Supporting Information (SI)

On-Chip Evaluation of Neutrophil Activation and Neutrophil-Endothelial cell Interaction during Neutrophil Chemotaxis

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I. EXPERIMENTAL SECTION

1. Cell Preparation: Whole human blood, anti-coagulated with ethylenediaminetetraacetic acid (EDTA), was obtained from Memorial Blood Center (St. Paul, MN) according to approved IRB protocol E&I ID#07809. All blood samples were collected on the day of experiments from healthy donors and were used immediately after the samples were obtained. Each condition has 3 biological replicates (3 different donors). Polymorphprep (Accurate Chemical & Scientific Corp., Westbury, NY) was used to isolate neutrophils by density gradient centrifugation as recommended by the manufacturer. Hank's buffered salt solution (HBSS, Sigma-Aldrich, St. Louis, MO) was used while washing and resuspending neutrophils during isolation, and finally, ~5 x 10^6 isolated neutrophils were resuspended in HBSS containing 2% human serum albumin (HSA, Sigma-Aldrich, St. Louis, MO). The final cell suspension was kept at 37 ℃ until use.

2. Device Fabrication: Device fabrication was done using standard soft lithography techniques and is described in detail in our previous paper.[1] Briefly, designs on transparent film (Cad/Art Service Inc., Bandon, OR) were transferred onto a chrome mask plate with a positive photoresist (Nanofilm, Westlake Village, CA) and patterned using standard photolithography techniques. The finalized chrome mask was used to make an SU-8 (Microchem, Newton, MA) master on a silicon wafer. The observation channel (Figure S1 for schematics) was 400 μm (width) x 100 μm (height) x 2500 μm (length). Once a master was prepared, a 10:1 ratio of Sylgard 184 elastomer and curing agent mixture (Ellsworth Adhesives, Germantown, WI) was cast and cured overnight at 80℃ to obtain channels in PDMS. Then, the device was completed by plasma bonding the PDMS layer with a glass substrate. The complete device was put on a hot plate at 125 ℃ for 1 minute, brought into a

biosafety cabinet, exposed to UV light for 30 minutes, and kept in the biosafety cabinet until use.

3. Endothelial Cell Culture in a Microfluidic Channel: A detailed description of how endothelial cells were cultured is given in our previous paper,[2] and we performed the same procedures with minor modifications. Briefly, the hy926 human endothelial cell line was purchased from ATCC and cultured in a T-flask as advised by the manufacturer. Cell culture medium was Dulbecco's modified eagle medium (DMEM) with high glucose (formula: 4mM L-glutamine, 4.5g/L L-glucose, and 1.5g/L sodium pyruvate (Gibco®, Carlsbad, CA)), supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Sigma Aldrich, Milwaukee, WI). Endothelial cells were cultured using this supplemented media (denoted as DMEM w/ FBS and PS throughout this article) in a T-flask at 5% CO_2 at 37℃. When necessary, endothelial cells in the T-flask were trypsinized and re-suspended into the culture media (~5 x 10^7 cells/mL), and then the cell suspension was injected into the microfluidic culture chamber inlet and incubated under 5% CO_2 and 37 ℃ for 4 hours. Then, fresh culture media was introduced into the culture chamber inlet to remove non-adherent cells, and the device was incubated for another hour. Finally, fresh culture media was introduced into the inlets at the top of the device to remove any remaining non-adherent cells. The endothelial cells were placed in a cell culture incubator for a maximum 2 days, with media replacement every 12 hours, until use.

4. Neutrophil treatment with cytokines: Before introducing isolated human neutrophils into a microfluidic device, they were pretreated with either IL-2 or IL-6 (Sigma Aldrich, St. Louis, MO) in HBSS at the final, physiologically relevant concentration of 10 ng/mL.[3-9]

5. Device preparation for experiments without endothelial cells: For experiments without endothelial cells, the channels were washed using a 70% v/v ethanol solution in sterilized Milli-Q water (Millipore, Billerica, MA) and then treated with a 250 μg/mL solution of

human fibronectin (Invitrogen, Carlsbad, CA) in sterilized Milli-Q water followed by an hour long incubation under 5 % CO_2 at 37 ℃. Following fibronectin incubation, the media in the channels was exchanged for fresh HBSS with 2% human serum albumin (HSA); then, 20 μL of neutrophil suspension (~5 x 10^6 cells/mL), either treated or not treated with interleukins, was injected into the cell inlet on the microfluidic device. After another hour of incubation to promote neutrophil adhesion, the device was connected to a syringe pump to introduce the chemoattractant gradient (0 – 10 ng/mL fMLP) into the observation channel. This 0 – 10 ng/mL concentration gradient was established by introducing HBSS containing no fMLP into the left inlet (0 ng/mL fMLP) and 10 ng/mL fMLP into the right inlet (shown in Figure 1).

