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Two-Dimensional Motility of a Macrophage Cell Line on Microcontact-Printed Fibronectin

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Abstract

The ability of macrophages to migrate to sites of infection and inflammation is critical for their role in the innate immune response. Macrophage cell lines have made it possible to study the roles of individual proteins responsible for migration using molecular biology, but it has not been possible to reliably elicit the motility of macrophage cell lines in two-dimensions. In the past, measurements of the motility of macrophage cell lines have been largely limited to transwell assays which provide limited quantitative information on motility and limited ability to visualize cell morphology. We used microcontact printing to create polydimethylsiloxane (PDMS) surfaces functionalized with fibronectin that otherwise support little macrophage adhesion. We used these surfaces to measure macrophage migration in two-dimensions and found that these cells migrate efficiently in a uniform field of colony-stimulating factor-1, CSF-1. Knockdown of Cdc42 led to a non-statistically significant reduction in motility, whereas chemical inhibition of PI3K activity led to a complete loss of motility. Inhibition of the RhoA kinase, ROCK, did not abolish the motility of these cells but caused a quantitative change in motility, reducing motility significantly on high concentrations of fibronectin but not on low concentrations. This study illustrates the importance of studying cell motility on well controlled materials to better understand the exact roles of specific proteins on macrophage migration.

Introduction

Macrophages are highly motile cells of the monocytic lineage and are important in a variety of biological processes including innate immunity, development, and disease (Pollard 2009). During the innate immune response, macrophages must move quickly and efficiently to sites of infection or inflammation in order to clear the site of pathogens and release cytokines (Pixley 2012). In order to do this, macrophages move towards cytokine signals released by inflamed tissue, such as macrophage colony stimulating factor-1 (CSF-1 also known as M-CSF1). M-CSF1 signals the cell through the CSF-1 receptor, a tyrosine kinase receptor, which dimerizes and autophosphorylates upon ligand-binding (Hamilton 1997). In addition

to cytokine signals, macrophage migration is regulated by proteins of the extracellular matrix (ECM) such as fibronectin and collagen through integrin-binding interactions. Signaling downstream of both the M-CSF1 receptor and integrins is controlled by a variety of proteins including several members of the Rho GTPase family as well as cytoskeletal proteins (Allen et al. 1997; Allen et al. 1998; Jones 2000). When properly regulated, macrophage motility is critical to maintain homeostasis but improper regulation of this migration can lead to a progression of diseases such as cancer, rheumatoid arthritis, and atherosclerosis (Pollard 2009). For example, tumor associated macrophages have been associated with a poor prognosis in several types of cancer and are often associated with high levels of metastasis and solid tumor angiogenesis (Mantovani and Sica 2010).

Macrophages, like other leukocytes, employ amoeboid migration. Macrophages do not form strong focal contacts to the substratum but rather create short-lived weak adhesions that allow them to move quickly through their environment (Pixley 2012). These adhesions may involve the formation of podosomes, which are comprised of actin-rich cores surrounded by rings of adhesion proteins such as vinculin (Calle et al. 2006). Podosomes are known to function in matrix remodeling and degradation and many of the same proteins found in functional podosomes are critical for macrophage migration; however, no direct link has been found between podosomes and macrophage migration (Dovas et al. 2009). It is crucial that we understand how macrophages move through their environments and how this movement is coordinated.

Immortalized macrophage cell lines, such as the subline of RAW264.7 (RAW/LR5) cell line, are invaluable tools for studying the specific role of various proteins because of the ability to change their proteomics through molecular biology. In the past, the motility of these cells has been investigated using transwell chambers (Dovas et al. 2009) and ruffling assays (Park and Cox 2010), but analysis of their 2D migration on specific extracellular matrix (ECM) proteins has not been possible. On most surfaces normally employed to study 2D motility, such as tissue culture plastic and glass, the cells polarize but do not crawl, making studies of directional motility in 2D impossible on those materials. Given the numerous mutants of RAW/LR5 cells that have been created, a means to effectively elicit and measure the 2D motility of these cells would allow us to better understand how motility in macrophages is controlled molecularly.

We used microcontact printing to prepare surfaces specifically coated with fibronectin and quantified the motility of RAW/LR5 macrophages undergoing chemokinesis. Previously, our laboratory showed that microcontact printing fibronectin allowed elucidation of the mechanisms of neutrophil motility (Henry et al. 2014). With RAW/LR5 cells, we found that these materials elicit robust migration, which we attribute to the effective blocking of non-specific adhesion on these materials. We then used these surfaces to compare the migration of wild-type RAW/LR5 cells to the migration of RAW/LR5 cells with chemically inhibited ROCK or PI3K and of RAW/LR5 cells with reduced endogenous levels of the GTPase Cdc42. Cells without PI3K activity lost their ability to polarize and showed no migratory capabilities. Cells with reduced Cdc42 levels showed no significant change in motility compared to wild type RAW/LR5 macrophages but showed increased ruffling behavior. Finally, cells in which ROCK signaling was inhibited were highly sensitive to fibronectin

concentration showing two different motile phenotypes with correspondingly different random motility coefficients on high versus low concentrations of fibronectin, with motility most significantly reduced on high concentrations of fibronectin. These results illustrate the importance of studying cell motility on well defined surfaces and allow us to realize the potential of these modified cell lines for the study of the molecular mechanisms of macrophage migration.

Materials and Methods

Reagents

Bovine fibronectin was obtained from Sigma (St. Louis, MO) and recombinant murine CSF-1 was obtained from PeproTech (Rocky Hill, NJ). We used the inhibitors LY294002 at 50 μ M from Cell Signaling (Boston, MA), Wortmannin at 10 μ M from Sigma (St. Louis, MO), and Y27632 at 10 μ M from Millipore (Billerica, MA).

