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Supporting Information

Recapitulation of *in vivo*-like Neutrophil Transendothelial Migration using a Microfluidic Platform

*Xiaojie Wu, Molly A. Newbold and Christy L. Haynes**

Department of Chemistry, University of Minnesota, 207 Pleasant Street SE, Minneapolis, Minnesota, 55455, United States

* To whom correspondence should be addressed

E-mail chaynes@umn.edu, Tel. +16126261096

Methods

Scanning Electron Microscope (SEM) Imaging

Two drops of polymerized collagen gel solution were added on a small piece of silicon wafer for water evaporation and the dried gel was coated with 5-nm-thick platinum. The sample was observed at 10 kV using a field emission scanning electron microscope (Jeol 6700F, JEOL USA, Peabody, MA).

Endothelial Cell-Conditioned Medium Experiment

To obtain the endothelial cell conditioned medium, 100 μL of endothelial cells at 3 - 4 × 10^6 cells/mL were seeded on a 96 well plate and mixed with 100 μL of chemoattractant solution (20 ng/mL, 40 ng/mL or 100 ng/mL) to make sure that the final concentration of endothelial cells and chemoattractants were equal to those used in single gradient conditions. The plate was placed in the 5% CO_2 incubator at 37 ^oC overnight. Then, the

conditioned medium containing all the secreted molecules and chemoattractants was collected and added into the right side channel of the microfluidic device with 30 μL in each reservoir. After achieving a stable chemical gradient, 5 μL of 4 - 5 × 10^6 cells/mL neutrophils were injected into the bottom channel, and neutrophil migration was tracked using the bright-field microscope.

Receptor Expression

The prepared device with endothelial cell layer is incubated with desired chemoattractant solution in the right side channel or both side channels for 7 h (the first 2 h for chemoattractant diffusion and the following 5 h for endothelial cell activation) without the addition of neutrophils. After that, all the channels were washed twice using 1× phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) solution by filling one reservoir with 40 μL PBS and letting it flow along the channels to fill the other one. Then, 30 μL of allophycocyanin (APC) conjugated p-selectin antibody (5 μg/mL) or ICAM-1 antibody solution (2.5 μg/mL) (eBioscience, San Diego, CA) was added into each reservoir of the bottom channel while 1× PBS was added into the other reservoirs for 2 h adhesion molecules labeling. Finally, fluorescence agents were washed using 1× PBS twice and the devices were imaged on the microscope. The fluorescence intensity of receptor molecule expression was measured with a 20x objective (Nikon, Melville, NY) using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Numerical Simulation and Fluorescence Imaging

Chemical gradients of 50 ng/mL fMLP in the gel chamber were verified using the

molecular diffusion module in simulation software COMSOL 4.3b. Diffusion coefficients for fMLP in the collagen gel, free solution and endothelial cell layer were assumed to be 6.99×10^{-11} m^2/s, 4.2×10^{-10} m^2/s, and 9.55×10^{-11} m^2/s, respectively.[1] In addition, 100 μM of Rhodamine 6G in HBSS buffer was employed to replace chemoattractants solution in the right side channel for the visualization of chemical gradients. The fluorescence intensities in the gel chamber were recorded using MetaMorph ver. 7.7.5 imaging software at different time points. For the endothelial cell layer permeability experiment, 10 μM of FITC-dextran (Sigma-Aldrich, St. Louis, MO) was placed in the bottom channel while HBSS buffer was added into side channels. After 2 h diffusion of fluorescence solution, the flux balance at the endothelial cell- gel interface was developed to calculate the permeability:

$$J = -D\frac{\partial C}{\partial x}$$

where, J is flux, D is diffusion coefficient of FITC-dextran (6.75×10^{-11} m^2/s) in the collagen gel and x is the position.

$$Flux = -P\Delta C$$

where, P is permeability and C is concentration.

