

Electronic Supplementary Information (ESI) †

Article title: A versatile assay for monitoring *in vivo*-like transendothelial migration of neutrophils

Authors: Sewoon Han¹, Ji-Jing Yan², Yoojin Shin¹, Jessie Jeon³, Jihee Won¹, Hyo Eun Jeong¹, Roger Kamm³, Young-Joon Kim² and Seok Chung¹

Affiliations of institutions: ¹Department of Mechanical Engineering, Korea University, South Korea

²Department of Integrated Omics for Biomedical Science, WCU Program of Graduate School, Yonsei University, South Korea

³Department of Mechanical Engineering, Massachusetts Institute of Technology, USA

Corresponding author: Professor Seok Chung

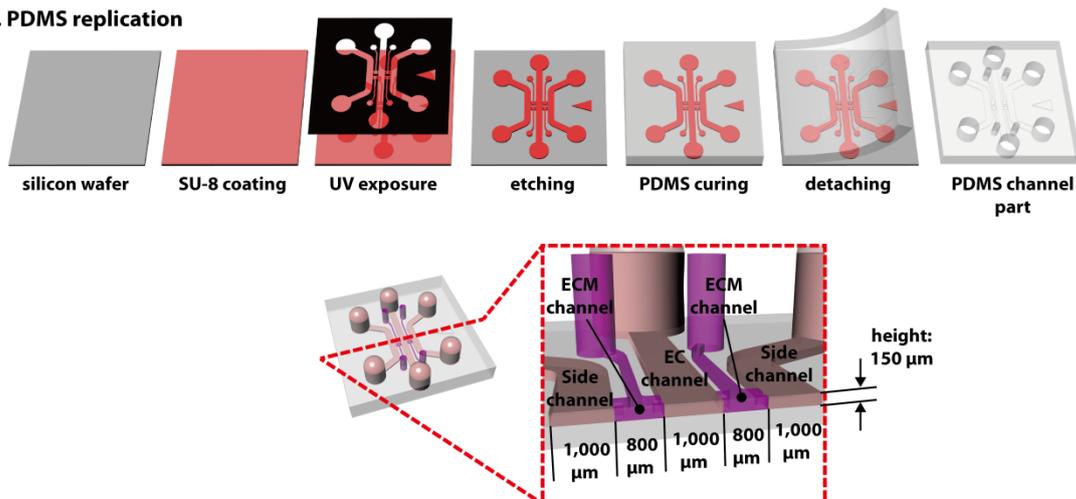
Address: #512B Innovation Hall Korea University Anam-dong, Sungbuk-gu, Seoul, South Korea

