The Role of p38 MAPK in Neutrophil Functions: Chemotaxis and Surface Marker Expressions

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Supporting Information (SI) – Experimental Procedures and Supplemental Data

I. Experimental Procedures

1. Cell Preparation
   Human blood samples from healthy donors were collected and treated with ethylenediaminetetraacetic acid (EDTA) anti-coagulant by professionals at the Memorial Blood Center (St. Paul, MN) according to the approved IRB protocol E&I ID#07809. Once the blood samples were obtained, neutrophils were isolated using a protocol documented elsewhere.[1 e745] Once isolated, the cells were re-suspended in Hank's buffered salt solution (HBSS) containing 2% human serum albumin (Sigma-Aldrich, St. Louis, MO). Then, the cell density was controlled by diluting/adding desired stimulants described below.

2. Device Fabrication
   Schematics and design of microfluidics were described in a previous paper.[2] Device design was transferred from a transparent film (CAD/Art Services Inc., Bandon) to a blank chrome mask plate (Nanofilms, Westlake Village, CA for the blank masks), and then to a SU-8 photoresist (Microchem, Newton, MA) on a silicon wafer using standard photolithography techniques. Device dimensions were as follows: 50 μm (width) x 100 μm (depth) x 2330 μm (length) for the mixing channel and 400 μm (width) x 100 μm (depth) x 2500 μm (length) for the cell culture chamber. Once the master is obtained, a 10:1 weight ratio of polydimethylsiloxane resin and curing agent mixture (Sylgard 184, Ellsworth Adhesives, Germantown, WI) was cast onto the master, cured at 80 ℃ overnight, cut and bound to a clean glass substrate by treating surfaces with oxygen plasma. Inlet and outlets were punched right before the plasma treatment step and the entire fabrication was done in the Nanofabrication Center at the University of Minnesota. Once completed, devices were brought into a biosafety cabinet, exposed to UV light for an hour, and kept in the cabinet until use.

3. Device preparation for experiments
   During cell isolation, the channels were washed with a 70% v/v ethanol solution in sterilized Milli-Q water (Millipore, Billerica, MA) three times, dried by injecting air, and then incubated with a 250 μg/mL solution of human fibronectin (Invitrogen, Carlsbad, CA) in sterilized Milli-Q water under 5 % CO_2 at 37 ℃ for an hour. Once cells were ready, the channels were filled with HBSS containing 2% of HSA, and 20 μl of neutrophil suspensions (~ 10^6 cells/mL) were injected through the cell inlet on the microfluidic device. After one hour of incubation under 5 % CO_2 at 37 ℃, the channel was washed with fresh media to remove non-adherent cells. For the SB203580 pre-incubation condition, neutrophils were treated with SB203580 prior to this injection, injected through the cell inlet, and then incubated. This results in hundreds of cells in a device ready for experiments.

4. Solution Preparation
   Chemoattractants solutions, 10 ng/mL of fMLP (22.9 nM), CXCL2 (1.3 nM), CXCL8 (1.0 nM), and LTB4 (29.7 nM), whether or not containing 5.3 μM SB203580, were prepared in HBSS. CXCL2, CXCL8 and fMLP were purchased from Sigma-Aldrich (St. Louis, MO), LTB4 was purchased from

Cayman Chemical (Ann Arbor, MI), and SB203580 from Sigma-Aldrich (St. Louis, MO). Anti-CD11b conjugated with AlexaFluor 700 (Life Technologies, Grand Island, NY), anti-CD66b conjugated with AlexaFluor 647 (BD Pharmingen, San Diego, CA), anti-FPR2 conjugated with AlexaFluor350 (Bioss Inc., Woburn, MA), anti-BLTR conjugated with AlexaFluor 647 (AbD Serotec, Raleigh, NC), and anti-CXCR1 conjugated with fluorescein isothiocyanate (Novus Biologicals, Littleton, CO) for human were used as instructed by the manufacturer.