Confocal Imaging and Dark-field Imaging

After the formation of endothelial cell layer on the side wall of collagen gel, DMEM medium in all reservoirs was removed, and the channels were rinsed with 1× PBS solution using the same method detailed in the receptor expression experimental procedure. PBS was aspirated from all the reservoirs, and the wash step was repeated

twice. Following the PBS wash, 30 μL of 4% (wt/vol) paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO) was added to each reservoir to fix the cells for 15 min. PFA was washed with PBS using the same steps above and then 30 μL of 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich, St. Louis, MO) was placed in each reservoir for permeablization of endothelial cell membranes, allowing fluorescence reagent to enter the cells more easily. After incubation with Triton X-100 for 5 min, endothelial cells were washed with PBS twice and 30 μL of the mixture containing 4,6-diamidino-2-phenylindole dilactate (DAPI dilactate) at the working concentration (5 μg/mL) and rhodamine phalloidin (6.6 μM) (Life Technologies, Carlsbad, CA) was added in each reservoir for staining the cells. The petri dish containing devices was wrapped with aluminum foil and placed in the dark place for 1 h incubation. Before imaging, microfluidic devices were washed with PBS again to remove excess fluorescence reagents. The 3D configuration of endothelial cell layer was imaged using a Nikon A1R MP confocal microscope. Finally, all the slices were deconvoluted using AutoQuant X 3.0.4 software (Media Cybernetics, Rockville, MD) and processed with Imaris software (Bitplane AG, Zurich, Switzerland).

For 3D dark-filed imaging, the preparation work is almost the same as that used in confocal imaging while only DAPI was employed to label endothelial cells in this part. The 3D configuration of endothelial cell layer was imaged on a CytoViva dark-field microscopy system (CytoViva, Auburn, AL) equipped with a NanoScan Z stage (Prior Scientific, Rockland, MA) and a QIamging Exi Blue camera (QImaging, Surrey, BC, Canada). Finally, all the slices were deconvoluted using a point spread function (PSF) in

ImageJ software (National Institutes of Health, Bethesda, MD) to reduce out-of-focus light.

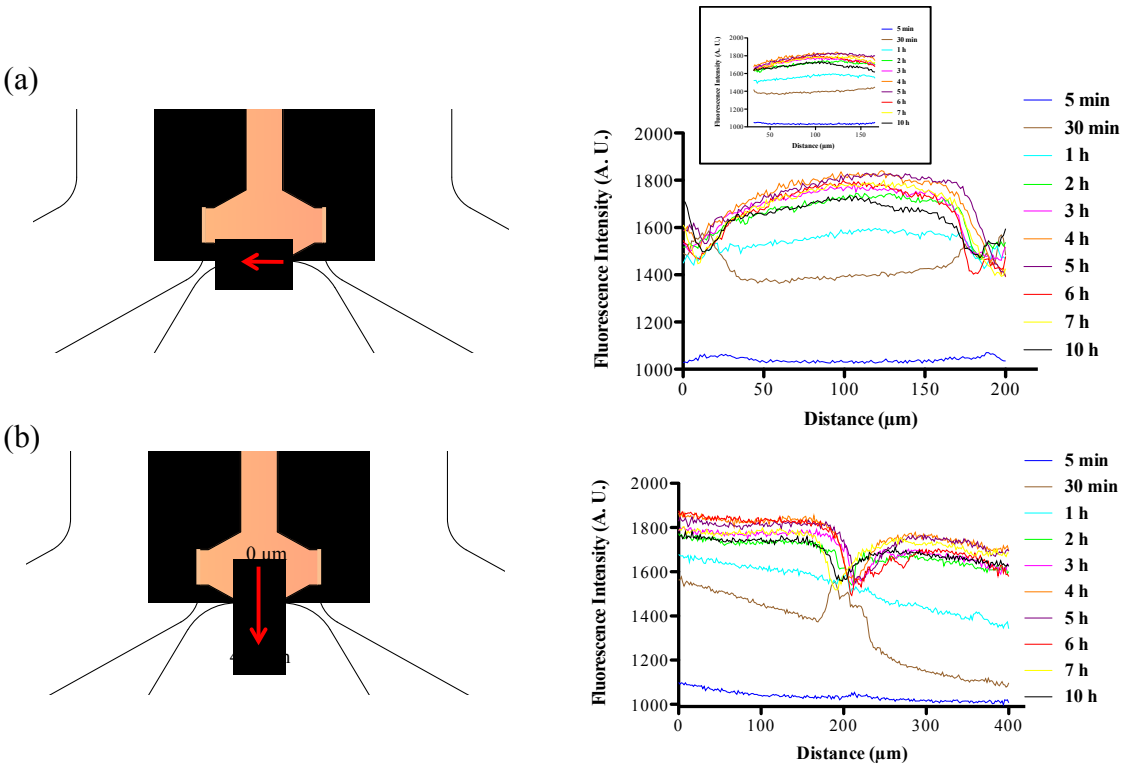

Figure S1. Fluorescence gradients along different directions in the gel chamber. The fluorescence gradients (a) at the interface of the gel chamber and the endothelial cell layer and (b) in the vertical direction from the gel chamber to the endothelial cell channel (The burst change of fluorescence intensities are caused by the autofluoresence of endothelial cells and the autofluoresence was subtracted from the gradient in the inset picture).