Email: sidchung@korea.ac.kr

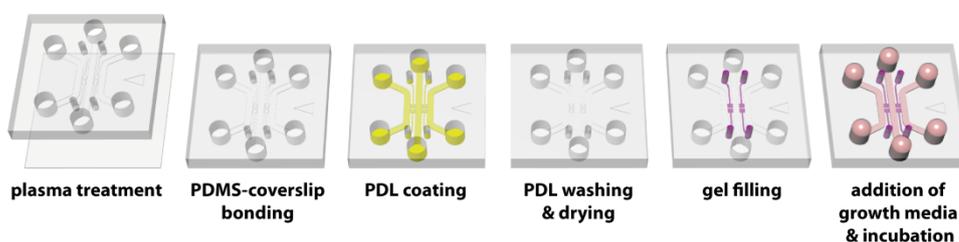
Phone: +82-2-3290-3352

Fax: +82-3290-3352

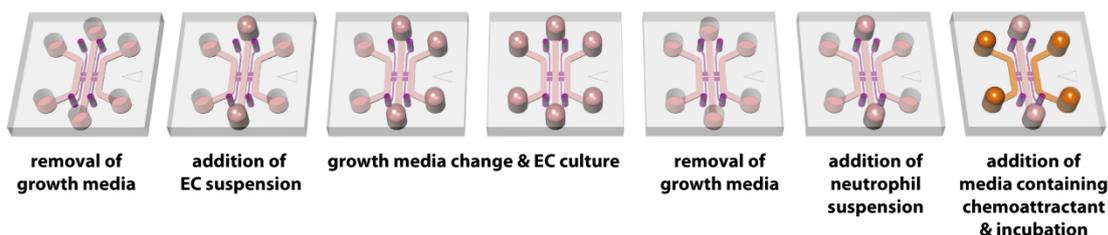
a. PDMS replication



b. surface treatment & gel filling



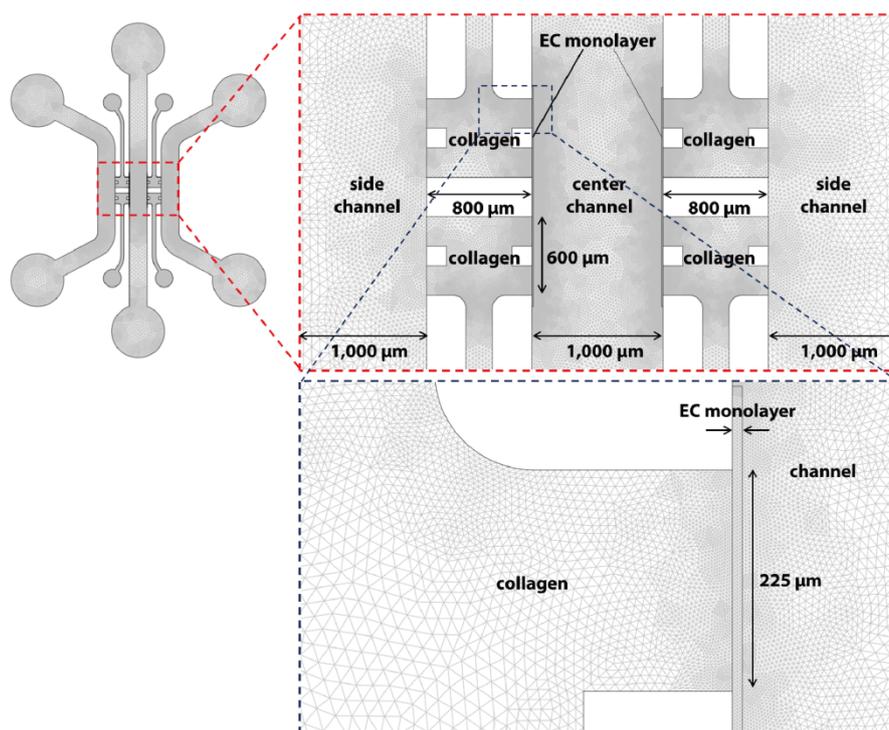
c. cell seeding & chemical stimuli



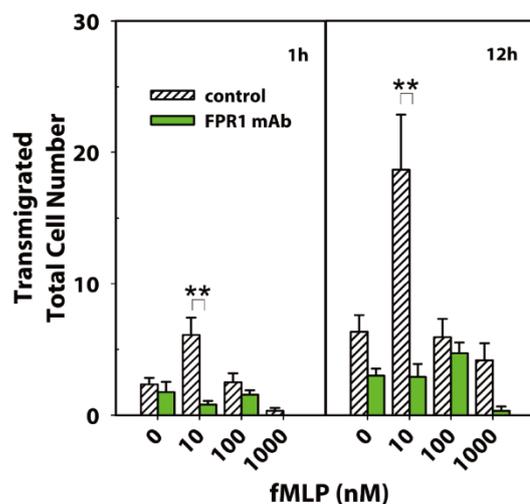
ESI Figure 1† Device preparation and cell seeding/culture in the microfluidic device. **(a)** PDMS replication.

The channels of the device were patterned onto a photoresist (SU-8 100, MicroChem)-coated silicon wafer by photolithography. The PDMS channel component was then cast with PDMS solution (10:1 weight ratio of base to curing agent) on the prepared SU-8 mold at 80 °C for 1 hour. Media ports and gel injection ports were opened with 4- and 1-mm biopsy punches, respectively. The cross-sectional sizes of the microchannels are 1,000 μm (width) × 150 μm (height) for endothelial cell and the side channels, and 800 μm (width) × 150 μm (height) for ECM channels. **(b)** Surface treatment and gel filling. The PDMS channel component and glass coverslip were irreversibly bonded by treatment with oxygen plasma (CUTE, Femtoscience). Immediately after bonding, 60 μl of a 1-mg/ml poly-D-lysine (PDL; Sigma) solution (MW: 30,000–70,000) in sterile distilled deionized water (DDW) was pipetted into the media port. After incubation at 37 °C for 4 hours, the coating solution was