6. Device preparation for experiments with endothelial cells: For experiments on endothelial cells, the endothelial cells cultured in the device were visually inspected to confirm uniformity of the endothelial cell layer. Once confirmed, 20 μL of neutrophil suspension (~5 x 10^6 cells/mL), either un-activated or activated with interleukins, was injected into the microfluidic cell culture chamber inlet. After an hour of incubation to promote neutrophil adhesion, the device was connected to a syringe pump to introduce the chemoattractant gradient (0 to 10 ng/mL fMLP) over the cells in the observation channel.

7. Sample preparation for fluorescence imaging: For fluorescence imaging, 100 μL of neutrophil suspension (~5 x 10^6 cells/mL) was injected into each well of a 96-well plate, incubated for 1 hour to promote adhesion to the surface, washed three times with HBSS, and subject to interleukin exposure, endothelial cell-conditioned media, and/or fMLP. Endothelial cell-conditioned medium was prepared from the medium in an endothelial cell culture. Rather than a regular media change, the medium in a culture was collected and centrifuged at 150xg for 10 minutes to remove non-adherent cells. Then, the supernatant was collected and kept frozen until use; this supernatant was used to examine the effect of soluble species produced by endothelial cells. For single stimulation conditions, cells were challenged with IL-2, IL-6,

or fMLP at the final concentration of 10 ng/mL or endothelial-cell conditioned medium for an hour. For co-stimulation conditions with both interleukins and fMLP, cells were subject to IL-2 or IL-6 for an hour first and then challenged with fMLP for another hour. For co-stimulation conditions with endothelial cell-conditioned medium and fMLP, cells were subject to endothelial cell-conditioned medium for an hour first and then challenged with fMLP for another hour. For co-stimulation conditions with interleukins, endothelial cell-conditioned medium, and fMLP, cells were subject to interleukins with endothelial cell-conditioned medium, to be consistent with on-chip chemotaxis investigation, for an hour and then challenged with fMLP for another hour. After stimulation, cells were washed with HBSS three times and then subject to anti-CD11b conjugated with AlexaFluor 700 (Life Technologies, Grand Island, NY) and anti-CD66b conjugated with AlexaFluor 647 (BD Pharmingen, San Diego, CA) following instructions from manufacturers, incubated for 1 hour, washed with HBSS three times, and then surface expression of those adhesion molecules on individual cells were fluorescently monitored.

8. Time-Lapse/Fluorescence Microscopy: Migration and CD11b and CD66b surface expression of neutrophils was monitored using collected time-lapse images (Supple Video 1 and 2) from Metamorph Ver. 7.7.5 imaging software and an inverted microscope (Nikon, Melville, NY) equipped with a CCD camera (QuantEM, Photometrics, Tucson, AZ). For migration studies, images of neutrophils in the observation channel were acquired every 10 seconds for 20 minutes. For fluorescence imaging, three images were collected from a section of a well with 1 second exposure time, 1 second time interval, and 2 seconds collection time, and once a stack of images was collected, individual cells were randomly selected and assessed for their maximum fluorescence intensity in arbitrary units (denoted as $A.U._{max}$) using MetaMorph 7.7.5 software.

9. Analysis of migration data: From each experiment, individual neutrophils were randomly chosen and individually tracked using Metamorph Ver. 7.7.5 software. Once the imaging software extracted the trajectory information for individual cells from the collected image stack (ESI Fig. 1), these raw data were imported into Microsoft Excel and further processed to calculate two unitless numeric parameters: motility index (MI) and chemotactic index (CI) as previously described [1,10]. Briefly, MI is the ratio of final displacement (d) and maximum displacement (d_{max}) of a neutrophil:

$$MI = d/d_{max}$$

where d_{max} is defined as the average migration speed times the total migration time.

CI quantifies the cells' orientations:

$$CI = x/d_{total}$$

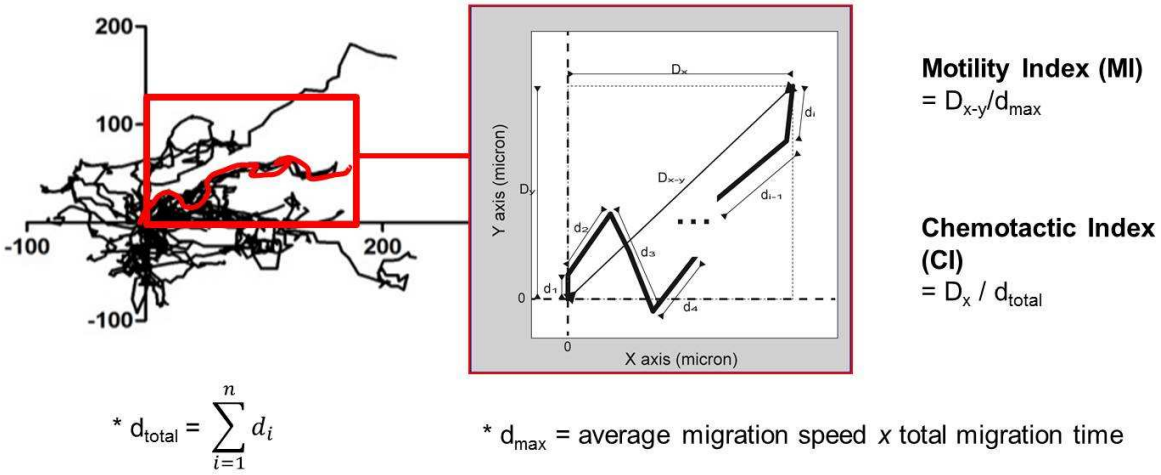
where x is the final displacement along the direction of the gradient and d_{total} is the total migration distance.

In this report, movement toward the right side of the observation channel, toward a higher concentration of fMLP, results in positive CI. In addition to these parameters, % population was calculated simply by taking the number of cells with negative CI values divided by the total number of cells. Thus, this % population represents cells with overall migration opposite to the direction of the fMLP gradient. Also, the angular displacement of individual cells were digitized by giving a score of "+1" to cells in each frame that moved with $-90^\circ < \text{angle} < 90^\circ$, and giving a score of "-1" to cells in each frame moved with angle $> 90^\circ$ or with angle $< -90^\circ$. The angle value was obtained with respect to the x-axis (parallel to the gradient direction); thus, this parameter is descriptive of how much deviation from the parallel to the fMLP gradient occurred during migration. An angle distribution value of "+1" would indicate that the cell moved straight toward the fMLP signal in every collected image. To assess individual neutrophils' response in a time-resolved manner, this angle parameter was calculated for the

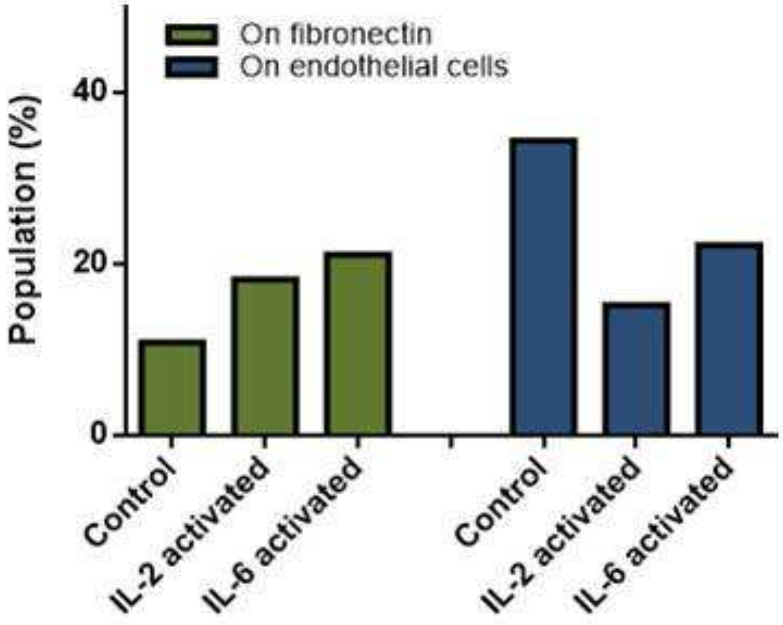
first 1, 4, and 7 minutes, and for the entire duration of the experiment. All angle distribution plots provided in this manuscript contain data for the first 4 minutes and for the entire duration. T-tests were used for statistical comparison, and errors are reported as +/- standard error of mean, SEM, throughout the manuscript.

