the terms stiffness, elasticity and compliance to describe how stiff the external environment appears to migrating DCs. Geometry refers to both the physical structure of the substrate as well as the patterning of ligand. We will specify ligand geometry when we are explicitly focusing on that parameter. The word discretization is used to describe a surface with discontinuous geometry. Finally, we use the word topography to describe all of these physical characteristics collectively.

The goal of the present study was to determine how DCs respond to the physical characteristics of their surroundings. We observed that DC migration was unaffected by variations in stiffness over the range of 1 to 1,000 kPa, but was sensitive to the geometry of the substrate. We further confirmed the effect of geometry on the dynamics of motility by

testing patterns of ligands printed on continuous PDMS surfaces, and confirmed that the changes in motility are due to changes in the geometry of ligand presentation. Further, we saw that DC responses to geometry are regulated by actomyosin contractility and integrin-based adhesions. Taken together, this work supports the observation that DCs are able to adapt to a variety of microenvironments. A greater understanding of how DCs modulate their migration in different environments can be applied to the development and administration of immunotherapies to optimize treatment efficacy and delivery and for the design of *in vitro* devices to manipulate immune cell behavior.

Materials and Methods

Reagents

Recombinant murine CCL19 was purchased from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS; L4516), DMSO, Pluronic F127, silane (Trichloro(1H,1H,2H,2H-perfluorooctyl)silane) and bovine fibronectin were obtained from Sigma-Aldrich (St. Louis, MO). PBS was obtained from Thermo Fisher Scientific (Hampton, NH). Poly(dimethylsiloxane) (Sylgard 184 Silicone Elastomer) was purchased from Dow Corning, Midland, MI. Fetal bovine serum was obtained from Atlanta Biologicals (Flowery Branch, GA). Recombinant GM-CSF was produced from the B78Hi/GM-CSF.1 cell line provided by T. Laufer (University of Pennsylvania, Philadelphia, PA).

Mice

C57BL/6J mice from Jackson Laboratories and GFP-Lifeact mice bred on the C57BL/6J background²² were housed under pathogen-free conditions in the Children's Hospital of Philadelphia animal facility. All studies involving animals were reviewed and approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

Culture of Bone Marrow Derived DCs (BMDCs)

Primary BMDCs were prepared following the protocol of Sixt and Lämmermann²⁵. Mice were euthanized with CO₂ gas and cervical dislocation. Leg bones were harvested and bone marrow was flushed with sterile PBS. Cells were spun at 1500 rpm for 10 minutes at 4°C and resuspended at 2.5×10^6 cell/mL. 1 mL of cells, 9 mL R10 media (RPMI 1640 (1x with L-Glutamine and 25 mM HEPES) + 50 mL heat inactivated FBS + 5 mL penicillin-streptomycin (Thermo Fisher Scientific, final concentration 100 U/mL pen. and 100 µg/mL strep.)) and 100 µL GM-CSF were added to 10 cm Petri dishes. On day three of culture, an additional 10 mL R10 and 100 µL GM-CSF were added. On day six, 10 mL media was gently removed from each dish and replaced with 10 mL fresh R10 and 100 µL GM-CSF. Between days seven and nine DCs were transferred to 6 cm tissue culture dishes and matured for 24 hours in the presence of 200 ng/mL LPS. DCs were maintained at 37°C under 5% CO₂ throughout the culture period and experiments.

Unpatterned PDMS-coated coverslip Preparation

The following methods were adapted from our previous work². 2D migration experiments were conducted on PDMS-coated coverslips printed with fibronectin. These surfaces were uniform, flat and devoid of any micropatterning. Clean 25 mm round glass coverslips

(Thermo Fisher Scientific) were spun with a thin layer of 10:1 PDMS (weight, base:cure) on a Laurell spinner (4000 rpm, 1 minute) and cured overnight at 65°C. The PDMS layer on coverslips prepared in this manner is ~10 µm thick¹¹. Coverslips were mounted onto the bottom of laser-cut one-well dishes with 10:1 PDMS and cured for at least 3 hours. 60 g of 10:1 PDMS was cast against a flat silicon wafer and cured overnight at 65°C. The PDMS was carefully peeled away from the silicon wafer and cut into 1 cm² stamps. Stamps were sonicated in 200 proof ethanol for 10 minutes, rinsed twice in diH₂O and dried with N₂ gas. The clean stamps were linked with 10 µg/mL fibronectin and incubated at room temperature for 2 hours. The PDMS-coated coverslips were treated with UV/ozone for 7 minutes (UVO Cleaner Model 342, Jelight, Irvine, CA). The fibronectin-coated stamps were gently rinsed twice in diH₂O and dried with N₂ gas. Fibronectin was transferred from the stamps to the PDMS-coated coverslips through microcontact printing⁷ and the PDMS-coated coverslips were blocked with 0.2 w/v% Pluronic F127 for at least one hour. The PDMS-coated coverslips were rinsed 2x with PBS and incubated with PBS at 4°C for at least one hour. Mature DCs were centrifuged for 10 minutes at 1500 rpm and 4°C and added to the PDMS-coated coverslips at a concentration of 100,000 cells/mL. Any inhibitors were added at this point (for concentrations and incubation times, see below). The cells were incubated at 37°C and 5% CO₂ throughout the entirety of the experiment. 10 nM CCL19 was used to stimulate the cells immediately prior to imaging.