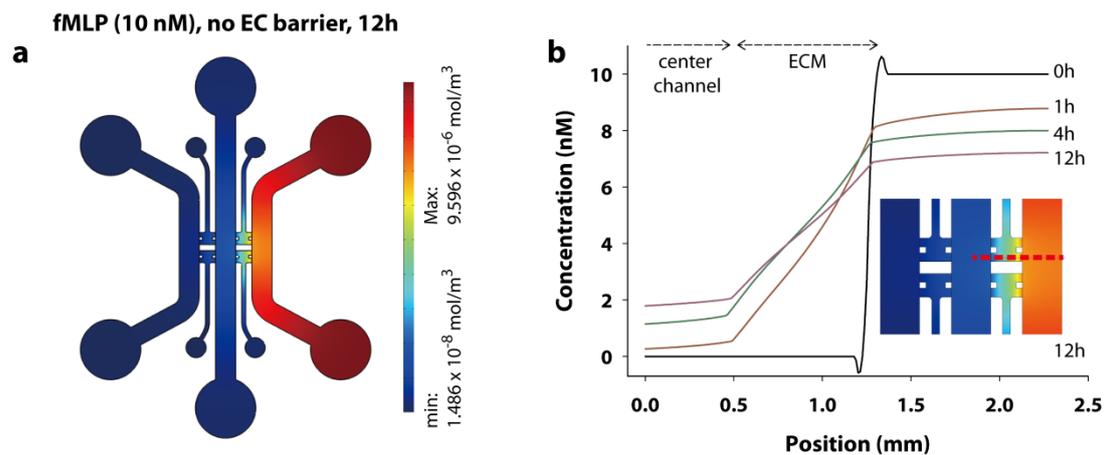
removed with an aspirator, and excess PDL was removed by washing twice with DDW. For gel-filling of ECM channels, the hydrophobicity of the device was restored by incubating in a dry oven at 80 °C for 24 hours. The ECM-filled device was then placed in a pipette tip box containing DDW in the lower chamber. After 30 minutes gelation, growth medium was forcibly injected into endothelial cell and side channels; a blunted pipette tip was used for this because of its hydrophobicity. **(c) Cell seeding and chemical stimuli.** After briefly removing growth media from each port, 60 µl of endothelial cell suspension at a cell density of 2×10^6 cells/ml was added into the endothelial cell channel, and then the device was placed in a humidified 37 °C CO₂ incubator for 1 hour. After cells had adhered, the growth medium in each channel was replaced with fresh medium, and replenished every 24 hours thereafter. Endothelial cells were cultured in the device for 4 days. At this time, the growth medium in each port was gently aspirated and the neutrophil suspension was added into the endothelial cell channel. Growth media containing chemoattractant were then directly added into the side channels.



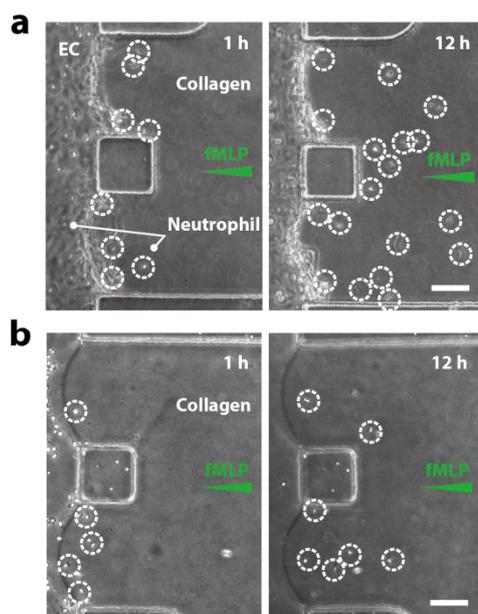
ESI Figure 2† Mesh structure of the device. The endothelial cell monolayer worked as a diffusion barrier with diffusion coefficients of $9.55 \times 10^{-11} \text{ m}^2/\text{s}$ for fMLP (MW: 400 Da; Sigma) and $6.32 \times 10^{-12} \text{ m}^2/\text{s}$ for IL-8 (1 kDa; R&D Systems) [S5-S7]. This structure was automeshed, and mesh refinement was performed twice. Chemoattractants were supplied via side channels.