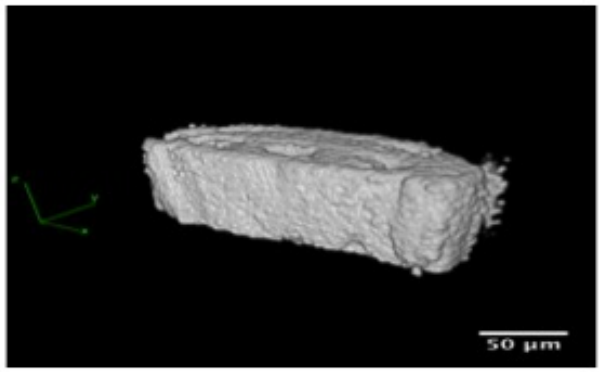

Figure S2. Dark-field 3D imaging of endothelial cell layer cultured on the side wall of gel region.

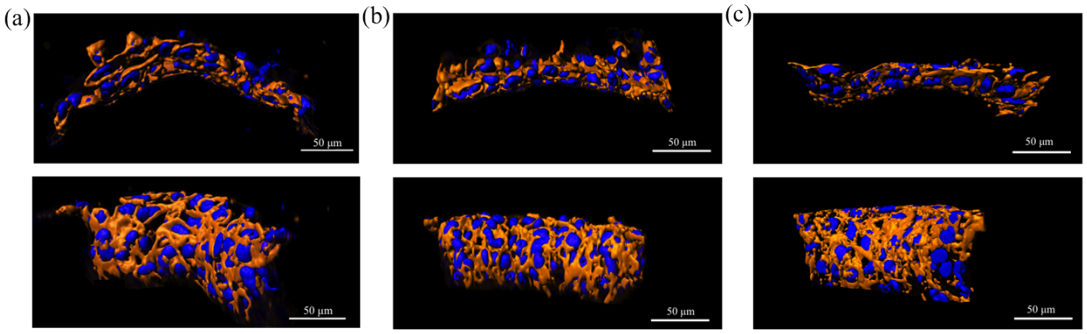

Figure S3. Confocal images of endothelial cell layer in three different microfluidic devices: (a) top view (top) and side view (bottom) of cell layer in device 1; (b) top view (top) and side view (bottom) of cell layer in device 2; (c) top view (top) and side view (bottom) of cell layer in device 3. (blue indicates cell nucleus stained by DAPI and orange represents cytoskeletal F-actin labeled by rhodamine phalloidin).

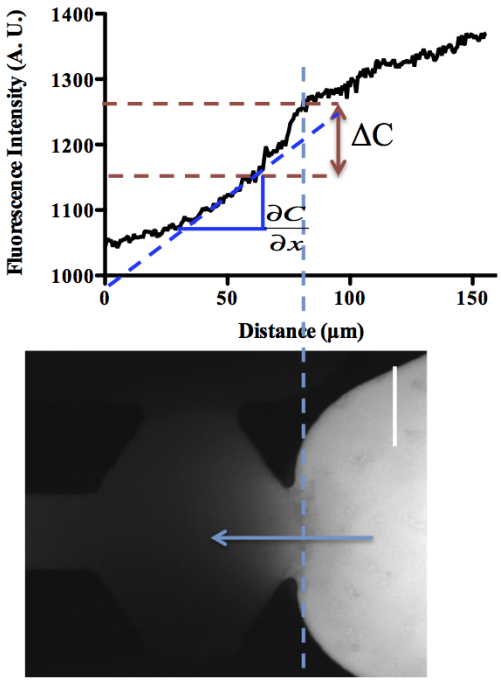

Figure S4. Measurement of endothelial cell layer permeability. The permeability of endothelial cell layer was measured using the fluorescence intensity profile of FITC-dextran solution after 2 h diffusion from bottom channel to gel scaffold. The blue arrow indicates the direction of fluorescence gradient. (scale bar: 200μm)