mPAD Preparation

mPADs were prepared following the protocol of Yang et al.³¹. Negative molds were created by casting degassed PDMS (10:1 base to cure) against a silanized silicon master mold and curing for 12 minutes at 110°C. The negative molds were gently peeled away from the silicon master, plasma treated (SPI Supplies Plasma Prep II, West Chester, PA) for 7 seconds, and silanized overnight. PDMS (10:1 base to cure) was added to the negative molds and degassed for 30 minutes to remove any air bubbles. 25 mm round glass coverslips were cleaned with N₂ gas and plasma treated for 90 seconds. The molds were placed, fresh PDMS side down, onto the cleaned coverslip and cured at 110°C for 20 hours. The mPADs were gently peeled away from the negative molds, immediately submerged in 200 proof ethanol and supercritical dried (SAMDRI-PVT-3D, Tousimis Corporation, Rockville, MD) in liquid CO₂. Circular holes were laser cut from the bottom of one well dishes. mPAD coverslips were secured to the bottom of the one well dishes with 10:1 PDMS and cured overnight at 65°C.

10 µg/mL fibronectin was microcontact printed onto the post tips, following the same procedure as above with minor modifications. After stamping the mPADs, 1 mL of 200 proof ethanol was added and the stamp was gently flicked off the micropost array. The ethanol was immediately diluted to 60% with diH₂O. The mPADs were gently rinsed three times with diH₂O. The post tips were blocked with 0.2 % Pluronic F127 for 1 hour at room temperature, rinsed three times with sterile diH₂O and stored at 4°C overnight. The following day, 1 mL of mature DCs (100,000 cells/mL) was added to the mPAD. Any inhibitors were added at this point (for concentrations and incubation times, see below). Motility was stimulated with 10 nM CCL19 immediately before moving the dish to the

microscope stage, and cells were allowed to settle on the micropost array before imaging began.

Spring constants for the mPADs were calculated using the known geometry of the posts and confirmed by finite element method analysis. The effective stiffness was computed from the spring constant, and gives a description of the stiffness of the surface as perceived by the cell.

Ligand Patterning on PDMS-Coated Coverslips

Patterned stamps were created by casting polymer against a patterned silicon wafer, yielding a discontinuous stamp, with a surface complementary to the silicon mold. First, a rigid polymer solution was created by combining 3.4 grams of (7.0–8.0% Vinylmethylsiloxane)-Dimethylsiloxane Copolymer, Trimethylsiloxy Terminated (Gelest, Inc.); 1.0 grams of (25–35% Methylhydrosiloxane)-(Dimethylsiloxane) Copolymer (Gelest, Inc.); 4 drops of modulator 2,4,6,8-Tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane (Sigma Aldrich); and 4 drops of catalyst Platinum(0)-2,4,6,8-tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane complex solution (Sigma Aldrich). The solution was mixed thoroughly and cast against a patterned silicon wafer. Large, surface-trapped air bubbles were removed by gently blowing N₂ gas over the layer of polymer before baking for 40 minutes at 50°C. A soft PDMS layer (22 grams of 10:1 PDMS (weight, base:cure)) was poured over the rigid polymer and the stamp was baked overnight. The next day, the polymer was gently peeled away from the wafer and the stamps were trimmed. Microcontact printing was performed as described above. The only difference was the use of fluorescently labeled fibronectin (488-conjugated) to visualize stamped patterns. The conjugated fibronectin adsorbed only to the pattern on the top of the stamp, thus leading to the dot pattern observed by microcontact printing. The rigid polymer used to create the top of the stamp (the patterned portion) ensured stability of these small features and prevented stamp collapse.

