

Bioinspired Microfluidic Assay for In Vitro Modeling of Leukocyte–Endothelium Interactions

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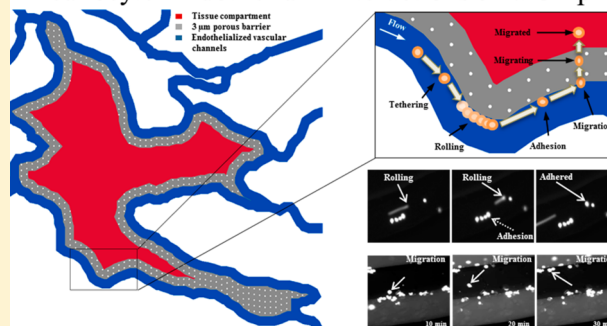
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S Supporting Information

ABSTRACT: Current in vitro models of the leukocyte adhesion cascade cannot be used for real-time studies of the entire leukocyte adhesion cascade, including rolling, adhesion, and migration in a single assay. In this study, we have developed and validated a novel bioinspired microfluidic assay (bmFA) and used it to test the hypothesis that blocking of specific steps in the adhesion/migration cascade significantly affects other steps of the cascade. The bmFA consists of an endothelialized microvascular network in communication with a tissue compartment via a 3 μm porous barrier. Human neutrophils in bmFA preferentially adhered to activated human endothelial cells near bifurcations with rolling and adhesion patterns in close agreement with in vivo observations. Treating endothelial cells with monoclonal antibodies to E-selectin or ICAM-1 or treating neutrophils with wortmannin reduced rolling, adhesion, and migration of neutrophils to 60%, 20%, and 18% of their respective control values. Antibody blocking of specific steps in the adhesion/migration cascade (e.g., mAb to E-selectin) significantly downregulated other steps of the cascade (e.g., migration). This novel in vitro assay provides a realistic human cell based model for basic science studies, identification of new treatment targets, selection of pathways to target validation, and rapid screening of candidate agents.

Leukocyte-Endothelium Interactions on a Chip



Inflammation is a physiological response of the body to protect tissues from infection (bacteria, virus, parasitic worms, or other pathogens), injury, or disease (cardiovascular, immune, etc.). The primary role of the inflammatory response is to eliminate the causative agent and to repair the surrounding tissue.¹ Usually, this starts with the signaling cascade at the affected areas, followed by the production and release of chemical agents. The evolving signals then recruit leukocytes to the inflammation site, activating an acute inflammation process.² This process involves the initiation of the leukocyte adhesion cascade mediated by a series of interactions between receptors and ligands on the endothelium and the leukocytes, respectively. Specifically, circulating leukocytes tether and roll along the vessel wall by establishing transient selectin-mediated interactions with endothelial cells. This initial contact facilitates the binding of leukocyte integrins (beta2 and/or alpha4 integrins) to their counter-receptors on the activated endothelium, which allows leukocytes to firmly adhere to the endothelium and resist disruptive hemodynamic shear forces. Ultimately, arrested leukocytes extravasate to inflamed tissues across endothelial cells (diapedesis or transendothelial migra-

tion) via a multistep process controlled by concurrent chemoattractant-dependent signals, adhesive events, and hemodynamic shear forces.^{3,4}

Due to the significance of the leukocyte–endothelium interactions in pathogenesis of disease⁵ and drug delivery,⁶ among many others, several in vitro models have been developed to study different aspects of the leukocyte adhesion cascade. Traditionally, most studies involving the inflammatory process were performed using in vitro static well plates or animal experiments. However, in recent years a number of fluidic devices have been used for studying the functional significance of variables such as adhesion molecules and shear forces in the leukocyte adhesion cascade. Currently available fluidic devices, including the Glycotech parallel plate flow chamber⁷ and recent entries from Cellix and Fluxion Biosciences, are used to study leukocyte rolling and adhesion. However, these devices are typically idealized in that they lack