5. Time-Lapse/fluorescence Microscopy

Metamorph Ver. 7.7.5 imaging software on an inverted microscope equipped with a 20 x objective (Nikon, Melville, NY) and a CCD camera (QuantEM, Photometrics, Tucson, AZ) was used to collect time-lapse images for chemotaxis assessment. Images of neutrophils in the observation channel were acquired every 10 seconds for 30 minutes. For the surface marker expression assessment, after cell exposure to a chemoattractant gradient, the channel was washed with fresh HBSS solution without serum. Then, the device was incubated for an hour in antibody-containing solution. After another washing step with fresh HBSS solution without serum, fluorescence images were collected using the MetaMorph software with one second exposure time. Then, individual cells were randomly chosen and their maximum fluorescence intensity was assessed for individual cells. All chemotaxis experiments were done in 3 biological replicates, and surface marker studies were done in 3 analytical replicates for the 3 biological replicates.

6. Analysis of data

For the assessment of chemotaxis, neutrophils were randomly chosen from a stack of images and individually analyzed. Time 0 is when the gradient is established in the device because we found that results may be biased if the image collection starts after all cells in the field of view start to migrate. Also, to avoid subjective bias in analysis, our goal was to include as many as cells as possible with minimal movement threshold. Our rules for choosing cells are as follows: (1) cells are randomly chosen for analysis, (2) cells that move less than 10 μm in total are discarded, and (3) cells that move for less than 10 minutes are discarded. There were a significant number of cells that repeatedly stopped and then moved again as well as some cells that moved back and forth repeatedly. These cells were included in the data set as long as their total migration distance was longer than 10 μm and they moved longer than 10 minutes. The total number of analyzed neutrophils per condition is summarized in a table below. Detailed migration-relevant information of individual cells in each frame was obtained from the software, and further processed using Microsoft Excel. The numerical parameters, motility index (MI) and chemotactic index (CI) were defined as:

$$MI = d/d_{max}$$
$$CI = x/d_{total}$$

where d is the final displacement, d_{max} is the maximum displacement, x is the final displacement along the direction of the gradient, and d_{total} is the total

migration distance of an individual cell, respectively.[2, 3] As expected from the definitions, MI represents how active individual cells move under the condition while CI represents how directional movement of individual cells is under the condition. The migration rate of individual cells was given in pixel/second by the MetaMorph software, and the % population represents the number of cells in % that moved AWAY from the fMLP signal. Lastly, in each frame, angular displacement of individual cells was given either a score of "+1" or "-1" depending on which direction the cell moved (+1 when toward a higher concentration of fMLP). This data was then averaged for the entire collection time to indicate how much a cell was distracted on the way toward the fMLP signal. This angle distribution parameter was measured for the first 1, 4, and 7 minutes, and for the entire measurement to analyze temporal response of neutrophils to set the direction of their migration. As the angle distribution and CI described above may have y-axis direction bias caused by flow within the device, these parameter consider only x-axis direction movement, parallel to the chemoattractant gradient direction. In addition, the flow rate was kept at 100 μL/min, which generates minimal shear-induced impact on neutrophils.

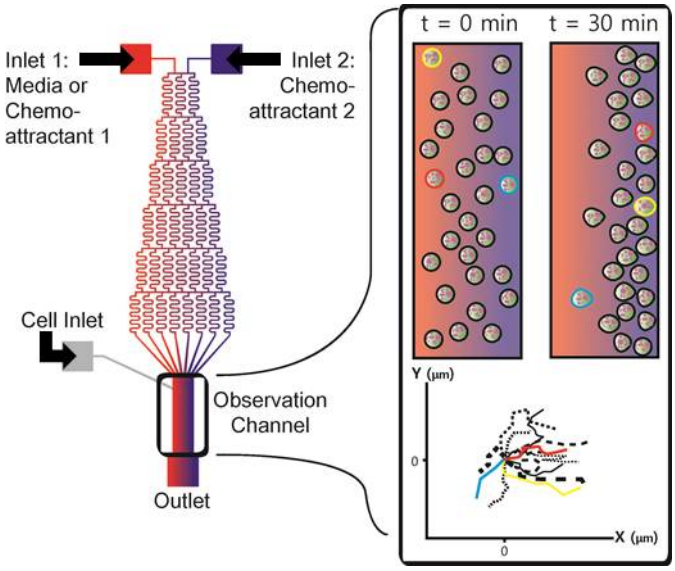
Conditions	Total # of analyzed cells
fMLP	50
p38-blocked, fMLP	50
qCXCL8-fMLP	52
p38-blocked, CXCL8-fMLP	45
CXCL2-fMLP	48
p38-blocked, CXCL2-fMLP	57
LTB4-fMLP	42
p38-blocked, LTB4-fMLP	52

7. Statistical Test

T-test and one-way ANOVA was used for statistical tests, and the error was represented as the standard error of the mean in this manuscript.