Inhibitors and Antibodies

Inhibitors and antibodies were added to one-well dishes along with mature DCs (at 100,000 cells/mL) prior to imaging. Cells were maintained at 37°C unless otherwise noted. The following concentrations and incubation times were used: blebbistatin (B0560; Sigma-Aldrich, St. Louis, MO) at 2 µM for 1 hour; CK666 (SML006; Sigma-Aldrich, St. Louis, MO) at 10 µM for 1 hour; α_5 and β_1 antibodies (14-0493-85 and 14-0292-85, respectively; eBioscience, San Diego, CA) at 5 µg/mL for 10 minutes at room temperature then 10 minutes at 37°C.

Live Cell Imaging

Mature DCs were imaged at 10x by phase microscopy on a Nikon Eclipse TE300 (Nikon, Melville, NY) with MetaMorph software (MetaMorph Inc., Nashville, TN). Images were collected at multiple positions for one hour at one minute intervals using a motorized stage. Cells were maintained at 37°C and 5% CO₂.

Image Analysis and Quantification

Cell migration was analyzed with the Manual Tracking plugin in ImageJ (<http://rsbweb.nih.gov/ij>). Cells that were apoptotic or completely stationary were excluded, as well as any cells in contact with other cells. The list of x- and y-coordinates obtained from ImageJ was further analyzed with a custom-written MATLAB (Mathworks, Natick, MA) script. The MATLAB script computed the MSD of the compiled list of cell tracks and used the Dunn equation (Equation 1) to fit speed (S) and persistence (P)⁸. The random motility coefficient was calculated from the speed and persistence, as shown in Equation 3.¹¹

Statistical Analysis

We used a one-way ANOVA along with Tukey's Post Hoc Test to determine statistical significance. Statistically significant differences at $p < 0.05$ were marked with an asterisk (*) and differences at $p < 0.01$ were marked with a double asterisk (**).

Atomic Force Microscopy

All experiments were carried out at room temperature using a Bruker Bioscope Catalyst AFM (Billerica, MA, USA). We used a spherical tip from Novascan (Boone, IA, USA) with a nominal spring constant of 0.06 N/m and a nominal tip radius of 500 nm. The cantilever spring constant was calibrated using Nanoscope software (Bruker). The AFM was operated in the fluid contact mode at a force distance curve acquisition frequency of 2 Hz.

Results

Dendritic Cell Migration is Affected by the Underlying Substrate

We have previously quantified DC chemokinesis on continuous PDMS surfaces, formed by uniformly spin coating PDMS on a glass coverslip, and we have measured DC force generation on discrete PDMS microposts^{21,2}. Although we have always considered these experiments to be complementary, important differences between the two experimental systems leave open key questions about whether migration is affected by the stiffness and/or the geometry of the underlying substrate. To address these questions, we measured the random migration of DCs on mPADs, and compared it to our previous results for DCs randomly migrating on PDMS-coated coverslips. In both cases, the surfaces were printed with fibronectin to ensure cell adhesion, and blocked with Pluronic F127 to restrict cell attachment to the printed region. 10 nM CCL19 was also added to DCs to stimulate motility. DCs were able to move and had similar morphologies on the two surfaces (Figure 1A and B, Supplementary Videos 1 and 2).

Sample trajectories of DCs on PDMS-coated coverslips and mPADs are shown in Figure 1C and D, respectively. As a first step of analysis, we used the trajectories to calculate the mean squared displacement (MSD) as a function of time. When plotted on a log-log scale, the slope of this curve provides a quantitative description of the form of migration. For DCs migrating randomly on either PDMS-coated coverslips or microposts, we found that the MSD versus time had a slope of 1, indicating purely diffusive migration (Figure 1E). We next analyzed this data using the Dunn equation (Equation 1) to determine the speed (S) and persistence time (P)⁸. We used speed and persistence time to calculate two additional

quantitative features of motility—the persistence length (P) and random motility coefficient (μ)—given by Equations 2 and 3, respectively.