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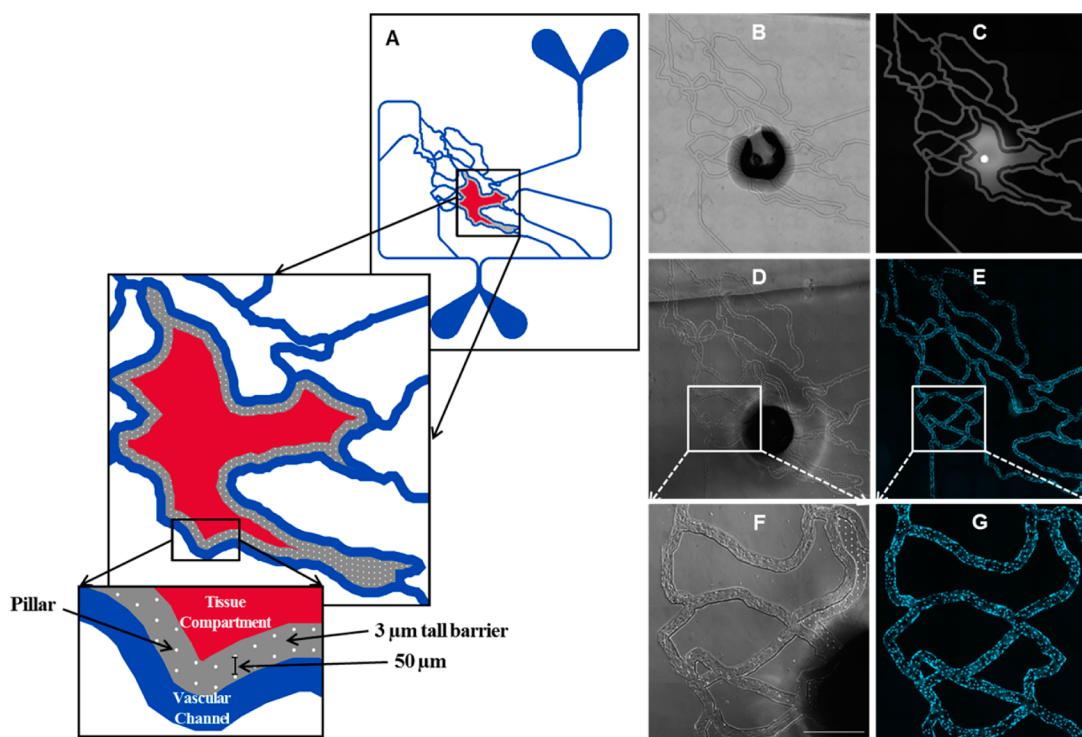


Figure 1. Overview of the bMFA. (A) Schematic of the bMFA. The vascular channels ($100\ \mu\text{m}$ width; $100\ \mu\text{m}$ height) are separated from the tissue compartment by a $3\ \mu\text{m}$ pore size and $100\ \mu\text{m}$ wide barrier. (B) Fabricated bMFA with a $3\ \mu\text{m}$ pore size barrier. (C) FITC dye-perfused bMFA. (D) Confluent culture of endothelial cells in bMFA shows an elongated shape in the direction of the flow. (E) Endothelial cells in bMFA are viable as indicated by Hoechst staining. (F–G) Magnified images of endothelial cells in phase contrast and fluorescence (scale bar is $500\ \mu\text{m}$). Dark shadows in (B and D), as well as the bright fluorescent spot in (C), are from the tubes connecting the tissue compartment to the syringe pump.

correspondence with *in vivo* geometry, including scale/aspect ratios (microvasculature vs large vessel models) and require large reagent volumes.⁸ Several investigators have developed microfluidic devices using either a better representation of cross-sectional areas in vessels^{9–11} or computer generated ideal geometries that roughly approximate microvascular networks.¹² However, these devices do not realistically mimic the *in vivo* geometrical features (e.g., successive bifurcations, vascular morphology) and flow conditions (e.g., converging or diverging flows at bifurcations) and are inadequate for studying adhesion event differences between healthy and diseased vasculature. On the other hand, devices such as the Boyden chamber (or Transwell) have been used for decades to study the leukocyte migration process. However, these assays require the passage of leukocytes through membrane pores which do not allow direct observation of leukocytes migration. Furthermore, these *in vitro* assays require a setup oriented in parallel to the direction of gravity for use during extended periods of time. As a result, the process of leukocyte adhesion to the cultured endothelium may be influenced by gravity in addition to adhesion molecules and/or the concentration gradient of chemoattractants under investigation. The attempts to integrate the Boyden chamber with shear flow conditions^{13,14} to monitor cell migration have been limited by the high cost (large volumes of media) and inability of capturing the dynamic behavior of cell migration (since these attempts rely on end-point measurements). Three-dimensional models of leukocyte migration can better mimic the microenvironment using extracellular matrix components, such as synthetic nanofiber scaffolds, Puramatrix from 3DM Inc., collagens, or Matrigel.^{15,16} However, this leads to additional problems, such as cell clumping; making it nearly

impossible to quantify the number of cells migrated. Additional problems, such as difficulty in setup and the influence of the stiffness of matrix on the migration process,¹⁷ also limit the application of these devices.

Although there are devices available to study rolling, adhesion, and migration separately, there is no *in vitro* model that can simultaneously study the entire leukocyte adhesion cascade comprising of rolling, adhesion, and migration. Previously, our group has developed a microfluidic device that reproduces the geometry of microvessels on a chip.^{18,19} In this study, we developed a novel bioinspired microfluidic device that enables a single assay to capture the entire leukocyte adhesion cascade in a synthetic microvascular network mimicking *in vivo* physiological conditions. We have validated this novel assay against *in vivo* data and used it to test the hypothesis that blockers/suppressors of specific steps in the adhesion/migration cascade can affect other steps of the adhesion/migration cascade. This *in vivo* mimetic assay has realistic microvasculature geometry with physiological shear conditions and allows direct observation and quantification of leukocytes rolling, adhesion, and migration over time.