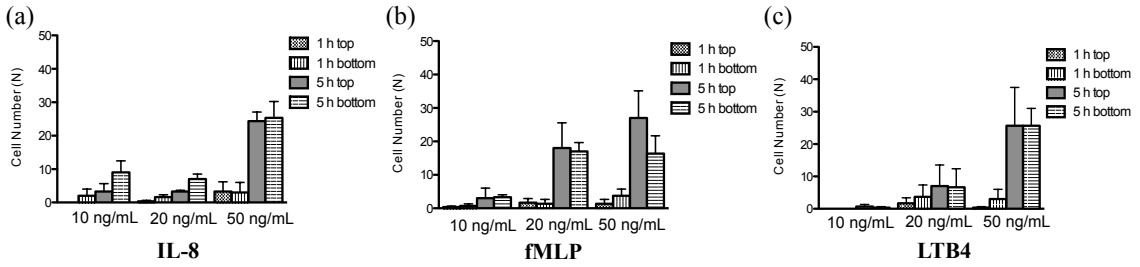

Figure S5. Quantitative analysis of neutrophil TEM at 1 h and 5 h after neutrophil injection under various single chemoattractant gradients. The number of neutrophils in the top and bottom parts under (a) 10 ng/mL, 20 ng/mL, and 50 ng/mL of IL-8 gradient; (b) 10 ng/mL, 20 ng/mL, and 50 ng/mL of fMLP gradient; and (c) 10 ng/mL, 20 ng/mL, and 50 ng/mL of LTB4 gradient. There was no statistical significance among t-tested pairs in this data set.

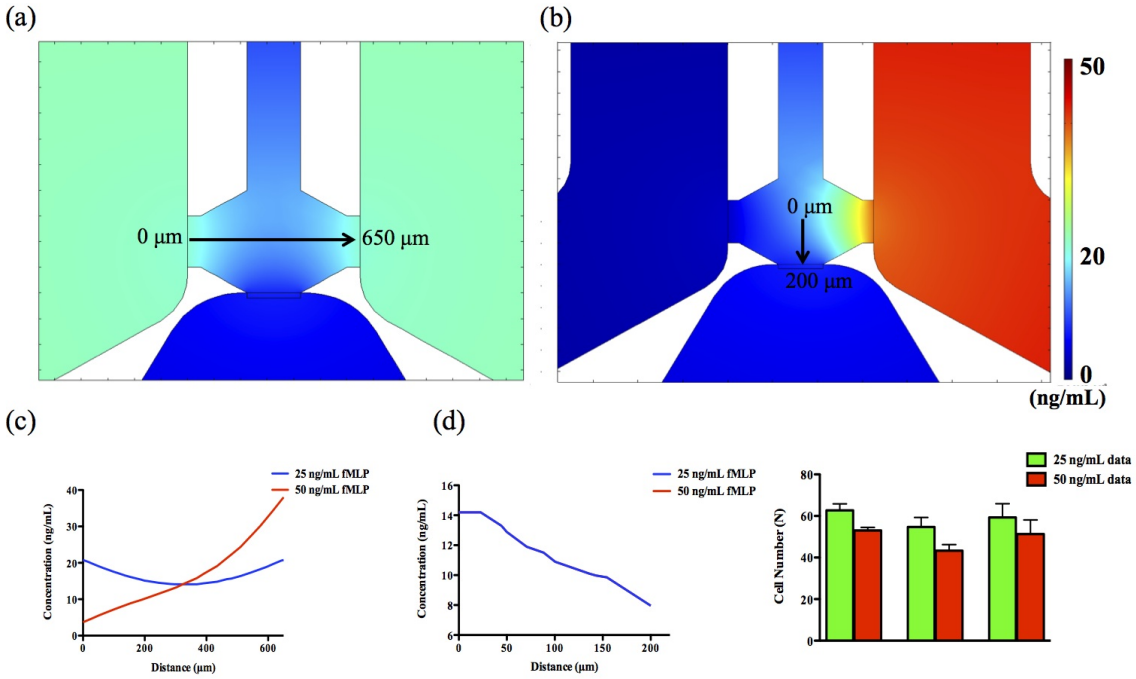

Figure S6. Comparison of gradient profiles in single chemoattractant gradients and no gradient conditions. The COMSOL simulation results of fMLP solution in the microfluidic device after 5 h diffusion: (a) 25 ng/mL of fMLP solution in both side channels; (b) 50 ng/mL of fMLP solution in the right channel. The gradient profiles of two conditions along the (c) center line of gel chamber (indicated in (a)) in the horizontal direction and (d) the center line (indicated in (b)) in the vertical direction. (e) The total numbers of neutrophils located in the gel scaffold after 5 h transmigration for these two conditions.