$$\langle MSD(\tau) \rangle = 2S^2P [\tau - P(1 - \exp(-\tau/P))] \quad (1)$$

$$P_l = (P)(S) \quad (2)$$

$$\mu = \frac{S^2P}{2} \quad (3)$$

The results from these calculations are shown in Figure 2. We previously performed a thorough analysis of the migration of WT DCs on PDMS-coated coverslips, and report these values here as reference². While DCs migrated diffusively on both PDMS-coated coverslips and mPADs, the method by which DCs achieved their random migration was quite different. DCs on PDMS-coated coverslips migrated at a speed of $4.00 \pm 0.18 \mu\text{m}/\text{min}$ and persisted for $4.88 \pm 0.46 \text{ min}$ before changing directions (Figure 2A, B). DCs on mPADs exhibited a modest, but significant increase in speed ($4.92 \pm 0.24 \mu\text{m}/\text{min}$), and persisted for a significantly shorter time ($2.35 \pm 0.25 \text{ min}$) before changing direction. The significant reduction in persistence time also led to a reduction in persistence length (Figure 2C). While there is a modest reduction in the random motility coefficient of DCs on mPADs, it does not reach significance (Figure 2D). By examining the cell trajectories on the PDMS-coated coverslip and mPAD surfaces, we can see that the majority of DCs on the PDMS-coated coverslips migrate over large distances. In contrast, there appear to be two separate populations of DCs on mPADs, with some DCs exhibiting long trajectories and some DCs exhibiting more circuitous trajectories huddled around the origin. This large variance in the phenotype of DC migration on mPADs accounts for the failure of the decrease in random motility coefficient to reach significance. Taken together, these measurements indicate that DCs are able to undergo random migration on mPADs, but the characteristics of their migration are quite different.

Dendritic Cells Respond to Geometry and Not Stiffness

Next, we sought to determine whether the DCs were responding to differences in geometry or elasticity of their substrate. The coverslips are coated with PDMS, the same material used to generate the mPADs. However, while the coverslip is coated with a continuous layer of PDMS, the mPAD is molded into a discrete array of posts with adjustable geometry (diameter and post-to-post spacing) and adjustable elasticity (set by the height of the posts)³¹. Both substrates present a 2D, fibronectin-printed surface to migrating DCs, with the mPAD presenting discrete islands of fibronectin. However, the stiffness of the PDMS-coated coverslip is two orders of magnitude greater than that of the tall mPAD we generally use, and the ligand is presented in a continuous pattern instead of a discrete array.

Both the geometry and stiffness of the microenvironment have been known to influence the migration of many different types of cells³. Since most immune cells show functional dependence of motility on substrate stiffness, we hypothesized that DCs would also be responsive to this parameter^{13,17,14}. To test whether the elasticity of the mPADs affects the migration of DCs, we repeated the migration experiments using two additional mPADs, called “short” and “medium.” (Table 1). Each mPAD has the same 2D cross sectional geometry (diameter 1.83 μm and post spacing $\sim 3 \mu\text{m}$) but different heights, resulting in different effective stiffnesses³¹. “Tall” mPADs are approximately 1 kPa, “medium” mPADs are 10 kPa, and “short” mPADs are 10^3 kPa. In experiments on coverslips, we spun the surface with a layer of PDMS, which leads to a stiffness (elastic modulus) on the order of 100 kPa (Supplementary Figure 1). By quantifying DC migration on these mPADs of different stiffnesses, we can effectively tease out the effect of stiffness while keeping substrate geometry constant. As shown in Figure 3, the average speed and persistence time of DCs are similar on all three mPADs. Consistent with our earlier finding, DCs exhibit diminished persistence time on all mPADs as compared with PDMS-coated coverslips. These results indicate that DC migratory behavior is unaltered by changes in stiffness in the range tested.

Next, we wanted to determine if this geometry dependence was due to physical topography of the substrate (i.e. discrete micropillars vs. continuous surface) or the geometry of ligand printed on the surfaces. We addressed this question by spatially patterning ligand on PDMS-coated coverslips (Figure 4A), which allowed us to probe ligand geometry independently of the physical structure of the substrate. DC migration on these patterned PDMS-coated coverslips was quantified as described above, and average speed and persistence time were compared to the values for migration on the continuously-printed PDMS-coated coverslips and mPADs (Figure 4B, C). DCs migrating on these patterned surfaces migrated significantly faster than DCs on continuously printed PDMS-coated coverslips, while their speed was indistinguishable from that of DCs on mPADs (Figure 4B). DCs on patterned PDMS-coated coverslips also displayed a significant difference in persistence time, matching the values we calculated for persistence time on mPADs. These results indicate that DC migration responds to printed ligand geometry and is not affected by the physical structure of the substrate.