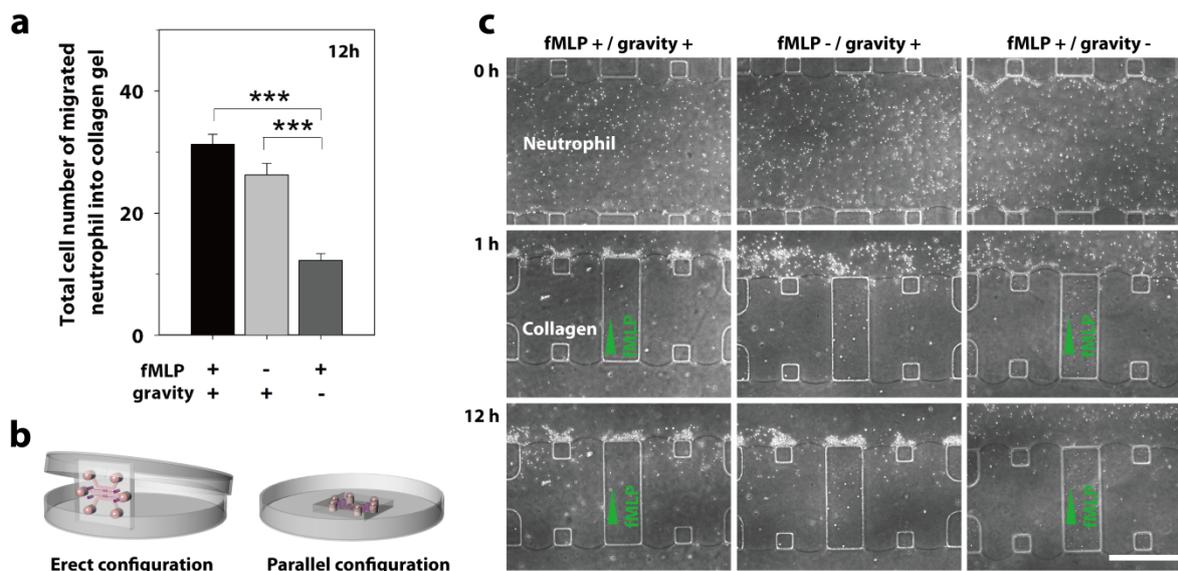
ESI Figure 3† A monoclonal anti-human fMLP receptor 1 antibody (FPR1 mAb, 1 $\mu\text{g/ml}$ in RPMI-1640 medium containing 10% fetal bovine serum; R&D Systems) was used to block the fMLP receptor activity of neutrophils. Antibody-treated neutrophils were placed in a CO_2 incubator at 37°C for 30 minutes, after which, the cell suspension was incubated in endothelial cell growth medium. The seeding density of neutrophils was 1×10^6 cells/ml. The TEM of neutrophils in the presence of the fMLP gradient (10 nM fMLP in endothelial cell growth medium delivered via side channels) was influenced by FPR1 activity (** $P < 0.01$). Significance was analyzed by Student's t -test. Error bars, \pm SEM ($n = 12$).