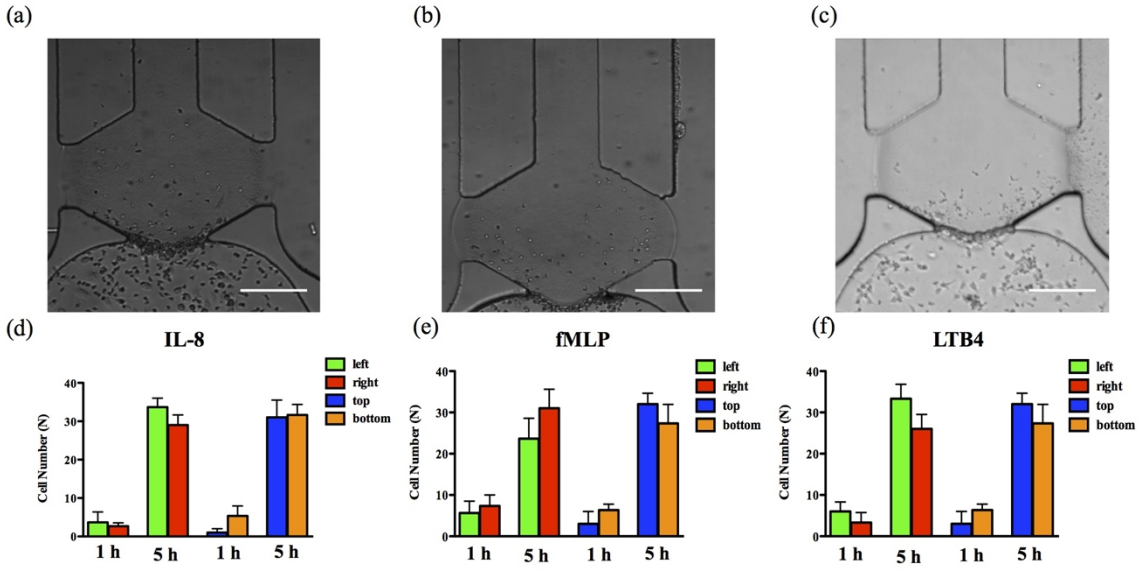

Figure S7. Neutrophil migration results under no gradient conditions. Bright-field images of neutrophil TEM at 5 h after neutrophil injection: (a) 25 ng/mL IL-8 in both side channels; (b) 25 ng/mL fMLP in both side channels; (c) 25 ng/mL LTB4 in both side channels. Quantitative analysis of neutrophil numbers in different parts of the gel chamber at 1 h and 5 h after neutrophil injection: (d) 25 ng/mL IL-8 in both side channels; (e) 25 ng/mL fMLP in both side channels; (f) 25 ng/mL LTB4 in both side channels.

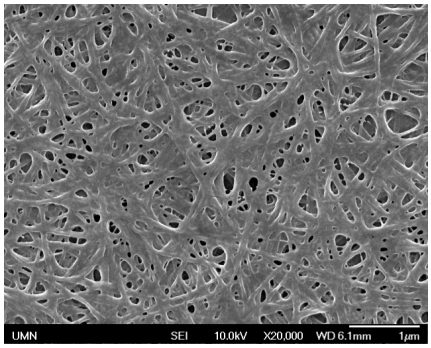

Figure S8. SEM image of collagen gel porous fiber structure. (scale bar: 1 μm)

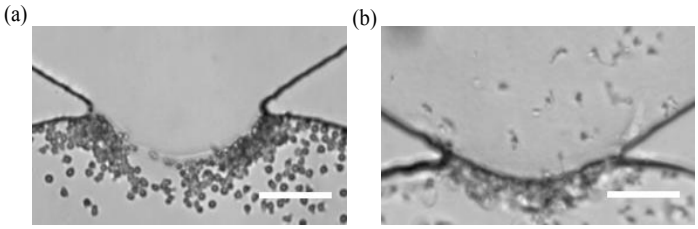

Figure S9. The morphological changes of neutrophils (a) without endothelial cell layer and (b) with endothelial cell layer. (scale bar: 100 μm)