Cell Culture

The RAW/LR5 and RAW/LR5 shCdc42 cell lines have been previously characterized and were obtained from Dianne Cox's lab (Albert Einstein College of Medicine, Bronx, NY) (Cox et al. 1997; Park and Cox 2009). Murine bone marrow derived macrophages (BMMs) were isolated and prepared according to (Park and Cox 2009). The RAW/LR5 Lifeact-mCherry cell line was created by retroviral transduction using the vector pTK93_Lifeact-mCherry (Addgene plasmid 46357), which was generously deposited by Dr. Iain Cheeseman. Retrovirus was packaged using 293T cells, purified by ultracentrifugation, and titered by flow cytometry as previously described (Peltier and Schaffer 2010). RAW/LR5 cells were infected at a multiplicity of infection of 0.25 and treated with 3 μ g/ml puromycin for 6 days to select for transduced cells. All cells were cultured in supplemented RPMI medium containing 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO) and 1% penicillin-streptomycin (MediaTech, Manassas, VA). All cells were maintained at 37°C and 5% CO₂.

PDMS Microcontact Printing of Fibronectin

Poly(dimethyl siloxane) (Dow Corning, Midland, MI) or PDMS was made at a 10:1 ratio of polymer to cross-linker. Round glass 25 mm coverslips were cleaned in 0.2N hydrochloric acid and then rinsed twice with Milli-Q water and once with 99% ethanol. Coverslips were dried with pressurized N₂. The coverslips were then spincoated with PDMS using the Laurell Spinner (4000 rpm, 1 minute). Coverslips were allowed to cure for 1 hour in a 62°C oven. To generate stamps, flat PDMS was cured against a silicon wafer to ensure a uniform topology. Small 1cm² cubes were then cut from the PDMS block. The stamps were inked with fibronectin (Sigma, St. Louis, MO). The PDMS spincoated coverslips to be stamped were then treated with UV ozone for 7 minutes to create a hydrophilic surface for optimal protein transfer. The stamps were washed with water and carefully dried with pressurized N₂. The stamps were then placed on coverslips prepared under UV ozone and protein transfer occurred almost immediately. The stamps were then removed, and the stamped coverslips were blocked with 0.2% Pluronic-F127 (Sigma, St. Louis, MO) for 30 minutes.

The coverslips were rinsed 3x with 1xPBS and incubated in PBS overnight (Desai et al. 2011).

Chemokinesis Assay

Stamped coverslips were attached to a 6-well plate for chemokinesis experiments. Cells were plated in each well at 4.2×10^4 cells/mL. Cells were incubated overnight in RPMI supplemented with 1% fetal bovine serum (Sigma, St. Louis, MO) and 1% penicillin-streptomycin. After incubation, the cells were washed with RPMI to remove any unattached cells. Chemokinesis media consisted of serum-free RPMI supplemented with the indicated concentration of CSF-1 (PeproTech, Rocky Hill, NJ). Using a custom-built LabView (Texas Instruments, Austin, TX) software, 24 fields of view were imaged at 20 $\times$ magnification by phase microscopy on a Nikon Eclipse TE300 (Nikon, Melville, NY). Images were captured every two minutes for four hours using time-lapse microscopy. Cell trajectories were captured using the ImageJ ManualTracking plugin. Chemokinesis parameters were calculated using a custom written MATLAB (Mathworks, Natick, MA) script which fits the speed (S) and persistence time (P) to the Dunn Equation:(Dunn 1983) $\langle d^2 \rangle = nS^2[Pt - P^2(1 - e^{-t/P})]$. The random motility coefficient is a relative diffusion coefficient for the cells in a uniform chemokine field. The random motility coefficient, μ , is calculated using the fit

parameters in the following equation, $\mu = \frac{1}{n} S^2 P$.

Chemical Inhibition

Pharmacological inhibition of cells, if any, was performed by pre-incubation of cells with inhibitor for 1 hour at the designated concentration before the experiment and continued incubation at the same concentration during the experiment.

Immunofluorescence

Cells were fixed for 7 minutes in 3.7% paraformaldehyde and permeabilized for 4 minutes in 0.1% Triton X-100. Actin was detected using Phalloidin coupled to Alexa-Fluor 568 (Invitrogen, Carlsbad, CA). Vinculin was detected using the monoclonal antibody hVIN1 (ab11194, Abcam, Cambridge, UK). The secondary antibody used was Alexa-Fluor-488-labeled goat anti-mouse (Invitrogen, Carlsbad, CA). Coverslips were mounted using Fluoromount G (SouthernBiotech, Birmingham, AL) as an anti-fading reagent.

Images of fixed samples were acquired with a confocal microscope (Leica SP5) equipped with a 63 $\times$ oil objective. Images were processed using the Leica Application Suite (Leica, Wetzlar, Germany) and ImageJ software.

Results

Microcontact Printing of Fibronectin

Microcontact printing was used to prepare the surfaces for all experiments described in this paper. A schematic overview of the printing technique is shown in Figure 1A. A flat 1 cm² polydimethylsiloxane (PDMS) stamp was incubated with a specific concentration of the ECM protein fibronectin. Separately, a glass coverslip was spin-coated with a thin layer of

PDMS. This PDMS-coated coverslip was then treated with UV ozone to render the surface hydrophilic. Excess protein was removed from the stamp by carefully washing it with water and the stamp was dried with pressurized nitrogen gas. The dry, hydrophobic stamp was then brought into contact with the hydrophilic surface and the protein preferentially transferred to the surface. Finally, the surface was blocked with the polymer Pluronic-F127 which binds to unreacted groups on the PDMS, functionally blocking the surface. Stamping PDMS offers many advantages over traditional techniques for creating molecularly coated surfaces for imaging cell motility in two dimensions. First, the stamping method allows for precise spatial control of a protein ligand, as illustrated with fluorescently-tagged fibronectin in Figure 1B. The ligand is also allowed to bind uniformly across the surface because it is transferred from a hydrophobic inked surface to a hydrophilic surface (Desai et al. 2011). PDMS is also convenient for imaging because it is transparent to optical wavelengths (Ye et al. 2006).