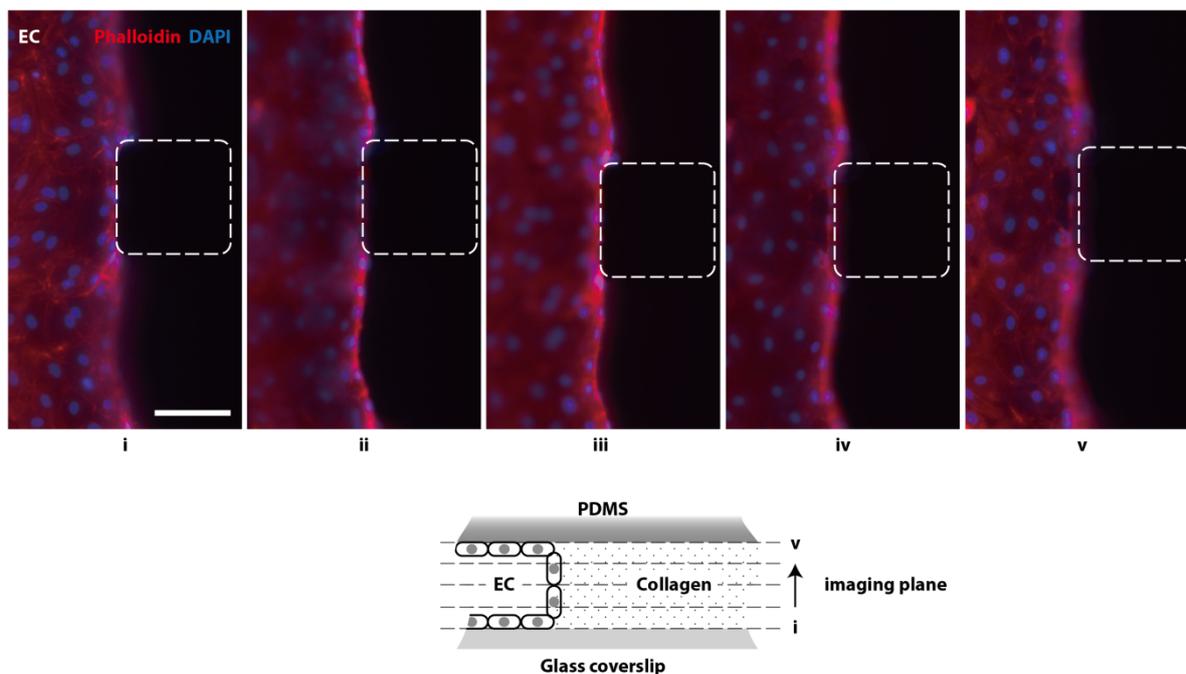
ESI Figure 4† Diffusion profiles of fMLP without an endothelial cell monolayer were characterized using COMSOL Multiphysics software. **(a)** Concentration profile 12 hours after adding fMLP (10 nM) into the side channels. **(b)** Simulated fMLP diffusion characteristics in the device without an endothelial cell monolayer at 0, 1, 4 and 12 hours. Inset images depict the fMLP concentration in the center region of the device at 12 hours. Each concentration profile was acquired from the section indicated by the red dotted line. These results show that the molecular weight of the fMLP is low enough to allow fMLP to cross the endothelial cell monolayer without disturbing its diffusion (see **Fig. 2a**).



ESI Figure 5† Phase-contrast images of transmigrating/migrating neutrophils in the collagen gel at 1 (left) and 12 (right) hours with **(a)** and without **(b)** an endothelial cell monolayer in the presence of an fMLP gradient. fMLP (10 nM) was applied to the side channels. The seeding density of neutrophils was 1×10^6 cells/ml. Scale bar, 100 μm .



ESI Figure 6† Gravity affected neutrophil migration in the collagen gel in the absence of a chemotactic gradient. **(a)** The total number of neutrophils migrating inside the collagen gel with and without an fMLP gradient (10 nM fMLP in endothelial cell growth medium) was significantly affected by gravity ($***P < 0.001$). Significance was analyzed by Student's *t*-test. Error bars, \pm SEM ($n = 4$). The seeding density of neutrophils was 2×10^6 cells/ml. **(b)** Schematic of the experimental design. A gravitational force was applied to neutrophils by simply erecting the device. **(c)** Phase-contrast images of migrating neutrophils in the collagen gel at 0, 1 and 12 hours, with and without an fMLP gradient and/or gravity. Scale bar, 500 μ m.



ESI Figure 7† Fluorescent images of a perfect endothelial cell monolayer cultured in the device. The white dotted square indicates a PDMS post. The images were taken from the bottom (i) to the top (v) of the device. Actin and nuclei were stained with rhodamine phalloidin (red) and DAPI (blue), respectively. Scale bar, 100 μm .

ESI Video 1† 3D visualization of transmigrating neutrophils shown in Figure 6. Red voxel: 3D volumetric image of the endothelial cell monolayer (F-actin); blue voxel: nuclei of endothelial cells; green voxel: neutrophils.

ESI Video 2† Live-cell imaging of the TEM process. Chemoattractant: fMLP (10 nM); phase-contrast microscopy: time-lapse over 3 hours and 34 minutes (1,340 frames, 15 frames/s; 10x objective).