EXPERIMENTAL SECTION

In Vitro Experimental Procedures. *Fabrication of the bMFA.* Previously, our group has developed a methodology for digitization and fabrication of microfluidic devices based on *in vivo* microvascular networks.¹⁹ A modified Geographic Information System (GIS) approach was used to digitize the microvascular networks. The largest tissue area from the network was selected, and the vascular wall adjacent to the area was modified in AutoCAD to include a $3\ \mu\text{m}$ pore size, the

most common and optimum for studying leukocyte migration.^{20–22} Briefly, the fabrication of the microfluidic devices starts with lithographically patterning SU-8 photoresist on Si wafers. To achieve the multiple heights associated with the vascular channels, barrier, and tissue compartment area, multiple layers of SU-8 are spin-coated and patterned. Microfabricated pillars (10 μm diameter) were used to fabricate the 3 μm pores with a width of 100 μm connecting the vascular and tissue compartments. Once the SU-8 microfluidic features were patterned, the 1:10 w/w ratio of Sylgard 184 silicone elastomer base and curing agent (Dow Corning, Midland, MI) was poured over the master and cured. Subsequently, the cured polydimethylsiloxane (PDMS) was peeled from the SU-8 master, followed by punching of inlet/outlet ports, and plasma bonded to a glass slide cleaned to remove organic species. The schematic of the device used in this study is shown in Figure 1A.

Testing of the bMFA. Fluorescent dye, fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO) at a concentration of 100 $\mu\text{g}/\text{mL}$ was injected into the vascular channels of the device to evaluate the integrity of the fabricated 3 μm pore size barrier. As shown in Figure 1C, the vascular network and the tissue compartment are fluorescent with a darker bridge between them, indicating an intact 3 μm barrier connecting the two compartments.

Seeding of Human Umbilical Vein Endothelial Cells into the bMFA. With the use of our established protocol,²³ the fabricated device was degassed and then washed with sterile DI water. The device was then incubated with fibronectin (100 $\mu\text{g}/\text{mL}$) for 1 h. Human umbilical vein endothelial cells (HUVEC) (Lonza, Walkersville, MD) were introduced and incubated at 37 $^{\circ}\text{C}$ and 5% CO_2 . Media was replaced every 24 h until the cells were confluent in the device. As shown in Figure 1, panels E and G, endothelial cells were stained with Hoechst 33342 (1 $\mu\text{g}/\text{mL}$; Invitrogen, Carlsbad, CA) to show viability. Confluent endothelial cells were activated with 10 units/mL of TNF- α for 4 h before the experiments.

Neutrophil Isolation and Labeling. Human blood was obtained via venipuncture from healthy adult donors and collected into a sterile tube containing sodium heparin (BD Biosciences) after informed consent was obtained as approved by the Institutional Review Board of Temple University. Neutrophils were then isolated using a one-step Ficoll-Plaque gradient (GE Healthcare, Piscataway, NJ). After isolation, neutrophils were counted and resuspended in Hanks Balanced Salt Solution (HBSS) (3×10^6 cells/mL). Neutrophils were labeled in suspension using CFDA SE probe (Molecular Probes, Carlsbad, CA) for 10 min at room temperature. The labeled neutrophil solution was washed with HBSS and resuspended in prewarmed endothelial cell media containing 10 units/mL of TNF- α and kept in the incubator for 10 min prior the experiments.

Physiological Shear Experiments. The tissue compartment of the device was filled with a chemotactic concentration of fMLP (1 μM ; Sigma, St. Louis, MO) prior to the experiments.²⁴ The flow rate at the inlet was fixed at 1 $\mu\text{L}/\text{min}$ using a syringe pump (Harvard Apparatus, PhD Ultra) for injection of about 3000 labeled neutrophils per minute. The neutrophils were kept at 37 $^{\circ}\text{C}$ using a custom-made syringe warmer. Previously, we have developed a computational fluid dynamics (CFD)-based model to calculate various flow parameters (e.g., shear stress, velocity, etc.) at different locations in the network;¹⁸ this CFD model was used in the current study to

calculate flow parameters (shear rates less than 500 1/sec) in different channels of the network. Video clips were recorded at 30 fps using a Rolera Bolt camera (QImaging, Surrey, BC, Canada). After 10 min of flowing neutrophils into the device, PBS was flown through the syringe from the inlet port for about 5 min to completely wash off unbound neutrophils. A scan of the entire device at the 10 \times objective was performed using an automated stage on an epifluorescence microscope (Nikon Eclipse TE200, Melville, NY). The acquired images and videos were postprocessed using Nikon Elements software.