We first stamped PDMS with fluorescently labeled fibronectin and then blocked the surfaces with either 0.2% Pluronic-F127 (Figure 1D) or 1% bovine serum albumin (Figure 1C) to illustrate the fidelity of the stamping method and the importance of functionally blocking the surface. In Figure 1D, the cells only adhere to the surface on the fibronectin-patterned area, indicated by the fluorescent signal associated with fibronectin. The cells did not interact with the surface blocked solely with pluronic, indicating that cells do not bind to the polymer; therefore, all RAW/LR5 motility is due to binding interactions with fibronectin. Conversely, in Figure 1C, when surfaces are blocked with BSA, cells adhere to the surface on both the patterned and unpatterned regions indicating that the cells are interacting with the blocking protein (BSA) and the motile behaviors seen by these cells are not specific to their interaction with the fibronectin ligand. This result is consistent with our recently published observations on the motility of neutrophils on PDMS substrates which showed that BSA, normally thought of as a blocking protein, acts as a ligand for Mac-1 (Henry et al. 2014). Our ability to functionally block the PDMS surface offers a distinct advantage over glass or tissue culture plastic, to which pluronic does not bind (Yang et al. 2011), because it ensures that cell response is due to cell-ligand interactions and not unintended or non-specific cell-surface interactions.

We assessed if RAW/LR5 macrophages would form structures such as podosomes on the microcontact printed surfaces. To visualize molecular organization at the cell-substrate interface, RAW/LR5 macrophages cells were seeded on fibronectin-printed PDMS surfaces blocked with Pluronic-F127 then fixed and stained for F-actin (Figure 2B) and vinculin (Figure 2C). The RAW/LR5 macrophages showed small punctate F-actin clusters surrounded by vinculin rings, indicative of podosomes. When these stained images are overlaid (Figure 2A), typical podosome structures are easily recognizable at the leading edge of polarized cells. We further investigated podosome dynamics in motile macrophages using live imaging of the RAW/LR5 mCherry-LifeAct cell line. Cells were imaged in phase to confirm normal motility and in fluorescence to observe actin dynamics. As illustrated by the representative cell shown in Supplemental Video 1, podosomes are highly active at the leading edge of motile macrophages. When a bifurcation of the leading pseudopod occurs, podosomes appear in both extensions until the leading edge is re-established and podosomes in the rear of the cell are disassembled. This indicates that podosomes are only stable at the

leading edge of the cell suggesting that they are involved in the directional sensing of the cell. We concluded that this method of preparing surfaces is optimal for analyzing two-dimensional migration of RAW/LR5 macrophages because cell-substrate interactions are clearly defined, cells retained their ability to form podosomes, and cells were motile.

RAW/LR5 Chemokinesis on Fibronectin-Printed PDMS

Two-dimensional migration of RAW/LR5 macrophages on a clearly defined surface has not been previously described; therefore, migration parameters such as speed, persistence time, and the random motility coefficients for this cell line have not previously been elucidated. Analysis of two-dimensional migration would allow us to determine the type of migration used by RAW/LR5 macrophages and how their migration compares to other amoeboid and mesenchymal cells. In one experiment, RAW/LR5 macrophages were seeded on PDMS printed with various concentrations of fibronectin and exposed to a uniform concentration, 20 ng/mL, of the chemokine CSF-1. In a second experiment, cells were seeded on PDMS printed with a uniform concentration of fibronectin, 5 μ g/mL, and exposed to various concentrations of the soluble chemokine CSF-1. RAW/LR5 cells were able to efficiently migrate on fibronectin stamped PDMS (Supplemental Video 2) and the random motility coefficient, a relative diffusion coefficient of migrating cells, showed biphasic motility as a function of the concentration of fibronectin (Figure 3A), and the concentration of soluble chemokine, CSF-1 (Figure 3B). Murine bone marrow derived macrophages (BMMs) were also able to migrate efficiently on PDMS printed with fibronectin and their motility was biphasic as a function of fibronectin concentration with peak motility at 2.5 μ g/mL (Supplementary Figure 1). This result proves that 2D migration of both macrophage cell lines and primary cells is supported by the PDMS printing technique outlined in this paper. The effect of ligand density on cell migration is commonly seen, because a low concentration of ligand does not provide sufficient traction and a high concentration of ligand makes cells too adhesive (Palecek et al. 1997). The effect of soluble chemoattractant concentration on migration is also expected to be biphasic, since low concentrations of ligand do not provide sufficient signal and high ligand concentrations overwhelm the cell's signaling pathway leading to lower response to the signal (Ricart et al. 2011). The peak concentration of CSF-1 for motility is consistent with the values reported previously for these cells (Cox et al. 1997). Plots showing the dispersion of cells were created (Figure 4A) for all conditions by tracking each cell and moving the start of each cell track to the origin of the axis. These plots show qualitatively that at high and low concentrations of fibronectin, the RAW/LR5 macrophages migrate to a lesser extent than they do on a moderate fibronectin concentration. The plots also confirm that migration of these cells was random with no bias in one direction. The mean-squared displacement of the cells on each fibronectin concentration was plotted versus time (Figure 4B) and this plot was used to fit the speed and persistence time of the cells using the Dunn equation. At all fibronectin concentrations α was found to be nearly one indicating that RAW/LR5 macrophage chemokinesis is well-defined by the random walk model (Supplementary Table 1).

RAW/LR5 Chemokinesis with Leading Edge Inhibition

Several proteins are known to be important in the migration of cells, and the RAW/LR5 cells provide a unique opportunity to study how these molecules might affect macrophage

motility on surfaces that have been printed with fibronectin. Many of these proteins locate specifically to the leading edge of the cell during migration and are responsible for actin polymerization as well as maintenance of cell polarity and signaling downstream of integrin-fibronectin and chemokine-receptor signaling. We measured the motility of a RAW/LR5 derived cell line with reduced endogenous levels of the GTPase Cdc42. These cells were created using short-hairpin RNAi which led to an 80% reduction in Cdc42 levels compared to control cells (Park and Cox 2009). The shCdc42 cells qualitatively showed biphasic motility as a function of fibronectin concentration, much like the wild type RAW/LR5 cells, but there was no significant difference seen in the random motility at any fibronectin concentration (Figure 5A). The shCdc42 cells showed a non-statistically significant reduction in motility at each fibronectin concentration compared to the wild type cells but they were still able to efficiently migrate on the printed surfaces (Supplemental Video 3). These results indicate that Cdc42 is not required for efficient migration of RAW/LR5 macrophages but might have some contribution downstream of integrin-fibronectin binding. It is possible that the remaining Cdc42 in the knockdown cells provides sufficient signaling to maintain motility; however, it has been previously shown that the same cell line has reduced motility to the chemokine, CX3CL1 (Park and Cox 2010). The mean squared displacement was plotted as a function of time (Figure 5B), and the speed and persistence time for shCdc42 cells using the Dunn equation. The fit of the mean squared displacement yielded an α of 1.14, so the random migration is well-modeled as a persistent random walk. These cells were also stained for adhesion structures to determine if podosomes were present (Figure 5C). Consistent with previously published results (Dovas et al. 2009), the shCdc42 cells have actin-mediated protrusion, but do not show any of the hallmark structures of podosomes such as punctuate actin bundles or vinculin rings. This data suggests that in the RAW/LR5 cells, Cdc42 is required for podosome formation but that podosomes are not necessary for efficient random migration.