II. Supplemental Data

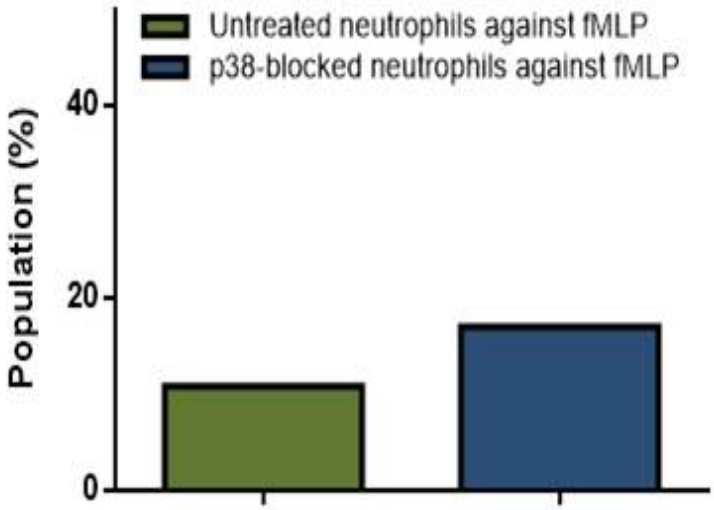
1. S1. Microfluidic Device Schematics



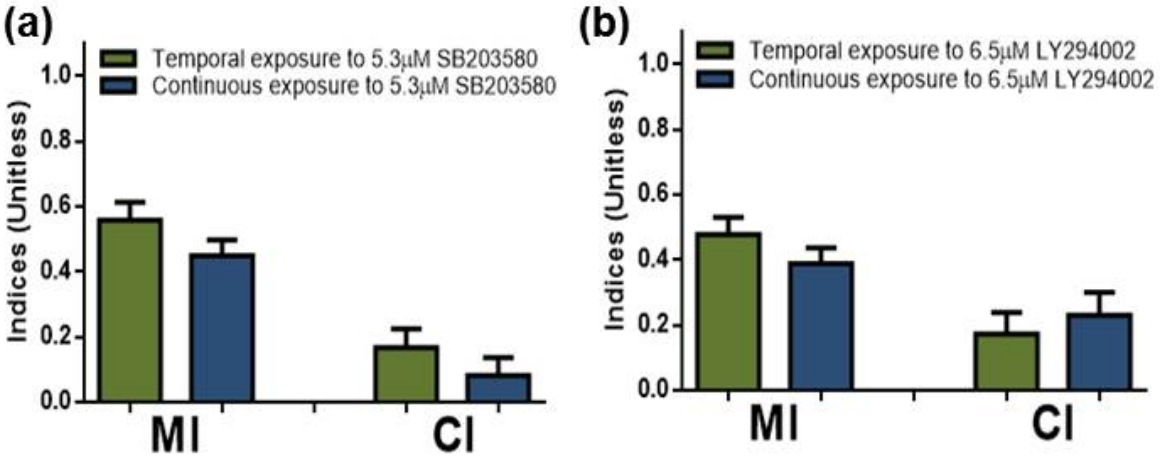
The device schematics are shown on the left side. Each serpentine channel acts a mixing channel. Once a chemoattractant-free buffer is injected through the inlet 1 and a chemoattractant solution into the inlet 2, a linear concentration gradient of the chemoattractant is achieved in the observation channel. If the concentration of the chemoattractant was 10 ng/mL, the gradient will be 0 – 10 ng/mL from the left to the right direction within the observation channel. For competing gradient experiments, chemoattractant-free buffer is replaced with a solution containing another chemoattractant. Then, 10 – 0 ng/mL concentration gradient of the second chemoattractant is established from the left to the right direction over the gradient of the first chemoattractant within the observation channel.