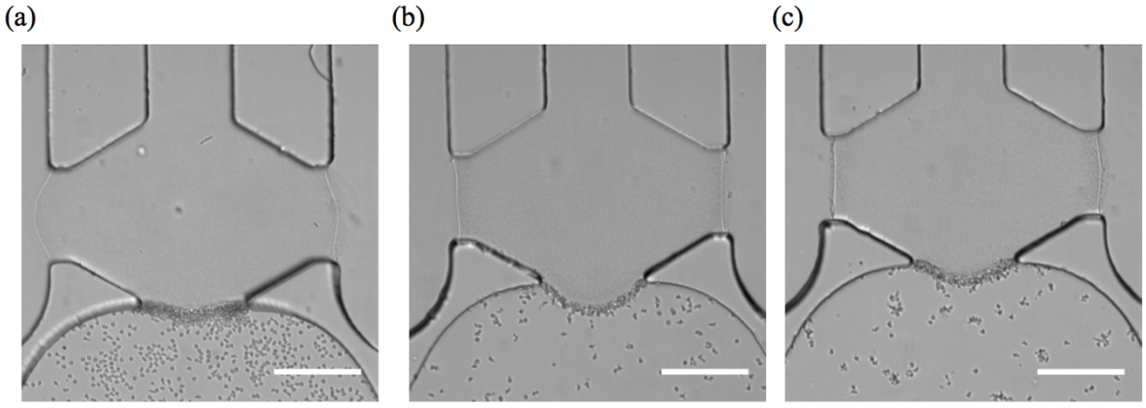

Figure S10. Bright-field images of neutrophil transmigration without endothelial cells at 5 h after neutrophil injection: (a) 50 ng/mL of IL-8 conditioned medium; (b) 50 ng/mL of fMLP conditioned medium; (c) 50 ng/mL of LTB4 conditioned medium. (scale bar: 200 μm)

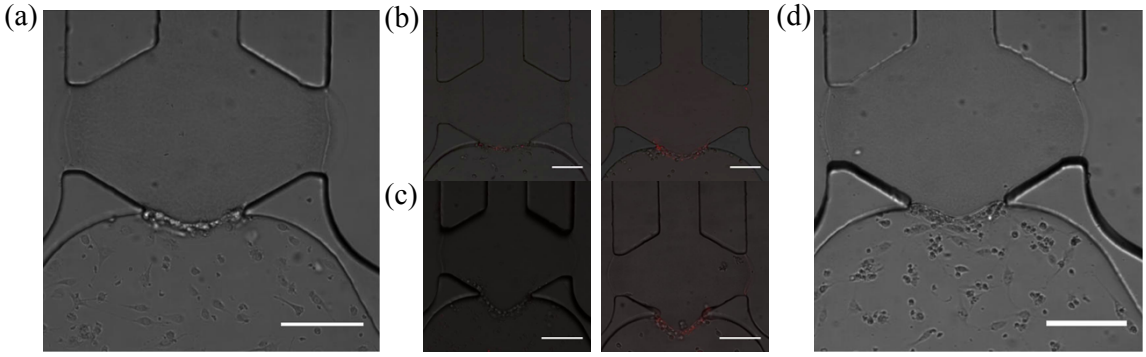

Figure S11. (a) Neutrophil TEM at 5 h in chemoattractant-free condition. (b) P-selectin expression in the absence of IL-8 (left) and in the presence of 50 ng/mL of IL-8 (right) using APC conjugated antibody fluorescence imaging. (c) ICAM-1 expression in the absence of IL-8 (left) and in the presence of 50 ng/mL of IL-8 (right) using APC conjugated antibody fluorescence imaging. The overlay of bright-field image and fluorescence image was taken using Adobe Photoshop CS software and the transparency of fluorescence image was set as 70%. (d) Neutrophil TEM at 5 h under 50 ng/mL IL-8 gradient with p-selectin and ICAM-1 antibodies. (scale bar: 200 μm)