We further investigated the effect of leading edge inhibition by targeting another protein, phosphoinositide 3-kinase (PI3K), which acts upstream in the signaling pathway from Cdc42. We inhibited this protein using two different chemical inhibitors, LY294002 and Wortmannin, and studied the migratory capacity of inhibited cells. We found that the use of either chemical inhibitor led to the complete loss of cell motility (Figure 5D). Under inhibition of PI3K, cells did not polarize to the same degree as uninhibited cells or migrate efficiently. The plot of mean squared displacement versus time (Figure 5E) further illustrates that these cells show minimal displacement over time for cells inhibited with either chemical inhibitor. Loss of PI3K activity also led to a disorganized cytoskeleton and the loss of podosomal structures seen in wild type cells (Figure 5F). In the inhibited cells, actin and vinculin remain cytoplasmic with no polarized distribution or organization. These data together indicate that PI3K is necessary for migration of RAW/LR5 macrophages and formation of podosome adhesion structures.

RAW/LR5 Chemokinesis with Cell Contraction Inhibition

The trailing edges of migrating cells rely on myosin II contraction to release the rear of the cell from the substratum and allow the cell to advance forward. Several proteins are important in myosin contraction. RhoA is the Rho family protein primarily involved in

myosin contractility and it stimulates this contractility through the RhoA kinase, ROCK. ROCK has been linked to the motility of many different cell types (Smith et al. 2007) and we wanted to determine its role in RAW/LR5 migration. The ROCK inhibitor Y-27632 was used at 10 μ M to abolish ROCK activity and myosin II contraction in migrating RAW/LR5 macrophages. In contrast to the biphasic motility seen in uninhibited macrophages, RAW/LR5 cells treated with the ROCK inhibitor showed a switch-like change in their random motility coefficient over a range of fibronectin concentrations (Figure 6A). At high concentrations of fibronectin, 50 μ g/mL and 10 μ g/mL, cells inhibited with Y-27632 had a low but constant random motility coefficient. However, at fibronectin concentrations lower than 10 μ g/mL, cells inhibited with Y-27632 had a higher and constant random motility coefficient similar to control treated cells. Under inhibition, the random motility coefficients at lower concentrations of fibronectin were not significantly different than each other but they were all significantly higher than the random motility coefficients at higher concentrations of fibronectin. This difference in random motility on high versus low fibronectin concentrations can be appreciated qualitatively by examining the dispersion plots for cells migrating on a high concentration and a low concentration of fibronectin (Figure 6B). The random motion of ROCK inhibited RAW/LR5 cells migrating on 50 μ g/mL fibronectin show far less dispersion and overall movement than the cells migrating on 2.5 μ g/mL fibronectin. The change in random motility was accompanied by a change in cell morphology between low and high fibronectin concentrations (Figure 6C and Supplemental Videos 4 and 5). On high concentrations of fibronectin, RAW/LR5 cells were unable to contract their trailing edges; this caused the cells to have long unretracted tails during migration (Figure 6C, top and Supplemental Video 4). In contrast, on low concentrations of fibronectin, RAW/LR5 cells showed a much more rounded morphology and showed long uropods that after sufficient migration would release and “snap” back to the cell body (Figure 6C, bottom and Supplemental Video 5). The reduced migration of ROCK inhibited RAW/LR5s on high concentrations of fibronectin is also illustrated in the plot of mean squared displacement versus time (Figure 6D). The cells on high concentrations of fibronectin have a far lower overall displacement over time. Finally, cells were stained to determine the effect of ROCK inhibition on the assembly of podosomes. ROCK-inhibited macrophages showed typical podosome structures (Figure 6E) with actin bundles surrounded by clear vinculin rings. These cells also show active actin-rich lamellipodia and display a long trailing tail which contains both actin and vinculin. Other researchers have found that stabilization of the actin cytoskeleton and integrin activation are tightly correlated with ROCK activity in monocytic cells (Worthylake and Burridge 2003) which complements our finding that fibronectin signaling levels alters the motile behavior of ROCK-inhibited RAW/LR5 cells. Overall, our data suggests that ROCK activity is not necessary for macrophage motility but plays an important role in organizing the response to fibronectin. Different levels of fibronectin lead to the activation of different signaling pathways or different levels of response to ROCK; this then leads to changes in the morphology and motility of RAW/LR5 macrophages.

RAW/LR5 Speed and Persistence Times in Motile Conditions

One major advantage of imaging cells and analyzing their motility in two dimensions is the ability to quantitatively compare cells moving under different conditions. We measured the

values of speed and persistence time for the motile RAW/LR5 cells investigated in this paper and determined what specific relationships could be determined by comparing cells with inactive ROCK or reduced endogenous levels of Cdc42 to wild-type RAW/LR5 macrophages. When the persistence time of the cells was plotted versus their speed we saw an inverse relationship for all motile cells (Figure 7A). This inverse relationship between speed and persistence time is seen in many motile cell types (Lauffenberger and Linderman 1993) and often lends insight into the type of migration a cell is undergoing and its physiological role. For example, endothelial cells which must move in a directed path during tissue development or wound repair move slowly but with high persistence (Stokes and Lauffenberger 1991) whereas neutrophils and other immune cells which must constantly be scavenging for pathogens and areas of inflammation move with high speed but low persistence (Henry et al. 2014; Jannat et al. ; Ricart et al. 2011). The persistence time of migrating macrophages was found to be biphasic as a function of fibronectin concentration for all motile cells (Figure 7B). The fibronectin concentration at which the maximum persistence time occurred for cells with reduced endogenous levels of Cdc42 was slightly lower compared to wild type cells. The fibronectin concentration required for maximum persistence time was even lower for cells with inhibited ROCK signaling. For all conditions, the maximal persistence time corresponded to the fibronectin concentration with the highest random motility coefficient, indicating that RAW/LR5 migration is driven by persistence even though the random motility coefficient only depends mathematically first order on persistence and second order on speed. Physiologically, it is important for cells to have some persistent motion because without persistent motion cells do not explore a wide enough territory to fully take advantage of the persistent random walk for investigating their surroundings.