Blocking Experiments. As mentioned before, a novel feature of this device is the ability to study the entire leukocyte adhesion cascade in a single assay. The two primary classes of adhesion molecules involved in the adhesion cascade are selectins (responsible for rolling) and integrins/immunoglobulins (responsible for adhesion). In order to demonstrate that our device can accurately mimic the entire leukocyte adhesion cascade, we inhibited the role of E-selectin and ICAM-1, which are key molecules from the selectin and integrin/immunoglobulin families, respectively, on HUVECs. We introduced mAb (anti-Eselectin or anti-ICAM-1) into the vascular channels of the bMFA at a concentration of 20 $\mu\text{g}/\text{mL}$ and incubated at 37 $^{\circ}\text{C}$ for 30 min prior to the experiments. We also used wortmannin, a fungal metabolite, which has been shown to inhibit fMLP-dependent leukocyte migration.²⁵ Wortmannin (50 nM) was mixed with neutrophils and incubated at 37 $^{\circ}\text{C}$ for 30 min prior to injection into the bMFA.

Data Analysis. The numbers of adherent, rolling, and migrating neutrophils were quantified in the bMFA using NIKON Elements. Any neutrophil traveling at a velocity below the critical velocity was considered a rolling cell. Critical velocity was estimated from a cell velocity flowing in the centerline (v_{cc}) as $v_{crit} = v_{cc} \times \epsilon \times (2 - \epsilon)$, where ϵ is the cell-to-vessel diameter ratio.²⁶ Any cell that did not move for 30 s was considered adherent. The distance of each of the adhered neutrophils to the nearest bifurcation was measured for each branch of bMFA. The distance of adhered neutrophils to the nearest upstream bifurcation was normalized by dividing by the diameter of the channel:

$$\text{normalized distance} = \frac{\text{distance to the nearest bifurcation}}{\text{diameter of the channel}}$$

In Vivo Experimental Procedures. All in vivo protocols were approved by the Institutional Animal Care and Use Committee of Temple University. Male C57BL mice (6–8 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME). The open cremaster muscle preparation surgical procedure used in this study has been described before.²⁷ Briefly, mice were treated with TNF- α (500 ng/animal) by testicular injection 4 h before surgery. An initial tracheotomy was performed on each mouse to facilitate breathing throughout the experiment. After, the mouse cremaster muscle was cut, flattened, and pinned on a surgery board, and a fluorescent dye, rhodamine 6G (0.4 mg/kg body weight; Sigma, St. Louis, MO), was injected through the cannulated right jugular vein to label the leukocytes.

Data Analysis. A Nikon Eclipse FN1 fluorescence microscope was used to obtain the images of labeled leukocytes. The images were taken under 10 \times magnification with a digital camera (Rolera Bolt, QImaging, Surrey, BC, Canada) and controlled by Micro-Manager software (National Institutes of Health, Bethesda, MD). As described above, any leukocyte traveling at a velocity below the critical velocity was considered

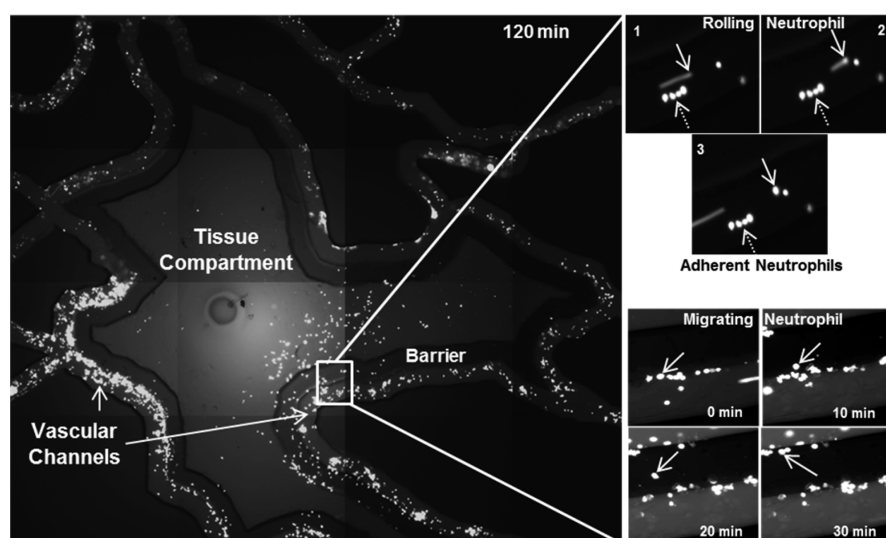


Figure 2. Rolling, adhesion, and migration of neutrophils in bMFA; migration of neutrophils (labeled with fluorescent dye) into the tissue compartment of bMFA after 120 min of continuous flow. (1 and 2) Solid arrows in the top right panels show a rolling neutrophil which (3) becomes adherent; dotted arrows in the top right panels show firmly adherent neutrophils. A neutrophil migrating from a vascular channel through the barrier into the tissue compartment over time (bottom right).

a rolling cell. Leukocytes that were immobilized for a minimum of 30 s were identified as firmly adhered leukocytes on the vessel wall. The distance to the nearest bifurcation for each adhering leukocytes was then measured in each network. As described above, the spatial distribution of leukocyte adhesion was measured in terms of the distance to the nearest bifurcation divided by vessel diameter.