Finally, we wanted to know how sensitive DCs are to variations in geometry. To address this question, we prepared mPADs with smaller dimensions (diameter and interpost spacing). While the mPADs we used previously have a diameter of 1.8 μm and post-to-post spacing of $\sim 3 \mu\text{m}$, the new smaller mPADs have a diameter of 0.8 μm and post-to-post spacing of $\sim 2 \mu\text{m}$ (Table I). These two mPADs have similar effective stiffnesses (Table I), ensuring that the only variable we are investigating is geometry. Quantification of persistence time revealed no differences in DCs migrating on larger and smaller diameter posts (Figure 5). Therefore, we conclude that migratory DCs are able to respond to large-scale differences in geometry (i.e. continuous vs. discrete ligand presentation) but are unable to discern differences in geometry on the scale of 1 μm .

DCs sense differences in geometry through myosin contractility, integrin-based adhesions and reorganization of the actin cytoskeleton

To gain an understanding of how substrate geometry affects subcellular organization and signaling during DC migration, we used GFP-Lifeact DCs. On PDMS-coated coverslips, F-actin was highly concentrated at the periphery of the cell, forming lamellipodia and filopodia (Figure 6A). When DCs were interacting with mPADs, the actin showed a very different distribution, with high levels of GFP signal surrounding each engaged post (Figure 6B). The process of ring formation is very dynamic, with the actin intensity in individual rings fluctuating on a time-scale of 1 minute (Supplementary Video 3), which is shorter than the persistence time (2.35 ± 0.25 min). The presence of these actin rings provides additional evidence that DCs detect differences in PDMS-coated coverslips and mPADs. This concentration of fluorescence appears to be specific to mPADs, as imaging LifeAct labeled DCs on printed patterns of fibronectin (ss used to make the measurements in Figure 4) did not show rings of actin, but rather local accumulation of actin near spots (see Supplemental Figure 2). Thus, the formation of actin rings seems specific to mPADs but does not affect the dynamics of DC migration (since there was no difference in migration between mPADs and printed spots).

We next aimed to clarify the mechanisms involved in DC geometry sensing through the use of small molecule inhibitors. We first used CK666 to investigate actin polymerization mediated by the Arp2/3 complex (orange bars in Figure 7), which is a protein we had previously shown to regulate DC speed on PDMS-coated coverslips². Treatment with CK666 had no effect on DC morphology on either PDMS-coated coverslips or mPADs (Figure 7C; see Supplementary Videos 1 and 2 for reference to normal morphology on both surfaces). Addition of CK666 to DCs on mPADs led to a significant decrease in speed and had no influence on persistence time (Figure 7A, B). Although speed is reduced in DCs on both PDMS-coated coverslips and mPADs when CK666 is present, the significant difference in speed between DCs on mPADs and PDMS-coated coverslips remains (Figure 7A, B). Thus, we conclude that while Arp2/3 complex activity is important for regulating migratory speed during DC chemokinesis, it does not contribute to geometry sensing.

Next we probed myosin contractility by directly inhibiting of myosin with blebbistatin (blue bars in Figure 7). At a concentration of 2 μ M, cells remained viable but displayed elongated morphology (Figure 7C). We saw no significant changes in average speed or persistence time under the influence of blebbistatin on mPADs (Figure 7A, B). We did however, see a significant decrease in persistence time of DCs on PDMS-coated coverslips in the presence of blebbistatin (Figure 7B). Indeed, the persistence time of blebbistatin-treated DCs on PDMS-coated coverslips was indistinguishable from that of untreated DCs on mPADs. This indicates that myosin contractility contributes to geometry sensing, since inhibition of this pathway eliminates the ability of DCs to sense differences in geometry between the two surfaces.

Finally, we used antibodies to impair integrin-mediated adhesions. Both of our surfaces were printed with fibronectin, so we chose to specifically block the two subunits of the major fibronectin integrin in DCs: $\alpha 5$ and $\beta 1$. We selected antibody concentrations that would only partially block integrin function (5 μ g/mL), thus allowing for continued adhesion but

reduced availability of active integrins. DCs were able to migrate on the PDMS-coated coverslips and mPADs under these conditions (Figure 8A). Blocking of either subunit had no effect on the average speed or persistence time of DCs on PDMS-coated coverslips (Figure 8A, B). In contrast, there was a significant decrease in average speed and a significant increase in persistence time on mPADs. Indeed, treatment with either antibody effectively eliminated the geometry-induced differences in DC migration. Taken together, these results show that the response of DCs to substrate geometry is a dynamic process mediated through myosin contractility, integrin-based adhesions and cytoskeletal reorganization.