The speed and persistence time of individual cell migration were compared to determine which parameter dominated the motility. The speed and persistence time of migrating wild type RAW/LR5 cells show no real trend on increasing concentrations of fibronectin other than an increased dependence of speed on fibronectin concentration at high fibronectin concentrations (Figure 7C). We used cells with reduced endogenous levels of Cdc42 and cells with inhibited ROCK signaling to clarify the roles of specific proteins on the speed and persistence of migrating RAW/LR5. We found that the motility of RAW/LR5 cells with reduced endogenous levels of Cdc42 is dominated by persistence time at low concentrations of fibronectin but is dominated by speed at high concentrations of fibronectin (Figure 7D). This indicates that Cdc42 is involved in signaling pathways downstream of integrins binding to fibronectin and the slight inhibition of this pathway leads to changes in the persistence of the cells. Macrophages with inhibited ROCK activity show a constant speed of about 2 $\mu\text{m}/\text{min}$ across all fibronectin concentrations (Figure 7E). It is possible that this occurs because cells without myosin II contraction are unable to significantly modulate their speed in response to variable integrin signaling. This data confirms the proposal that ROCK signaling is important in stabilizing the actin cytoskeleton during spreading and migration (Worthylake and Burridge 2003). Changes in the random motility coefficient for the ROCK inhibited macrophages, therefore, arise from differences in their persistence time. This data also indicates that ROCK signaling is important for modulating the speed of macrophages on differing fibronectin concentrations.

Discussion

The importance of properly regulated macrophage migration in maintaining biological homeostasis is well documented and, in the future, macrophages could be used as therapeutic targets because of their role in the progression of various diseases (Hamilton 1997; Pixley 2012; Pixley and Stanley 2004; Pollard 2009). Before we can properly target these cells, however, we must have a better understanding of the signaling pathways that control macrophage migration. In the past, the signaling pathways involved in macrophage migration have been studied with and without the contribution of extracellular matrix proteins (Allen et al. 1997; Allen et al. 1998; Jones 2000; Munugalavadla et al. 2005). We have extended this work by investigating the roles of Cdc42, ROCK, and PI3K in RAW/LR5 motility on a well-defined surface. Visualizing cell migration in two-dimensions using time-lapse imaging is a useful quantitative tool for understanding the way in which changes in signaling affect cell motility (Henry et al. 2014). Changes in the speed or persistence time of cells cannot be easily detected using methods such as transwell assays. A small difference in a cell's ability to persist or a small change in its velocity can significantly alter the cell's ability to use a persistent random migration to efficiently monitor surrounding tissues (Nishimura et al. 2012). We have shown that surfaces microcontact-printed with fibronectin and blocked with Pluronic-F127 are ideal for studying macrophage migration in response to changes in fibronectin concentration. Microcontact-printing allowed us to visualize macrophage migration in two-dimensions and determine the migration parameters of macrophages moving on various fibronectin concentrations. The functional blocking employed by this technique prevents any cell attachment to non-ligand bound surfaces ensuring all cell motility is a direct result of fibronectin-integrin binding without confounding cell-surface interactions. The completeness of our blocking is striking in contrast to traditional blocking moieties such as bovine serum albumin (BSA) to which macrophages can attach and migrate. BSA has been demonstrated to be a ligand to beta-2 integrins, which may explain the residual adhesion of macrophages to BSA (Henry et al. 2014).

Macrophages do not form classical focal adhesions or stress fibers like mesenchymal cells; instead, they form small punctuate complexes known as podosomes. These podosomes consist of an actin core surround by a ring of proteins typically found in mesenchymal focal adhesions such as talin, vinculin, and paxillin (Calle et al. 2006; Dovas and Cox 2011). We stained RAW/LR5 macrophages seeded on microcontact-printed surfaces for actin and vinculin and found small punctate actin cores surrounded by vinculin rings under the leading edge of polarized RAW/LR5 macrophages. This result indicated that the ability of macrophages to form podosomes was intact on our microcontact printed surfaces.

Extracellular matrix proteins, such as fibronectin, are found in all tissues in the body where macrophages reside; however, the specific role that integrin-fibronectin binding plays in macrophage migration is still not well known. By varying the concentration of fibronectin stamped onto our surface, we were able to determine that RAW/LR5 macrophages display biphasic motility with increasing ligand concentration. This result is further illustrated by the dispersion of cells on differing fibronectin concentrations. On the intermediate concentration that gave rise to the optimal motility, the cells are able to explore a much wider area than on

high and low concentrations of fibronectin. This type of motility profile is common among cells that rely on integrin-ligand binding and un-binding for migration (Palecek et al. 1997). This may be important in diseases where high levels of fibronectin are pathological, such as atherosclerosis, and changes in fibronectin concentration could contribute to increased macrophage recruitment (Libby 2009). We were able to show that all motile macrophages are displaying uncorrelated random walks with normal diffusion.

We were also able to show the importance of CSF-1 signaling on macrophage migration by varying the soluble CSF-1 concentration. The motility was again biphasic with increasing CSF-1 concentration at a fixed concentration of fibronectin. This indicates that at low concentrations of CSF-1 the cell is not completely stimulated, at very high concentrations the cell is desensitized, perhaps by receptor down regulation; both conditions lead to sub-optimal motility. The maximum in the random motility coefficient for alveolar macrophages with chemokine concentration was shown previously (Farrell et al. 1990) and is consistent with our results. The limited motility at high CSF-1 concentrations is likely because the CSF-1 receptor is internalized quickly after stimulation and is not recycled back to the membrane (Pixley and Stanley 2004) leading to reduced CSF-1 signaling after the initial stimulation.