ESI Methods

Cell differentiation and culture

Human promyelocytic HL-60 cells, obtained from the Korean Cell Line Bank (KCLB, South Korea), were grown in RPMI-1640 medium (Thermo Scientific, USA) supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 50 U/ml penicillin and 50 µg/ml streptomycin at 37 °C in a humidified 5% CO₂ incubator. HL-60 cells were induced to undergo differentiation along the neutrophil lineage by seeding aliquots of HL-60 cell suspension (2×10^6 cells/ml) onto tissue culture flasks and growing for 5 days in the presence of 1.25% dimethyl sulfoxide (DMSO). Complete differentiation of HL-60 cells into neutrophil-like cells was confirmed as follows: First, nuclear segmentation and granule formation in cells were analyzed by hematoxylin and eosin staining. Second, cell proliferation was assayed using 0.4% trypan blue exclusion. Third, myeloid cell maturation was determined by spectrophotometry at 540 nm. Fourth, surface expression of differentiation-related antigens was evaluated by flow cytometry using FITC-conjugated monoclonal antibodies against CD11b, CD16 and CD33 (BD Biosciences, USA). Thereafter, differentiated HL-60 cells were cryopreserved at a concentration of 2×10^6 cells/ml in FBS containing DMSO (10% v/v) until seeding into the endothelial cell channel of the microfluidic device. Third-passage, cryopreserved human dermal microvascular endothelial cells (hMVECs), endothelial cell basal medium-2 (EBM-2), and media supplements in the form of EGM-2-MV SingleQuots containing human epidermal growth factor (hEGF), hydrocortisone, gentamicin, vascular endothelial growth factor (VEGF), human fibroblast growth factor-basic (hFGF-B), R³-insulin-like growth factor-1 (R³-IGF-1), ascorbic acid, heparin and FBS were obtained from Lonza (USA). HMVECs were expanded by growing in microvascular endothelial growth media (EGM-2MV, mixture of EBM-2 and EGM-2-MV SingleQuots; Lonza, USA) on tissue culture flasks for no more than five passages. These hMVECs were then cryopreserved at a concentration of 1×10^6 cells/ml in EGM-2-MV containing DMSO (10% v/v) and FBS (10% v/v) until use.

Microfluidic device fabrication and preparation

The microfluidic device was fabricated by soft lithography. Briefly, SU-8-100 photoresist (MicroChem, USA) was spin-coated onto a 4-inch silicon wafer. The wafer was then baked on a hotplate at 95 °C for 1 hour. The microfluidic channels were patterned by UV photolithography. The UV-exposed pattern was developed in PGMEA photoresist developer (MicroChem, USA). A PDMS solution containing Sylgard 184 silicone

elastomer base and curing agent (weight ratio, 10:1; Dow Corning, USA) was cured on the patterned wafer at 80 °C for 1 hour in an oven. Inlet and outlet ports of endothelial cell and side channels were then opened with a 4-mm biopsy punch; injection ports for gel filling were opened with a 1-mm biopsy punch. After autoclaving the PDMS channel component and the glass coverslip (24 x 24 mm; Paul Marienfeld, Germany), the channel side of the PDMS component was irreversibly bonded to the coverslip using oxygen plasma (CUTE; Femtoscience, South Korea). Next, 60 µl of a 1-mg/ml poly-D-lysine (PDL, MW: 30,000–70,000; Sigma-Aldrich, USA) solution in distilled deionized water (DDW) was immediately pipetted into the bonded device, after which, the device was placed in the incubator for 4 hours. After washing away the excess PDL, the device was dried at 80 °C in an oven for more than 24 hours. A solution of Type I collagen (BD Biosciences, USA), prepared by dilution in a mixture of 10X phosphate buffered saline (PBS; Thermo Scientific, USA) and DDW, was injected into gel channels and gelated in the incubator for 30 minutes. The pH of collagen solutions was adjusted with a 0.5N NaOH solution. Prior to seeding hMVECs into the endothelial cell channel, all channels were filled with EGM2-MV.

Numerical characterization of chemoattractants transport

The concentration profiles of chemoattractants in the device were illustrated by generating computational models using COMSOL Multiphysics 4.3 (COMSOL, Sweden) software. The following diffusion coefficients were assumed: fMLP (400 kDa) and IL-8 (1 kDa) in endothelial cell growth medium, 4.2×10^{-10} and 8.4×10^{-11} m²/s, respectively; fMLP in the collagen gel and endothelial cell monolayer, 6.99×10^{-11} and 9.55×10^{-11} m²/s, respectively; and IL-8 in the collagen gel and endothelial cell monolayer, 6.75×10^{-11} and 6.32×10^{-12} m²/s, respectively [S1-S7]. The transient diffusion profiles of chemoattractants in the device from 0 to 12 hours were estimated by Fick's second law. The boundaries of the device were set to no-mass-flow condition.