RESULTS AND DISCUSSION

Device Characterization. In this study, we have developed a novel microfluidic based assay that includes vascular networks in communication with a tissue compartment filled with chemoattractant (fMLP) via a 3 μm porous barrier (Figure 1A). This bioinspired microfluidic assay is patterned upon a Geographic Information System (GIS)-based mapping of microvascular networks obtained by intravital microscopy of rodent vasculature^{28,29} and allows for the study of the entire leukocyte adhesion cascade. Figure 1B shows an image of the fabricated device and Figure 1C shows the FITC-perfused device indicating the fully functional barrier and the vascular and tissue compartments. Endothelial cells were successfully grown to confluence in the bMFA and oriented in the direction of flow (Figure 1, panels D and F). The viability of these endothelial cells was verified by staining with the Hoechst dye (Figure 1, panels E and G).

Due to the complex, changing nature of flow in the replicated network in this assay, the shear rate at different locations of the network cannot be directly calculated. However, we have developed a database of shear-based analysis for varying inlet flow conditions in these devices.¹⁸ We used these simulation flow profiles for analyzing the varying shear at different locations of the network and studying how it affects the neutrophil rolling adhesion and migration processes. At the flow rates used in this study, shear rates in the bMFA vessels ranged from 0–280 1/sec.

Rolling Step of the Neutrophil–Endothelial Interaction. Neutrophils displayed rolling behavior on a monolayer of activated endothelial cells in the bMFA similar to those observed in vivo [see Video clip S-1 (002.mov)] of the

Supporting Information]. Time lapse images of neutrophil rolling, adhesion, and migration in the bMFA (Figure 2) shows adhesive patterns seen in vivo. As shown in Figure 3, rolling

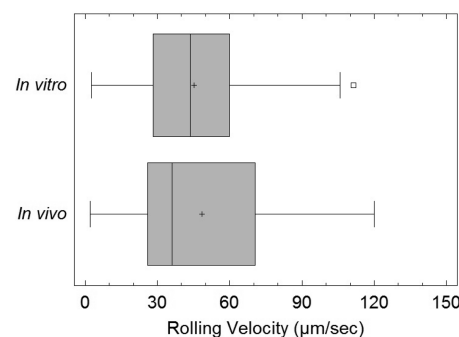


Figure 3. Neutrophil rolling in bMFA is similar to leukocyte rolling in vivo; box and whisker plots summarizing leukocytes rolling velocity measured in vivo in a mouse cremaster muscle model ($N = 5$) and rolling neutrophils velocity in vitro in the bMFA ($N = 3$). The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentile, and the whiskers indicate the highest and lowest values of the results. The “+” marked in the box indicates the mean. Rolling velocity of neutrophils measured in vitro in the bMFA was not significantly different from leukocytes rolling velocity in vivo ($p = 0.758$; Mann–Whitney Rank Sum Test). Instantaneous rolling velocities, average velocity, and velocity variance were measured using frame-by-frame analysis over 30 s intervals for each device (average of 600 neutrophils analyzed per device).

velocities of neutrophils measured in bMFA are in the same range of the rolling velocities of leukocytes measured in vivo. Our findings indicate that the average rolling velocity of neutrophils in bMFA ($45.2 \pm 1.7 \mu\text{m}/\text{sec}$, mean \pm SEM) was similar to the average rolling velocity of leukocytes in vivo ($48.0 \pm 1.3 \mu\text{m}/\text{sec}$), and that there was no statistically significant difference between the in vivo and in vitro groups ($P = 0.758$; Man-Whitney Rank Sum Test).

Adhesion Step of the Neutrophil–Endothelial Interaction. Previously, we have shown that both functionalized

particles *in vitro* and leukocytes *in vivo* preferentially adhere near bifurcations.²⁷ Neutrophils in bMFA also preferentially adhered within 1–2 diameters from the nearest bifurcation, and the number of adhered neutrophils decreased with an increasing distance from the nearest bifurcation (Figure 4).