Several Rho GTPases are thought to contribute to macrophage motility downstream of both integrin-binding and CSF-1R signaling. Cdc42 has been implicated in directional sensing of macrophages to a gradient of CSF-1 but has not been found to be necessary for random migration (Allen et al. 1997; Allen et al. 1998). We found that reduction of Cdc42 did not significantly change the random motility of cells except at the fibronectin concentration of 5 μ g/mL that was optimal for wild-type motility. These results suggest that the reduction of Cdc42 activity is mostly compensated for by other signaling molecules. We also saw slight morphological changes in migrating cells with reduced Cdc42 levels, indicating a link between Cdc42 signaling and the cytoskeletal network in macrophages. We found that these cells had broader lamellipodia and reduced uropods compared to wild-type RAW/LR5 macrophages. Their lamellipodia also showed increased ruffling. Similar morphological changes have previously been seen in Bac1.2F5 macrophages expressing a dominant negative Cdc42 (Allen et al. 1998). Staining of RAW/LR5 macrophages with reduced Cdc42 levels on fibronectin printed surfaces revealed a lack of podosome formation. Both actin and vinculin were found throughout the cell but were not organized into structures, consistent with the theory that Cdc42 regulates actin organization into podosomes (Allen et al. 1997; Jones 2000). It is still unclear what role podosomes have in macrophage migration, but our data with these macrophages suggests that podosomes are not required for random migration on fibronectin. It has been previously postulated that Cdc42 restricts the speed of migration in macrophages because expression of dominant negative Cdc42 in Bac1.2F5 cells leads to an increase in speed on glass surfaces (Allen et al. 1998). We found that on high concentrations of fibronectin the speed of RAW/LR5 macrophages with reduced endogenous levels of Cdc42 was significantly higher than their speeds on low concentrations of fibronectin, indicating that this restraint might be dependent on integrin-ligand binding. It is possible that an incomplete knockdown of Cdc42 in the cells left sufficient Cdc42 for motility signaling. However, the observation that these cells no longer form podosomes and the previous result with this cell line showing decreased phagocytosis,

podosome formation, and chemotaxis to the chemokine CX3CL1 (Park and Cox 2010) indicate that motility and cytoskeletal signaling pathways are altered by the reduction in Cdc42.

Phosphoinositide 3-kinase (PI3K) becomes activated by both integrins and the CSF-1R at the plasma membrane of macrophages (Jones 2000). PI3K has been shown to be upstream of many signaling pathways in macrophages and is important for macrophage migration (Munugalavadla et al. 2005; Papakonstanti et al. 2008; Vanhaesebroeck et al. 1999). Therefore, it is not surprising that we found no motility in RAW/LR5 cells inhibited with either LY29004 or Wortmannin, two PI3K inhibitors. Cells inhibited with either chemical inhibitor showed no polarization or ability to migrate on fibronectin printed surfaces and PI3K-inhibited cells showed no actin or vinculin organization. A requirement for PI3K signaling in macrophage migration has also been shown with Bac1.2F5 macrophages and primary murine macrophages (Munugalavadla et al. 2005; Vanhaesebroeck et al. 1999).

Our ability to visualize macrophages migrating in two-dimensions over time allowed us to discover a unique property of ROCK-inhibited macrophages migrating on fibronectin surfaces. We found that in the presence of the ROCK inhibitor Y-27632, RAW/LR5 macrophages showed a switch-like change in motility with increasing fibronectin concentration. Under ROCK inhibition, macrophages had significantly lower motility on high concentrations of fibronectin, (10µg/mL or higher) than on low concentrations of fibronectin, (5µg/mL or lower), but the random motility coefficient was constant within each regime. This sensitivity to fibronectin concentration was also accompanied by a change in morphology for migrating macrophages. On high concentrations of fibronectin, the cells showed a defect in contractility leaving long unretracted tails behind them. This accumulation of un-retracted tails has also been seen in THP-1 monocytes inhibited with Y-27632 (Worthylake and Burridge 2003). On low concentrations of fibronectin, however, ROCK-inhibited RAW/LR5 cells showed a much more rounded morphology with small but broad lamellipodia and almost no tails. Others have shown that loss of one ROCK isotype, ROCK1, leads to a significant increase in adhesion to the fibronectin fragment CH296 (Vemula et al. 2010). It has also been previously found in THP-1 monocytes that inhibition of ROCK leads to increased spreading and membrane activity on fibronectin (Worthylake and Burridge 2003). Therefore, it is reasonable to assume that the RAW/LR5 macrophages have reduced migration on high concentrations of fibronectin because of increased attachment to the surface compared to wild-type cells. Even with this increased attachment, however, the ROCK-inhibited cells show efficient motility on all concentrations of fibronectin, consistent with previous findings that RhoA is not required for forward migration, only efficient tail-retraction (Worthylake et al. 2001). We were able to show that podosome assembly still occurs in the absence of ROCK activity, consistent with the finding that ROCK inhibition leads to increased integrin-dependent phosphotyrosine signaling to podosome-associated proteins such as cofilin (Worthylake and Burridge 2003). We were able to discover this switch in random motility coefficient accompanied by a change in morphology for ROCK-inhibited RAW/LR5 cells on high versus low concentrations of fibronectin because of our unique ability to study motility in two dimensions.