Measurement of endothelial permeability

To determine endothelial permeability, FITC-dextran (10-kDa, 10 µmol/L in growth media) is added to the center channel of the device at 4 days after EC seeding for confluent monolayer, and the device was incubated in a 37 °C incubator for 3h. Fluorescent image was acquired at 3h after the FITC-dextran solution adding. Image analysis was carried out using ImageJ software to estimate EC permeability. The diffusion coefficient of 10-kDa FITC-dextran in the collagen gel was assumed to be 6.75×10^{-11} m²/s [S1-S4]. The governing equation for concentration distribution of the FITC-dextran solution is Fick's first law:

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

where, J is flux, D is diffusion coefficient and x is the position.

Fick's first law gives rise to the following formula:

$$Flux = -P\Delta C$$

where, P is permeability and C is concentration.

Culturing endothelial cells in a microchannel

Before seeding hMVECs into the endothelial cell channel, the vial of cryopreserved hMVECs was thawed in a water bath. The cells were then seeded into a T75 tissue culture flask, and incubated at 37 °C in a humidified CO₂ incubator until the cells reached 80% confluence. Growth medium was changed every 48 hours. hMVECs were detached by treating with a 0.05% trypsin/1 mM EDTA solution and prepared at a density of 2×10^6 cells/ml. The center endothelial cell channel was filled with 50 µl of this cell suspension, and the device was placed in a 37 °C incubator for 30 minutes to allow cell attachment. Thereafter, non-adherent cells and cell debris was removed by placing 50-µl media droplets at all inlets and outlets of the microfluidic channels. A confluent endothelial cell monolayer in the microfluidic channel was obtained by culturing hMVECs for 4 days. The growth medium in all microfluidic channels was replaced daily with EGM2-MV.

Seeding neutrophils into the endothelial cell channel

A cryovial of dHL-60 cells was quickly thawed in a 37 °C water bath. The dHL-60 cell suspension was prepared at a density of 1×10^6 cells/ml, and 100 µl of this cell suspension was added to the endothelial cell channel. Then, 100-µl media droplets containing fMLP or IL-8 were added to the side channels.

Scanning electron microscopic imaging of collagen gels

Collagen solutions (2.0 mg/ml; pH 5.0, 7.4, and 11) were gelled in a humidified incubator at 37 °C for 30 minutes. These gelled collagens were then fixed with 4% paraformaldehyde for 15 minutes. Thereafter, the collagen gels were dehydrated serially in 70% ethanol (1 hour), 90% ethanol (1 hour) and 100% ethanol (2 days), and air dried in a clean bench (3 days). The dehydrated gels were coated with platinum for 1 minute. The

specimens were examined at 10 kV in a field emission scanning electron microscope (FE-SEM, Nova 230; FEI Company, USA).

Image processing

All images were processed and composites were made using the Adobe Design Premium CS5 software package. When necessary, only contrast and brightness of the original images were minimally adjusted; all adjustments were within the linear range.

ESI References

- S1. M. Shaw and A. Schy, *Biophysical journal*, 1981, **34**, 375-381
- S2. V. Shenoy and J. Rosenblatt, *Macromolecules*, 1995, **28**, 8751-8758.
- S3. S. Ramanujan, A. Pluen, T. D. McKee, E. B. Brown, Y. Boucher and R. K. Jain, *Biophysical journal*, 2002, **83**, 1650-1660.
- S4. B. M. Gillette, J. A. Jensen, M. Wang, J. Tchao and S. K. Sia, *Advanced Materials*, 2010, **22**, 686-691.
- S5. A. Siflinger-Birnboim, P. J. del Vecchio, J. A. Cooper, F. A. Blumenstock, J. M. Shepard and A. B. Malik, *Journal of cellular physiology*, 1987, **132**, 111-117.
- S6. B. Eaton, V. Toothill, H. Davies, J. Pearson and G. Mann, *Journal of cellular physiology*, 1991, **149**, 88-99.
- S7. M. Plank, B. Sleeman and P. Jones, *Growth factors*, 2004, **22**, 1-11.