II. Supplemental Data

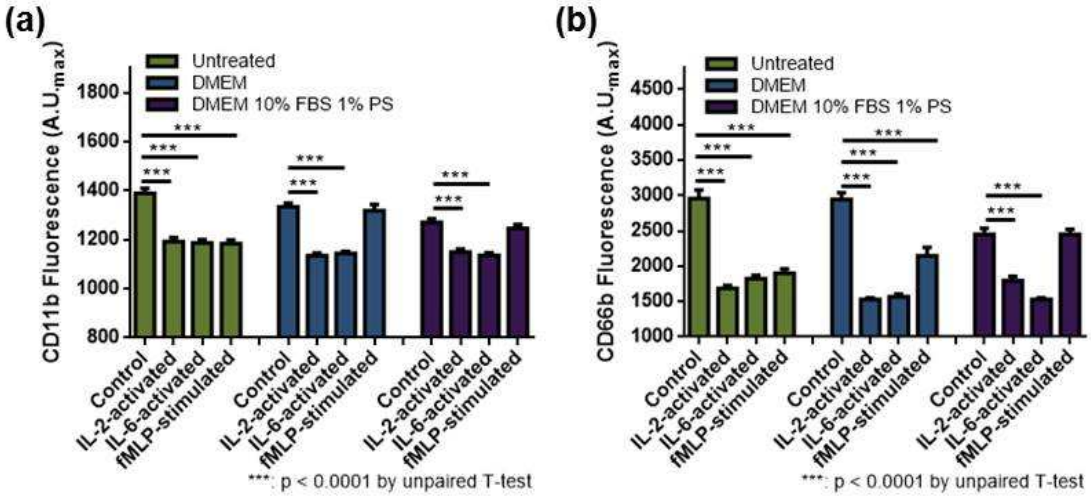
1. Figure S-1. Definition of motility index (MI) and chemotactic index (CI) used for trajectory analysis



2. Figures S-2. % Population of neutrophils on fibronectin (green) and on endothelial cells (blue) that moved away from the fMLP signal



3. Figure S-3. Surface adhesion molecule expression of untreated, DMEM-treated, and DMEM 10%FBS 1%PS-treated neutrophils.

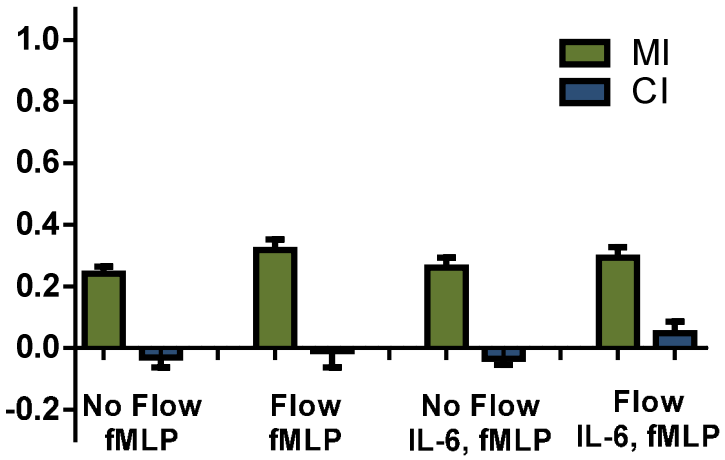


(a) Surface CD11b expression of neutrophils.

(b) Surface CD66b expression of neutrophils.

*DMEM-treated neutrophils are pre-incubated with DMEM (with high glucose) for an hour prior to stimulation and DMEM 10%FBS 1%PS-treated neutrophils are pre-incubated with DMEM (with high glucose) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin prior to stimulation. Untreated cells were pre-incubated with HBSS for an hour prior to stimulation.

4. Figure S-4. Chemotaxis controls



In this sub-set of experiments, four different control conditions were examined. The main purpose here is to examine the impact of either flow or the chemical gradient on neutrophil chemotaxis. Assessed conditions are as followed:

1. No flow, 10 ng/mL fMLP as chemoattractant (labeled as “No Flow fMLP” - no flow, no gradient)
2. Flow, 10 ng/mL fMLP as chemoattractant (labeled as “Flow fMLP” - flow, no gradient)
3. No flow, IL-2 as stimulant, fMLP as chemoattractant (labeled as “No Flow IL-2, fMLP” – no flow, no gradient)
4. Flow, IL-2 as stimulant, fMLP as chemoattractant (labeled as “Flow IL-2, fMLP” – flow, no gradient)

All controls were performed in the same microfluidic device used throughout the manuscript. For the “no flow fMLP” condition (furthest left), once neutrophils settled onto endothelial cells, the media was exchanged for fMLP solution, and the flow was stopped. For the “flow fMLP” condition (second from left), once neutrophils settled onto endothelial cells, they were exposed to a stream of fMLP. For the “no flow IL-2, fMLP” condition (second from right), once IL-6-activated neutrophils settled onto endothelial cells, the media was changed to fMLP solution, and then the flow was stopped. The “flow IL-2, fMLP” condition (furthest

right), once IL-6-activated neutrophils settled onto endothelial cells, the neutrophils were exposed to a stream of fMLP. In all of the above conditions, neutrophils were mobile but showed minimal directionality in migration pattern.

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