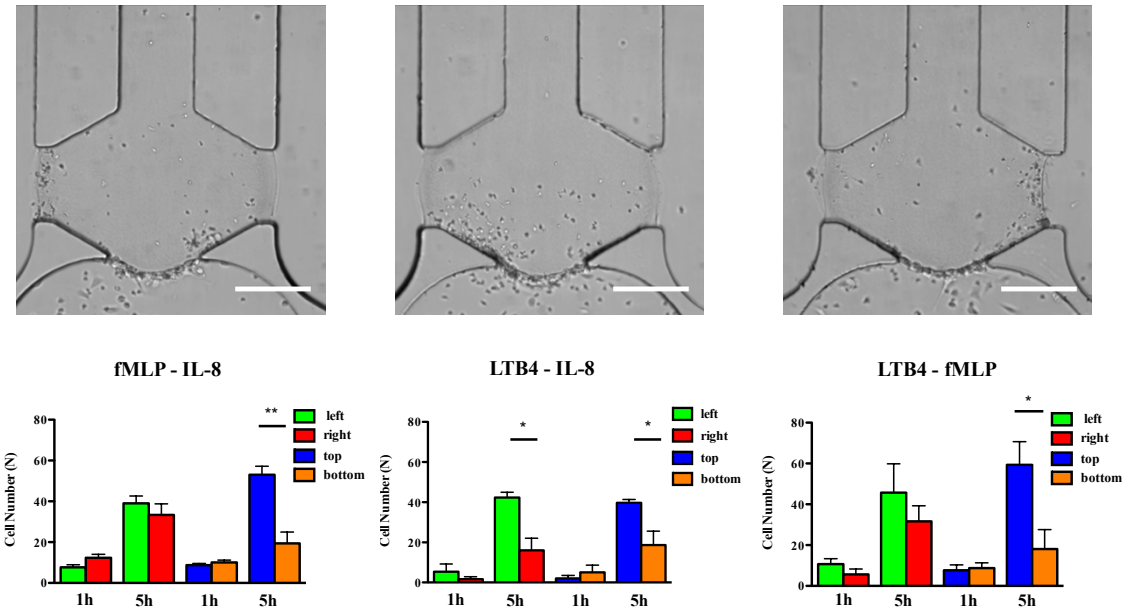

Figure S12. Synergistic effects of any two chemoattractants in separate channels. Bright-field images of neutrophil TEM 5 h after neutrophil injection: (a) 25 ng/mL fMLP vs. 25 ng/mL IL-8; (b) 25 ng/mL LTB4 vs. 25 ng/mL IL-8; (c) 25 ng/mL LTB4 vs. 25 ng/mL fMLP. Quantitative analysis of neutrophil count in different parts of the gel chamber 1 h and 5 h after neutrophil injection: (d) 25 ng/mL fMLP vs. 25 ng/mL IL-8; (e) 25 ng/mL LTB4 vs. 25 ng/mL IL-8; (f) 25 ng/mL LTB4 vs. 25 ng/mL fMLP (*, $p<0.05$, **, $p<0.005$, using a two-tailed unpaired t-test).

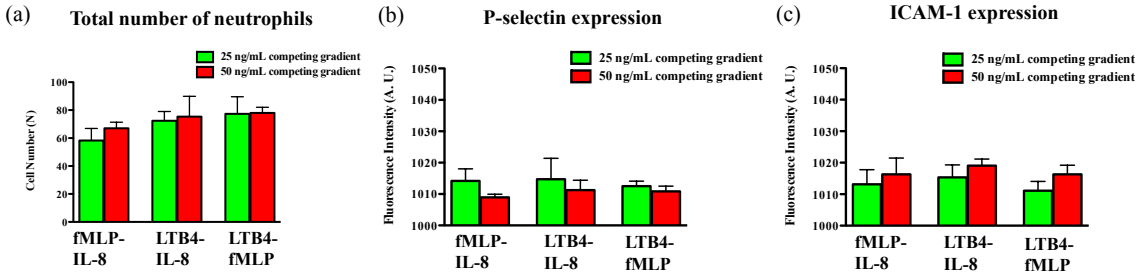

Figure S13. The comparison of 25 ng/mL and 50 ng/mL competing gradients conditions. (a) The total numbers of neutrophils located in the gel chamber after 5 h transmigration for two conditions. (b) The fluorescence intensity of P-selectin expression on the surface of endothelial cells for two competing gradients conditions. (c) The fluorescence intensity of ICAM-1 expression on the surface of endothelial cells for two competing gradients conditions.

References

(1) S. Han, J.-J. Yan, Y. Shin, J. J. Jeon, J. Won, H. Eun Jeong, R. D. Kamm, Y.-J. Kim, Y.-J. and S. Chung, *Lab Chip,* **2012**, *12*, 3861-3865.