In the upper section on the right side, schematic cartoons of the observation channel at time 0 and time 30 minute are shown. Each circular object represents a neutrophil. After collecting time-lapse images over the 30-minute duration, trajectories of individual cells can be tracked and analyzed. Example trajectories are shown in the lower section on the right side, with three representative neutrophils, in yellow, red, and blue.

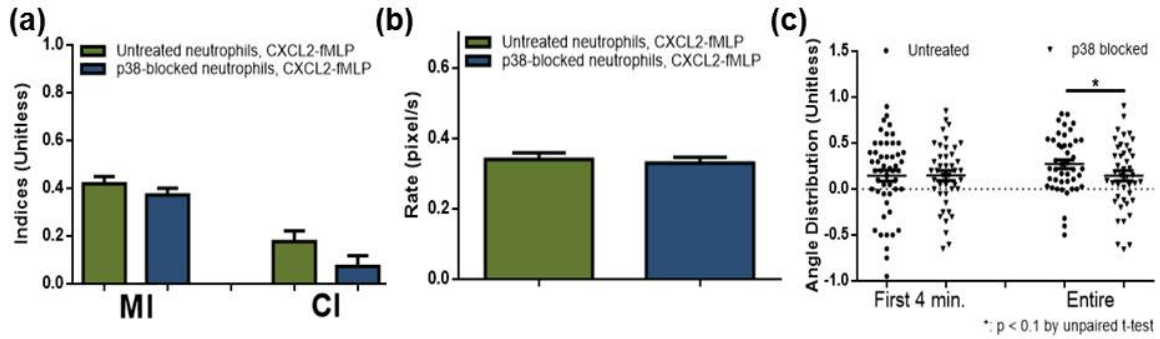
2. S2. % Population of untreated (green) and p38 MAPK-blocked (blue) neutrophils that moved away from fMLP.



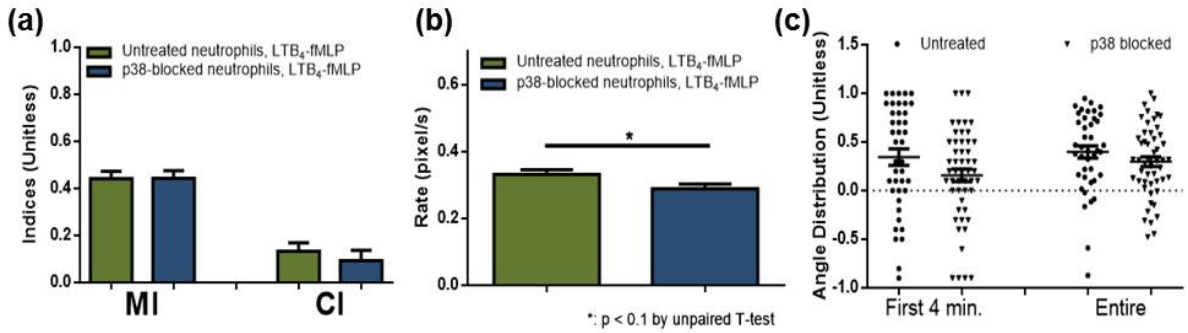
3. S3. Chemotaxis against the fMLP gradient. (a) Neutrophils temporally (green) and continuously (blue) exposed to p38 MAPK inhibitor, SB203580. (b) Neutrophils temporally (green) and continuously (blue) exposed to PI3K inhibitor, LY294002.