In conclusion, we have shown that microcontact printed PDMS surfaces serve as an ideal platform for studying macrophage migration in two-dimensions. We have shown that we can functionally block our surfaces, guaranteeing that all the macrophage motility seen in our experiments is specifically due to cell interactions with fibronectin. Using these surfaces, we were able to show that RAW/LR5 macrophages exhibit biphasic motility with increasing fibronectin or CSF-1 concentrations. We were also able to show that PI3K signaling but not Cdc42 or ROCK activity is required for migration of macrophages. This system for studying two dimensional migration has allowed us to discover unique migratory morphologies for ROCK-inhibited cells on varying fibronectin concentrations. It has also allowed us to quantitatively compare the migration of macrophages under various signaling-impaired conditions. In the future, this surface preparation can serve as a tool for studying highly adhesive cells such as macrophages in two-dimensions and directly relate their migration to integrin-binding interactions without confounding surface effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Allen WE, Jones GE, Pollard JW, Ridley AJ. Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J Cell Sci.* 1997; 110(Pt 6):707–20. [PubMed: 9099945]
- Allen WE, Zicha D, Ridley AJ, Jones GE. A role for Cdc42 in macrophage chemotaxis. *J Cell Biol.* 1998; 141(5):1147–57. [PubMed: 9606207]
- Calle Y, Burns S, Thrasher AJ, Jones GE. The leukocyte podosome. *Eur J Cell Biol.* 2006; 85(3–4): 151–7. [PubMed: 16546557]
- Cox D, Chang P, Zhang Q, Reddy PG, Bokoch GM, Greenberg S. Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. *J Exp Med.* 1997; 186(9):1487–94. [PubMed: 9348306]
- Desai RA, Khan MK, Gopal SB, Chen CS. Subcellular spatial segregation of integrin subtypes by patterned multicomponent surfaces. *Integr Biol (Camb).* 2011; 3(5):560–7. [PubMed: 21298148]
- Dovas A, Cox D. Signaling networks regulating leukocyte podosome dynamics and function. *Cell Signal.* 2011; 23(8):1225–34. [PubMed: 21342664]
- Dovas A, Gevrey JC, Grossi A, Park H, Abou-Kheir W, Cox D. Regulation of podosome dynamics by WASp phosphorylation: implication in matrix degradation and chemotaxis in macrophages. *J Cell Sci.* 2009; 122(Pt 21):3873–82. [PubMed: 19808890]
- Dunn GA. Characterising a kinesis response: time averaged measures of cell speed and directional persistence. *Agents Actions Suppl.* 1983; 12:14–33. [PubMed: 6573115]
- Farrell BE, Daniele RP, Lauffenburger DA. Quantitative relationships between single-cell and cell-population model parameters for chemosensory migration responses of alveolar macrophages to C5a. *Cell Motil Cytoskeleton.* 1990; 16(4):279–93. [PubMed: 2393911]
- Hamilton JA. CSF-1 signal transduction. *J Leukoc Biol.* 1997; 62(2):145–55. [PubMed: 9261328]
- Henry SJ, Crocker JC, Hammer DA. Ligand density elicits a phenotypic switch in human neutrophils. *Integr Biol (Camb).* 2014

- Jannat RA, Robbins GP, Ricart BG, Dembo M, Hammer DA. Neutrophil adhesion and chemotaxis depend on substrate mechanics. *J Phys Condens Matter*. 22(19):194117. [PubMed: 20473350]
- Jones GE. Cellular signaling in macrophage migration and chemotaxis. *J Leukoc Biol*. 2000; 68(5): 593–602. [PubMed: 11073096]
- Lauffenberger, DA.; Linderman, JJ. Receptors: Models for Binding, Trafficking, and Signaling. Oxford University Press; 1993.
- Libby P. Molecular and cellular mechanisms of the thrombotic complications of atherosclerosis. *J Lipid Res*. 2009; 50(Suppl):S352–7. [PubMed: 19096046]
- Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. 2010; 22(2):231–7. [PubMed: 20144856]
- Munugalavadda V, Borneo J, Ingram DA, Kapur R. p85alpha subunit of class IA PI-3 kinase is crucial for macrophage growth and migration. *Blood*. 2005; 106(1):103–9. [PubMed: 15769893]
- Nishimura SI, Ueda M, Sasai M. Non-Brownian dynamics and strategy of amoeboid cell locomotion. *Phys Rev E Stat Nonlin Soft Matter Phys*. 2012; 85(4 Pt 1):041909. [PubMed: 22680500]
- Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature*. 1997; 385(6616):537–40. [PubMed: 9020360]
- Papakonstanti EA, Zwaenepoel O, Bilancio A, Burns E, Nock GE, Houseman B, Shokat K, Ridley AJ, Vanhaesebroeck B. Distinct roles of class IA PI3K isoforms in primary and immortalised macrophages. *J Cell Sci*. 2008; 121(Pt 24):4124–33. [PubMed: 19033389]
- Park H, Cox D. Cdc42 regulates Fc gamma receptor-mediated phagocytosis through the activation and phosphorylation of Wiskott-Aldrich syndrome protein (WASP) and neural-WASP. *Mol Biol Cell*. 2009; 20(21):4500–8. [PubMed: 19741094]
- Park H, Cox D. Syk regulates multiple signaling pathways leading to CX3CL1 chemotaxis in macrophages. *J Biol Chem*. 2010; 286(17):14762–9. [PubMed: 21388954]
- Peltier J, Schaffer DV. Viral packaging and transduction of adult hippocampal neural progenitors. *Methods Mol Biol*. 2010; 621:103–16. [PubMed: 20405362]
- Pixley FJ. Macrophage Migration and Its Regulation by CSF-1. *Int J Cell Biol*. 2012; 2012:501962. [PubMed: 22505929]
- Pixley FJ, Stanley ER. CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol*. 2004; 14(11):628–38. [PubMed: 15519852]
- Pollard JW. Trophic macrophages in development and disease. *Nat Rev Immunol*. 2009; 9(4):259–70. [PubMed: 19282852]
- Ricart BG, John B, Lee D, Hunter CA, Hammer DA. Dendritic cells distinguish individual chemokine signals through CCR7 and CXCR4. *J Immunol*. 2011; 186(1):53–61. [PubMed: 21106854]
- Smith LA, Aranda-Espinoza H, Haun JB, Dembo M, Hammer DA. Neutrophil traction stresses are concentrated in the uropod during migration. *Biophys J*. 2007; 92(7):L58–60. [PubMed: 17218464]
- Stokes CL, Lauffenburger DA. Analysis of the roles of microvessel endothelial cell random motility and chemotaxis in angiogenesis. *J Theor Biol*. 1991; 152(3):377–403. [PubMed: 1721100]
- Vanhaesebroeck B, Jones GE, Allen WE, Zicha D, Hooshmand-Rad R, Sawyer C, Wells C, Waterfield MD, Ridley AJ. Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. *Nat Cell Biol*. 1999; 1(1):69–71. [PubMed: 10559867]
- Vemula S, Shi J, Hanneman P, Wei L, Kapur R. ROCK1 functions as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation and stability. *Blood*. 2010; 115(9):1785–96. [PubMed: 20008297]
- Worthylake RA, Burridge K. RhoA and ROCK promote migration by limiting membrane protrusions. *J Biol Chem*. 2003; 278(15):13578–84. [PubMed: 12574166]
- Worthylake RA, Lemoine S, Watson JM, Burridge K. RhoA is required for monocyte tail retraction during transendothelial migration. *J Cell Biol*. 2001; 154(1):147–60. [PubMed: 11448997]
- Yang MT, Fu J, Wang YK, Desai RA, Chen CS. Assaying stem cell mechanobiology on microfabricated elastomeric substrates with geometrically modulated rigidity. *Nat Protoc*. 2011; 6(2):187–213. [PubMed: 21293460]