Discussion

DCs have been shown to migrate well in a variety of settings including 2D surfaces, 3D gels and confinement chambers and are capable of migrating both with and without the use of integrins^{29,15}. In line with the adaptability of DC migration, we showed that DCs migrate well on different substrates and tune their speed and persistence to optimize their efficiency. It is unclear which mode of migration is used in vivo, but we hypothesize that the decreased persistence time could be beneficial to DCs in the lymph node where their goal is to probe surrounding cells and locate their cognate T cell. This initial observation motivated us to identify the specific biophysical parameters to which DCs respond. The two parameters we focused on the present study were stiffness and geometry. We performed experiments to explore each parameter individually and determine their relative contributions to DC migration. First, we used a set of mPADs with identical 2D geometry and different stiffness. In terms of physiological relevance, the softest mPAD (~1 kPa) is similar to the stiffness of endothelial tissue, the medium mPAD (~10 kPa) is similar to the stiffness of skeletal muscle and the stiffest mPAD (~1,000 kPa) starts to approach the compliance of rigid materials such as bone⁵. Somewhat surprisingly, we discovered that DCs are insensitive to stiffness. Since DCs are present throughout the body and in a variety of different tissues, each having a characteristic stiffness, it would be useful for DCs to be able to migrate well in a variety of different environmental compliances.

Next, we investigated the contribution of substrate geometry to DC migration. Substrate geometry can be split into two categories—the physical structure of the underlying surface and the patterning of adhesive ligand. It has often been difficult to probe the effect of these two parameters individually. For example, Frey et al. showed that 3T3 fibroblasts migrating on microposts moved faster and turned more frequently than fibroblasts on continuous surfaces¹⁰. Even though fibronectin was adsorbed to the entire substrate, the cells preferentially migrated on the post tips and not the interpost regions¹⁰. As a result, cells were exposed to discrete ligand and discrete substrate, and it was not determined whether the influence on migration was due to physical topography or the discrete pattern of ligand that the cells encountered. In a separate study, Wójciak-Stothard et al. exposed macrophages to grooved substrates containing regularly spaced, parallel lines³⁰. In this environment, macrophages were shown to migrate in straight lines, following the direction of the ridges and grooves, thus leading to a significantly increased persistence time³⁰. Again, it is not clear what aspect of the environment influences cell migration, as ligand patterning was restricted by the substrate structure. Our approach at printing patterns on a flat substrate permitted us to separate the effect of physical geometry from ligand geometry, and allowed

us to determine that DCs respond directly to ligand patterning and not substrate structure. It is unknown how sensitive DCs are to ligand patterning, but it is clear that they can robustly differentiate continuous from discrete ligand presentation. Overall, we found that DCs migrate faster and turn more frequently on punctate surfaces. Given that DCs migrate predominantly through filopodial extension, adhesion, and contraction²¹, the increased turning seems consistent with a cell that reaches out to find and touch spots; if the number of spots on the substrate is limited, the contact and turning frequency would be significantly enhanced.

Finally, we investigated signaling pathways relevant for DC geometry sensing. Since we previously showed that myosin is necessary for interactions with post arrays and transmission of traction forces²¹, it seemed likely that myosin would also be important for geometry sensing and migration. Our experiments reveal that myosin inhibition reduces persistence time of DCs on flat surfaces, to levels similar to that seen for DCs on posts. However, myosin inhibition of DCs on posts is unable to further decrease persistence time. This suggests that myosin contractility is indeed important for DC migration, in particular for regulation of persistence time. The normalization of migration on flat surfaces and posts in the presence of blebbistatin has also been shown for fibroblasts (although the authors focused on normalization in terms of speed and not persistence time)¹⁰.

Our experiments with GFP-Lifeact DCs revealed that the organization of the actin cytoskeleton is affected by substrate geometry. Geometry-dependent reorganization of actin has also been observed in macrophages migrating along a series of ridges and grooves³⁰. In these cells, actin was highly concentrated at the boundary of the ridge and groove interface, just as we see actin at the boundary of the posts on mPADs³⁰. Others have shown that the actin cytoskeleton is reorganized in cells adhered to discrete patterns of ligand²⁷, but unlike our observations, the actin is concentrated into solid circles over the patches and not into rings. These rings are dissimilar to other actin structures typically observed in DCs, such as podosomes, which have the appearance of punctate actin spots surrounded by a ring of adhesion protein⁶. However, the rings were not seen on surfaces in which fibronectin was stamped in spots on PDMS (see Supplemental Figure 5), which suggests the ring structure owes its origin in part to the curvature of the mPAD tips. Our observations suggest that actin is actively involved in the response of DCs to environmental geometry. The specific actin pathway responsible for this process has not been identified, but inhibition studies with CK666 have ruled out the Arp2/3 complex.