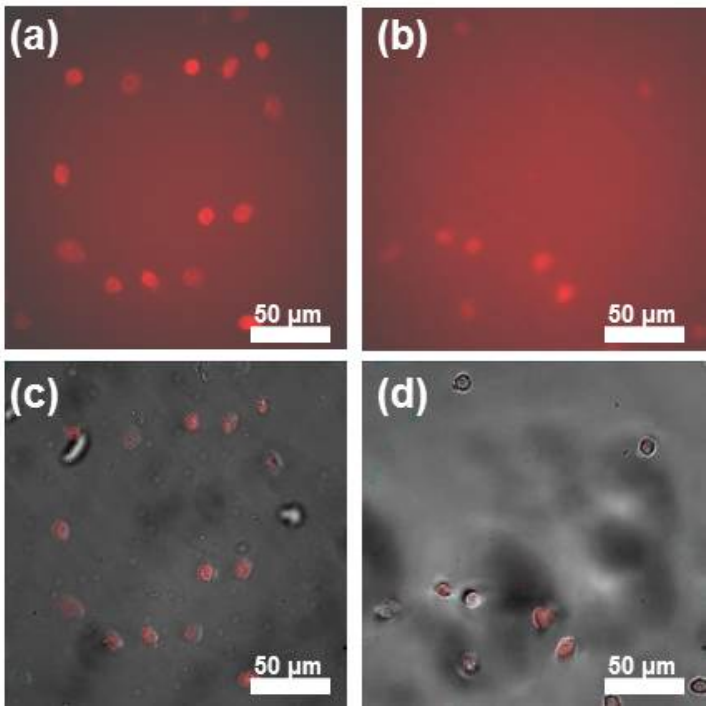
4. S4. Chemotaxis against a CXCL2-fMLP competing gradient (a) Motility index (MI) and chemotaxis index (CI) of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) Average migration rate of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (c) Angle distribution of untreated (circle) and p38 MAPK-blocked (triangle) neutrophils with a value of 1 indicating no deviation from the direction toward fMLP (each point represents angular distribution of a cell and the solid line indicates the average).



5. S5. Chemotaxis against LTB4-fMLP competing gradient (a) Motility index (MI) and chemotaxis index (CI) of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) Average migration rate of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (c) Angle distribution of untreated (circle) and p38 MAPK-blocked (triangle) neutrophils with a value of 1 indicating no deviation from the direction toward fMLP (each point represents angular distribution of a cell and the solid line indicates the average).

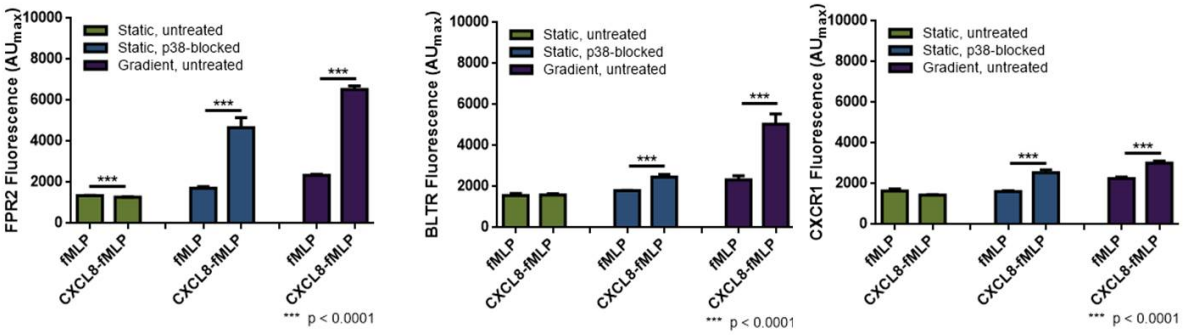


6. S6. Representative bright/fluorescence images of CD66b expression in untreated neutrophils. (a) Fluorescence image from untreated neutrophils exposed to no chemoattractant gradient. (b) Fluorescence image from untreated neutrophils exposed to an fMLP gradient. (c) Bright field/fluorescence image in untreated neutrophils exposed to no chemoattractant gradient (d) Bright field/fluorescence image of untreated neutrophils exposed to an fMLP gradient.



* Image processed using Adobe Photoshop 5.5. For the overlaid images (c and d), 50% of transparency was applied to the fluorescence image.

7. S7. Surface (a) FPR2, (b) BLTR, and (c) CXCR1 expression under static and flow conditions. Untreated neutrophils under static conditions are presented in green, p38 MAPK-blocked neutrophils under static conditions are presented in blue, and untreated neutrophils under gradient conditions are presented in purple.