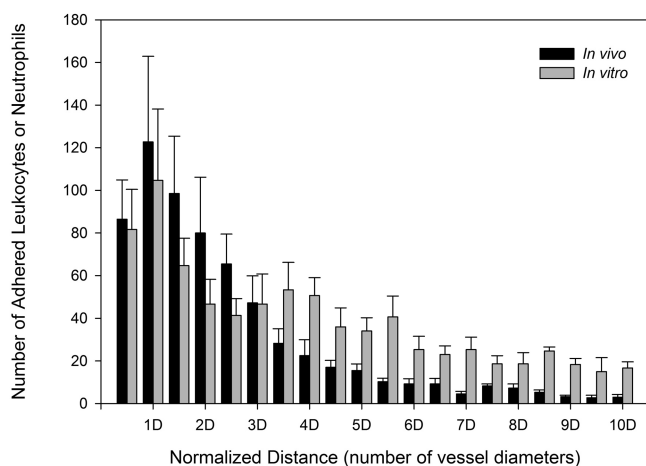


Figure 4. Neutrophil adhesion in bMFA is similar to leukocyte adhesion *in vivo*; distribution of the number of adhered leukocytes and neutrophils as a function of distance from the nearest bifurcation *in vivo* in mouse cremaster muscle model and *in vitro* in bMFA, respectively. Both histograms are skewed to the left, indicating that leukocytes and neutrophils preferentially adhere near bifurcations with the peak occurring at one vessel or channel diameter from the nearest bifurcation (mean \pm SEM; $N = 3$).

Furthermore, data shown in Figure 4 also indicate that the spatial pattern of neutrophil adhesion in bMFA was similar to leukocyte adhesion patterns *in vivo*. Furthermore, consistent with previous studies,^{23,30,31} neutrophil adhesion was found to be minimal in high shear regions (shear rate >120 1/sec) and maximal in low shear regions of bMFA, indicating that fluidic shear strongly influences cell adhesion in these microvascular networks (Figure 5).

Migration Step of the Neutrophil–Endothelial Interaction. Movement of neutrophils from the vascular to the

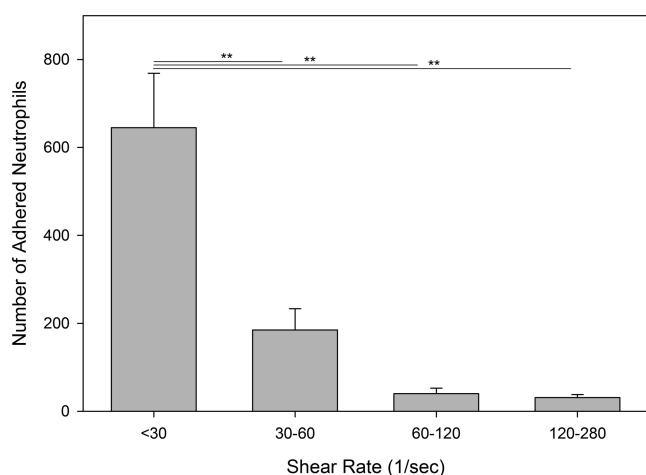


Figure 5. Neutrophil adhesion in bMFA is shear dependent; the number of adhering neutrophils in various channels of bMFA decreases significantly with shear stress in that channel (mean \pm SEM, $N = 3$) (** $P < 0.01$, ANOVA).

tissue compartment was monitored by obtaining images from the entire device every 3 min for 2 h [see Video clip S-2 (003.mov) of the Supporting Information]. Our findings indicate that neutrophil migration in the bMFA exponentially increased with time. In particular, 5% of the adhered neutrophils started to migrate into the chemoattractant-filled tissue compartment after 10 min, reaching 50% after 120 min (Figure 6). In control experiments, where the tissue compart-

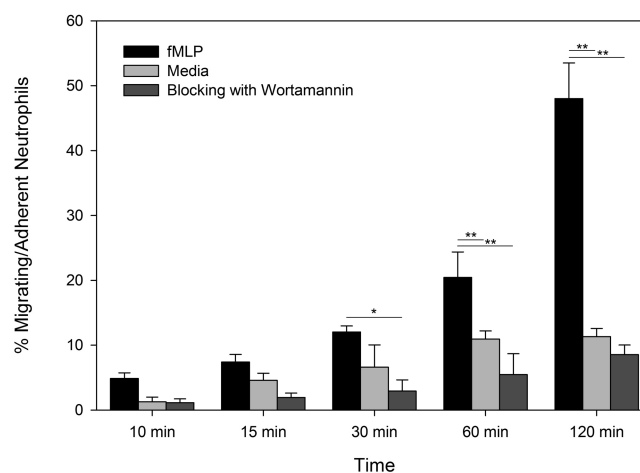


Figure 6. Neutrophil migration in bMFA is time dependent; adhered neutrophils migrate into the tissue compartment filled with fMLP over time. This migration exponentially increases with time, reaching 50% of adhering neutrophils having migrated into the tissue compartment after 120 min but is significantly blocked by treatment of neutrophils with wortmannin. The control experiments were performed filling the tissue compartment with media (mean \pm SEM; $N = 3$) (* $P < 0.05$, ** $P < 0.01$, ANOVA).

ment was filled with cell culture media, the percentage of migrated neutrophils was significantly lower than the case where the tissue compartment was filled with fMLP (Figure 6). Furthermore, during these experiments, we observed higher cell migration at a location in the network where the shear rate was in the range of 30–60 1/sec.