A final pathway we chose to investigate was the role of integrin-mediated adhesion. Since our surfaces are coated with fibronectin, we chose to inhibit each of the two subunits of $\alpha_5\beta_1$. While DCs in 3D environments are capable of migrating without the use of integrins, we and others have shown that DCs migrating in 2D require integrins¹⁵. When we partially inhibit either integrin subunit in DCs migrating on posts, we see a complete elimination of geometry dependence. Thus, although DCs are capable of migrating in a manner that is independent of integrins¹⁵, integrin engagement was critical for the ability of DCs to sense geometry while migrating in our 2D systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

DCs on PDMS-coated coverslips persist in the same direction for longer periods of time. (A) Sample phase contrast images of DCs on mPADs and (B) PDMS-coated coverslips. Colored lines show sample cell trajectories at each time point. Trajectories are longer and more linear for DCs on PDMS-coated coverslips than for DCs on mPADs. DCs were plated on 10 μg/mL fibronectin and stimulated with 10 nM CCL19. Sample cell tracks for DCs on mPADs (C) and PDMS-coated coverslips (D). In these specific samples, DCs on PDMS-coated coverslips cover a larger area during random migration. Over the entire population of cells tested for each condition, there was no statistically significant difference in this parameter (see random motility coefficient, Figure 4D). E) The Mean Squared Displacement of both cell populations shows a linear fit with time, on a log-log scale, with slope approximately equal to 1 (gray dashed line), indicating diffusive migration

Figure 2.

DCs migrated differently on PDMS-coated coverslips and mPADs. A) The average speed of DCs on mPADs is significantly greater than that of DCs on PDMS-coated coverslips.. B) The persistence time of DCs on mPADs is drastically reduced, as compared to the persistence time of DCs on PDMS-coated coverslips. C) The reduction in persistence time is carried through to a reduction in the persistence length. D) The combination of the increase in average speed and decrease in persistence time, results in a non-significant difference in the overall diffusivity. Figures represent average values $\pm$ SEM, for > 650 DCs from at least three independent experiments per condition. PDMS-coated coverslip values correspond to our previous data on characterizing DC migration on PDMS surfaces ². Statistical significance calculated with single factor ANOVA and post hoc Tukey test. Indicates significant difference compared to PDMS-coated coverslip. * $p < 0.05$, ** $p < 0.01$

Figure 3.

DCs respond to mPAD geometry, not stiffness. A) There is no trend in the average speed of DCs on the different mPADs. B) The persistence time of DCs is significantly reduced on all mPADs, regardless of stiffness. Figures represent average values $\pm$ SEM, for > 180 DCs from at least three independent experiments per condition. PDMS-coated coverslip values correspond to our previous data on characterizing DC migration on PDMS surfaces². Statistical significance calculated with single factor ANOVA and post hoc Tukey test. Asterisks indicate significant difference compared to PDMS-coated coverslip. * $p<0.05$, ** $p<0.01$. There is no statistically significant difference between the tall mPAD and the short and medium mPADs.

Figure 4.

DCs respond to geometry of printed ligand. A) Patterned stamps were used to transfer 488-conjugated fibronectin to PDMS-coated coverslips. Tracking was performed on the pattern located in the top right corner, which closely matches the mPAD geometry. This large dot pattern has the following dimensions: diameter of circular islands is 2 µm and centroid-to-centroid spacing is 5 µm. B) The average speed of DCs on PDMS-coated coverslips printed with islands of fibronectin is significantly greater than that on PDMS-coated coverslips printed with continuous fibronectin, but indistinguishable from that on mPADs. C) The persistence time of DCs on both mPADs and PDMS-coated coverslips printed with islands of fibronectin is significantly lower than that on PDMS-coated coverslips printed with continuous fibronectin. Figures represent average values $\pm$ SEM, for > 650 DCs from at least three independent experiments per condition. PDMS-coated coverslip values correspond to our previous data on characterizing DC migration on PDMS surfaces². Statistical significance calculated with single factor ANOVA and post hoc Tukey test. Asterisk indicates significant difference compared to PDMS-coated coverslip. *p<0.05, **p<0.01. There are no statistically significant differences between the tall mPAD and the patterned PDMS-coated coverslips.

Figure 5.