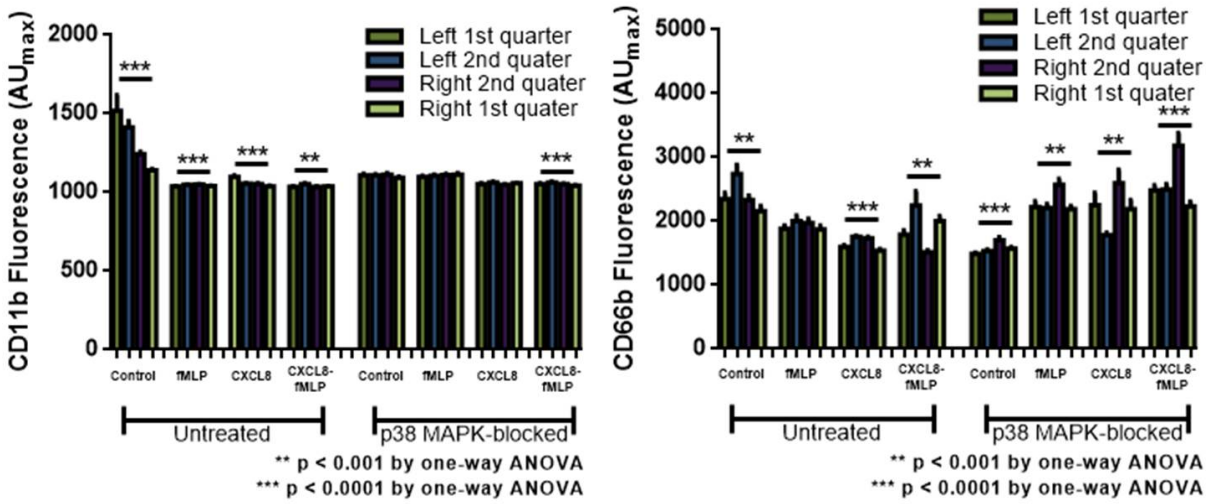


In this set of control experiments, the impact of gradient conditions on the expression of neutrophil surface markers was studied. While the results described in the manuscript prove that the on-chip evaluation of neutrophils can include both surface marker expression and chemotaxis, we also performed the control experiments where surface marker expression was measured in uniform (non-gradient) chemokines, both flowing and static. Thus, neutrophils isolated from healthy human donors were plated into the wells in a 96 well plate and either left untreated, were treated with fMLP only, or were treated with both CXCL8 and fMLP together; then, surface expression of FPR2, CXCR1, and BLTR was monitored using the same antibodies presented in the microfluidic device. The same conditions were repeated on the microfluidic platform for comparison. Neutrophils treated with fMLP within the microfluidic platform had lower expression of FPR2 and CXCR1 than those exposed to a competing "gradient" of CXCL8-fMLP on the microfluidic platform (Figure S7, purple bars). This is consistent with the results presented in the manuscript. On the other hand, neutrophils exposed statically to 10 ng/mL fMLP in a well showed similar levels of surface FPR2, CXCR1, and BLTR when compared to those exposed statically to 10 ng/mL fMLP and 10 ng/mL CXCL8 simultaneously (Figure S7, green bars). Interestingly, p38 MAPK-blocked neutrophils in a well showed a similar trend in surface marker expression as that of untreated neutrophils exposed to a "gradient" of chemoattractants on a microfluidic device. When neutrophils were treated with SB203580 to block p38 MAPK activity, surface FPR2, BLTR, and CXCR1 levels of neutrophils statically exposed to 10 ng/mL fMLP and 10 ng/mL CXCL8 were higher than those of neutrophils statically exposed to 10 ng/mL fMLP only (Figure S7, blue bars).

8. Table S1. The total number of cells analyzed for surface CD11b and CD66b expressions (* for lateral sub-section analysis)

	Total # of untreated cells				Total number of p38 MAPK-blocked cells			
	Control	fMLP	CXCL8	CXCL8-fMLP	Control	fMLP	CXCL8	CXCL8-fMLP
CD11b	60	48	46	41	57	60	46	48
CD66b	59	56	49	44	48	42	41	50

9. Fig. S8. Surface CD11b and CD66b expressions from lateral sub-section analysis



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2. D. Kim and C. L. Haynes, *Analytical Chemistry*, 2012, **84**, 6070-6078.
3. N. Li Jeon, H. Baskaran, S. K. W. Dertinger, G. M. Whitesides, L. Van De Water and M. Toner, *Nat Biotech*, 2002, **20**, 826-830.