Blocking Specific Steps of the Adhesion/Migration Cascade Significantly Affects Other Steps of the Cascade. To further validate the system, we tested the hypothesis that blocking of specific steps in the adhesion/migration cascade significantly affects other steps of the cascade. Treatment of activated HUVECs with the E-selectin antibody not only significantly reduced the number of rolling neutrophils to $\sim 60\%$ ($p < 0.05$) of its control value but also reduced adherent neutrophils to 40% ($p = 0.024$) and migrated neutrophils to less than 10% ($p < 0.001$) of their respective control values (Figure 7). Similarly, treatment with the ICAM-1 antibody not only reduced adhesion to 20% ($p = 0.007$) of its control value but also reduced rolling to 80% ($p < 0.05$) and migration to less than 10% ($p < 0.001$) of their respective control values (Figure 7). On the other hand, treating neutrophils with wortmannin significantly decreased the number of migrating neutrophils at time points of 30 min ($p = 0.009$), 60 min ($p < 0.001$), and 120 min ($p < 0.001$) (Figure 6) but did not result in any significant changes in rolling and adhesion of neutrophils (Figure 7).

The assay developed in this study provides a morphologically realistic environment and physiological flow conditions similar to *in vivo* microvascular networks and can be used to

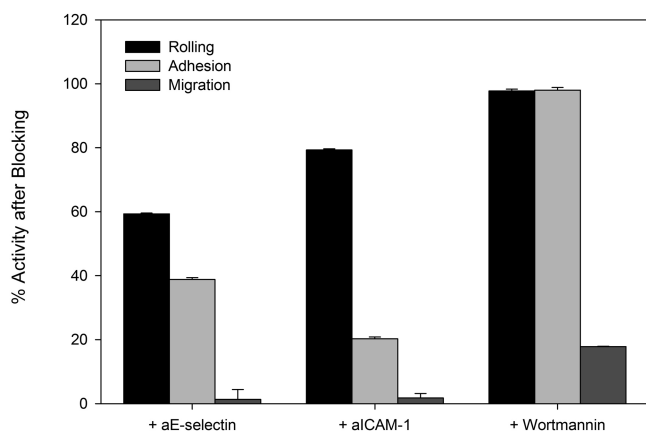


Figure 7. Antibody blocking of specific steps in the adhesion/migration cascade downregulates other steps of the cascade; monoclonal antibodies against E-selectin (aE-selectin), ICAM-1 (aICAM-1), and PI3K (wortmannin) significantly reduced the number of rolling, adhering, and migrating neutrophils in bMFA. The numbers represent the percentage of activity after treating cells with the respective blockers in comparison to their corresponding control values (mean \pm SEM; $N = 3$).

simultaneously study all the steps (rolling, adhesion, and migration) of leukocyte adhesion cascade in a single system. We characterized this assay by culturing a confluent monolayer of human endothelial cells and activating them with cytokines (TNF- α) to study the interactions of human neutrophils with inflamed endothelium. We validated this system by comparing our findings with an *in vivo* mouse model using intravital microscopy. This assay was used to validate the hypothesis that antibody blocking of specific steps in the adhesion/migration cascade (e.g., mAb to E-selectin) significantly downregulates other steps of the cascade (e.g., migration). The system developed in this study has many advantages, including the fact that a complete testing of the latter hypothesis in a single device would not be possible using traditional flow chambers and/or transwell systems.

Our *in vivo* findings indicate that leukocyte rolling velocity in inflamed cremaster muscle venules (diameter 40–110 μm) ranged from 2 to 120 $\mu\text{m}/\text{sec}$, with blood flow velocities ranging from 0.5 to 1 mm/sec, which are consistent with other reports.³² Furthermore, neutrophil rolling velocity in the bMFA was not significantly different from those measured *in vivo*. An advantage of our *in vitro* assay is that it allows for detailed investigation of the effects of shear forces on leukocyte–endothelial interactions.