DCs do not detect micron-scale differences in geometry. A) The average speed of DCs on smaller diameter mPADs is significantly greater than that on PDMS-coated coverslips, but indistinguishable from that on larger diameter mPADs. B) The persistence time of DCs on smaller diameter mPADs is significantly lower than that on PDMS-coated coverslips, but is again indistinguishable from that on larger diameter mPADs. Figures represent average values $\pm$ SEM, for > 290 DCs from at least three independent experiments per condition. PDMS-coated coverslip values correspond to our previous data on characterizing DC migration on PDMS surfaces². Statistical significance calculated with single factor ANOVA and post hoc Tukey test. Asterisks indicate significant difference compared to PDMS-coated coverslip. * $p < 0.05$, ** $p < 0.01$. There are no statistically significant differences between the tall mPAD and the small mPAD.

Figure 6.

The actin cytoskeleton is reorganized on mPADs. Representative GFP-LifeAct DCs plated on PDMS-coated coverslips (A) or mPADs (B and C). Cells were plated on 10 $\mu\text{g/mL}$ fibronectin and stimulated with 10 nM CCL19. Scale bars represent 10 μm . A) On PDMS-coated coverslips, actin is concentrated at the periphery. B) On mPADs, actin is redistributed into rings at the periphery of engaged microposts.

Figure 7.

DC response to substrate geometry is influenced by myosin contractility. A) The average speed of DCs on both PDMS-coated coverslips and mPADs is unaffected by myosin inhibition and is significantly reduced by inhibition of the Arp2/3 Complex. B) The persistence time of DCs on PDMS-coated coverslips is significantly reduced in the presence of the myosin inhibitor blebbistatin. Blebbistatin has no effect on DC persistence on mPADs. Arp2/3 Complex inhibition has no effect on persistence time for DCs on either PDMS-coated coverslips or mPADs. C) Sample phase contrast images of DCs inhibited with CK666 (left) or blebbistatin (right), on PDMS-coated coverslips (top) and mPADs (bottom). Colored lines indicate sample cell trajectories. Under these two inhibitor conditions DCs turn at similar frequencies. DCs were plated on 10 $\mu\text{g/mL}$ fibronectin and stimulated with 10 nM CCL19. Figures represent average values $\pm$ SEM, for > 290 DCs from at least three independent experiments per condition. PDMS-coated coverslip values correspond to our previous data on characterizing DC migration on PDMS surfaces². Statistical significance calculated with single factor ANOVA and post hoc Tukey test. Asterisks indicate significant difference compared to PDMS-coated coverslip without inhibitors. * $p < 0.05$, ** $p < 0.01$. Crosses indicate significant difference compared to tall mPAD without inhibitors. † $p < 0.05$, †† $p < 0.01$.

Figure 8.

DC response to substrate geometry is influenced by integrin-based adhesions. A) Blocking of either integrin subunit (α_5 or β_1) had no effect on the average speed of DCs on PDMS-coated coverslips, but significantly reduced the speed of DCs on mPADs. B) Likewise, blocking of either integrin subunit had no effect on persistence time of DCs on PDMS-coated coverslips, but significantly increased the persistence time of DCs on mPADs. C) Sample phase contrast images of DCs blocked with antibodies against α_5 (left) or β_1 (right), on PDMS-coated coverslips (top) and mPADs (bottom). Under these two inhibitor conditions, cells persist for long periods of time on mPADs and often exhibit an elongated morphology pointed in the direction of migration. Scale bars equal 20 μm . DCs were plated on 10 $\mu\text{g}/\text{mL}$ fibronectin and stimulated with 10 nM CCL19. Figures represent average values $\pm$ SEM, for > 290 DCs from at least three independent experiments per condition. PDMS-coated coverslip values correspond to our previous data on characterizing DC migration on PDMS surfaces². Statistical significance calculated with single factor ANOVA and post hoc Tukey test. Asterisks indicate significant difference compared to PDMS-coated coverslip without inhibitors. * $p < 0.05$, ** $p < 0.01$. Crosses indicate significant difference compared to tall mPAD without inhibitors. † $p < 0.05$, †† $p < 0.01$.

Table 1

Dimensions and stiffness of different mPADs. The mPAD used for DC force measurement (as well as the initial migration experiments and inhibitor studies) was the tall mPAD.

Large mPADs			
Diameter: 1.83 μm			
Post-to-Post Spacing: ~3 μm			
Name	Post Height (μm)	Spring Constant (nN/μm)	Effective Stiffness (kPa)
Short	0.97	4523.98	1218.44
Medium	5.70	22.30	17.21
Tall	12.90	1.92	1.49
Small mPAD			
Diameter: 0.80 μm			
Post-to-Post Spacing: ~2μm			
Small	4.77	1.39	2.49