Ye H, Gu Z, Gracias DH. Kinetics of ultraviolet and plasma surface modification of poly(dimethylsiloxane) probed by sum frequency vibrational spectroscopy. *Langmuir*. 2006; 22(4):1863–8. [PubMed: 16460119]

Figure 1.

Microcontact-printing of fibronectin on PDMS. (A) Schematic representation of stamping process used to prepare surfaces for motility experiments. (B) PDMS surface stamped with fluorescently-tagged fibronectin. (C) PDMS surface stamped with FITC-tagged fibronectin and blocked with BSA. (D) PDMS surface stamped with FITC-fibronectin and blocked with Pluronic-F127. In (C) and (D) cells were allowed to adhere to stamped and blocked surfaces for 2 hours prior to imaging. Scale bar indicates 50µm.

Figure 2.

Adhesion staining of RAW/LR5 macrophage on PDMS surfaces coated with fibronectin. (A) Merged image of actin and vinculin stains show podosomes. Area of inlay indicated by white box at leading edge of cells. (B) Phalloidin staining shows punctate actin. (C) hVIN1 staining shows rings of vinculin.

Figure 3.

Biphasic motility of macrophages. (A) Random motility coefficient versus fibronectin concentration shows biphasic motility of macrophages with increasing surface ligand density. ($n = 7$ experiments; an average of 447 ± 39 cells per condition). (B) Random motility coefficient as a function of CSF-1 concentration shows biphasic motility of macrophages with increasing soluble chemokine. ($n = 4$ experiments; an average of 246 ± 97 cells per condition). Error bars are standard error, * indicates $p < 0.05$.

Figure 4.

RAW/LR5 macrophage motility on PDMS surfaces coated with fibronectin. (A) Dispersion of RAW/LR5 macrophages migrating on 0.5 µg/mL fibronectin. (B) Dispersion of RAW/LR5 macrophages migrating on 5 µg/mL fibronectin shows increased total migration. (C) Dispersion of RAW/LR5 macrophages migrating on 50 µg/mL fibronectin. (D) Mean Squared Displacement versus time on all concentrations of fibronectin.

Figure 5.

Motility of RAW/LR5 cells with reduced endogenous Cdc42 and chemically inhibited PI3K.

(A) Random motility coefficients of wild type RAW/LR5 macrophages and shCdc42 cells on increasing concentrations of fibronectin. (n = 11 experiments; an average of 588±53 cells per condition). (B) Mean Squared Displacement versus time for RAW/LR5 and shCdc42 cells. (C) Staining of shCdc42 cells plated on 5μg/mL fibronectin; actin (red) and vinculin (green). (D) Random motility coefficients of wild type RAW/LR5 macrophages and macrophages chemically inhibited with LY294002 and Wortmannin to reduce PI3K signaling. (n = 5 experiments; an average of 237±30 cells per condition). (E) Mean squared displacement versus time for RAW/LR5 cells and cells chemically inhibited with LY294002 and Wortmannin. (F) Staining of RAW/LR5 macrophages plated on 5μg/mL fibronectin and chemically inhibited with LY294002; actin (red) and vinculin (green). Error bars are standard error, * indicates p < 0.05.

Figure 6.

Migration of RAW/LR5 macrophages with inhibited ROCK signaling. (A) Random motility coefficient of RAW/LR5 macrophages and macrophages inhibited with 10 μM Y27632 as a function of fibronectin concentration. (n = 9 experiments; an average of 501 $\pm$ 28 cells per condition). (B) Dispersion plots for RAW/LR5 macrophages plated on (i) 50 $\mu\text{g/mL}$ fibronectin and (ii) 2.5 $\mu\text{g/mL}$ fibronectin and inhibited with Y27632. (C) Phase images of RAW/LR5 macrophages plated on 50 $\mu\text{g/mL}$ fibronectin (top) show long unretracted tails and on 1 $\mu\text{g/mL}$ fibronectin (bottom) show rounded morphology. Scale Bar = 100 μm . (D) Mean Squared Displacement versus time for wild type macrophages and macrophages inhibited with Y27632 on 1 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, and 50 $\mu\text{g/mL}$ fibronectin. (E) Staining of RAW/LR5 macrophage plated on 5 $\mu\text{g/mL}$ fibronectin and inhibited with 10 μM Y27632 show podosomes at the leading edge of the cell; actin (red) and vinculin (green). Error bars are standard error. * indicates $p < 0.05$.

Figure 7.

Quantitative analysis of migration. (A) Persistence versus speed show inverse relationship for all motile conditions: RAW/LR5, shCdc42s, and RAW/LR5 macrophages inhibited with Y27632. (B) Persistence time versus fibronectin concentration for all motile conditions. (C) Speed and persistence time versus fibronectin concentration for wild type RAW/LR5 macrophages. (D) Speed and persistence time versus fibronectin concentration for shCdc42 macrophages shows that at low fibronectin concentrations motility is dominated by persistence time and at high fibronectin concentrations cell motility is dominated by speed. (E) Speed and persistence time versus fibronectin concentration for RAW/LR5 macrophages inhibited with Y27632 shows motility is dominated by persistence time. Error bars are standard error.