Leukocyte adhesion results from the balance between molecular adhesive forces and disruptive hemodynamic forces exerted by flowing blood.³³ We²³ and others³⁴ have previously shown that the presence of flow fluctuations in microvascular networks favors particle adhesion at junctions. Our findings in the current study indicate that neutrophil adhesion patterns in our *in vitro* bMFA predict the trends observed *in vivo*. Specifically, neutrophils preferentially adhere near bifurcations, supporting the hypothesis that the hemodynamic forces present *in vivo* can be replicated in an *in vitro* system characterized by the same geometric and fluidic features.^{27,35} This is the first two-arm study to show that neutrophil–endothelial adhesion patterns in an *in vitro* microvascular network is very similar to the adhesion patterns of leukocytes observed *in vivo*. Furthermore, these findings illustrate an important advantage of

bMFA in that characterizing the spatial pattern of particle/leukocyte adhesion to endothelium near bifurcations, for example, is not possible with traditional fluidic devices such as parallel plate flow chambers.

In order to further validate this assay, we blocked individual steps of the leukocyte adhesion cascade using specific inhibitors of rolling, adhesion, and migration. Our results indicate that rolling and adhesion of neutrophils in bMFA can be significantly reduced when endothelial cells are treated with antibodies against selectins or integrins. These findings are consistent with previous findings from our laboratory³⁶ and others³⁷ on the effects of mAb against selectins or integrins on rolling and adherent leukocyte in rat mesenteric or brain venules. In agreement with other studies^{38,39} which have shown that treatment of endothelial cells with mAbs against selectins or integrins also significantly reduces transendothelial migration of leukocytes, migration of neutrophils into the tissue compartment of bMFA was significantly reduced in the presence of antibodies to E-selectin or ICAM-1. One may hypothesize that the effects of antibody blocking on migration may simply be due to the lower number of adhered neutrophils in the proximity of the tissue compartment. However, in our study, $\sim 50\%$ of adhered neutrophils migrated into the tissue compartment in the control experiments while only $\sim 20\%$ of adhered cells migrated into the tissue compartment with anti-ICAM-1-treated endothelial cells. Thus, further studies are required to determine what other factors may be involved in this process. Consistent with other reports in the literature,^{25,40} wortmannin significantly reduced migration of neutrophils into the tissue compartment, suggesting that the migratory stage of neutrophil response during inflammation may be an appropriate target for therapeutics.

The multistep leukocyte adhesion cascade is a dynamic phenomenon, and its understanding often requires real-time monitoring of the entire process. Our novel microfluidic assay allows for the multistep analysis of the complex leukocyte adhesion and migration and integrates many aspects of leukocyte–endothelial interaction in a single assay. This assay can mimic physiological conditions and enables real-time monitoring of cell migration through endothelial cell junctions, which is generally considered the dominant route by which leukocytes penetrate the endothelial cell barrier under physiological flow conditions.⁴¹ The bMFA can also be used to study the influence of various compounds that simulate the extracellular matrix through which directional neutrophil migration can be accomplished. For example, multiple tissue compartment areas can be filled with different chemoattractant solutions and concentrations to understand in a realistic environment the balance of hydrodynamic shear forces, rolling, adhesion, and subsequent migration. Similarly, the role of various inflammatory molecules and blockers may be studied in real-time for elucidation of their mechanisms and development of next generation of anti-inflammatory therapeutics. Furthermore, this system permits studies of the effects of pulsatile flow on particle–cell interactions.

CONCLUSIONS

In conclusion, we have developed and validated a novel bioinspired microfluidic assay that closely mimics physiological conditions of leukocytes rolling, adhesion/migration cascade observed *in vivo* during the inflammatory process. This realistic fluidic model allows for *in vitro* reconstitution of the microvascular environment using human cells and thus allows

for screening of therapeutics on human cells. The bMFA has a number of other advantages, including flow and morphologically realistic environment, ability to model adhesion and migration in the same system, quantitative real-time visualization, reduced reagents, and use of disposable chips. Furthermore, this system can be further developed for high throughput analysis.

■ ASSOCIATED CONTENT

● Supporting Information

TOC of video clips (001.pdf). Video clip S-1 (002.mov): during the first minute after flowing into the vascular channels, neutrophils display rolling, adhesion and migrating behavior on a monolayer of activated endothelial cells in the vascular channels of the assay similar to those observed in vivo. Video clip S-2 (003.mov): Time lapse images (every 5 min for 60 min and then every 15 min for the next 60 min) of the movement of neutrophils from the vascular channels to the tissue compartment of the fluidic assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

G.L. designed and performed the experiments, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript. B.P. designed and developed the experimental protocols, contributed new analytical tools, interpreted data, and wrote the manuscript. C.G. contributed new analytical tools and analyzed data. A.S. contributed new analytical tools and analyzed data. K.P. designed the research, contributed new analytical tools, interpreted data, and wrote the manuscript. B.W. designed the research, analyzed and interpreted data, and wrote the manuscript. M.F.K. designed the research, analyzed, and interpreted data, performed statistical analysis, and wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): Four of the authors (B.P., C.G., A.S., K.P.) are employed by CFD Research Corp., which commercially markets the microfluidic device used in this study.

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