

Supporting Information

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SI Text

SI Materials and Methods. Supplementary cell culture methods.

The human mammary epithelial cell line MCF10A (Brugge Lab, Harvard Medical School, Boston, MA) was cultured as described previously (1) and grown until 70% confluence, before seeded inside the three-dimensional (3D) ECM as described in the section of macrophage permeability measurements. All tumor, macrophage, epithelial, and endothelial cells used were from fresh frozen stocks and the macrophage and tumor cells were passed less than six times, whereas the endothelial cells (EC) less than three times.

Live cell imaging and immunofluorescent staining. For live cell imaging, microvascular endothelial cells (MVEC) and human umbilical vein endothelial cells (HUVEC) were stained with 5 μ M green cell tracker 5-chloromethylfluorescein diacetate (CMFDA) (Invitrogen) and mCherry-HT1080 cells were constructed using an in-lab modified murine stem cell virus (pMSCV) (Clontech) vector including the mCherry sequence. For the endothelial barrier function measurements confocal image stacks (slice thickness 7 μ m) were captured every 60 min with a Leica TCSP5 confocal microscope and a 10 \times (N.A. = 0.3) objective. For the tumor-endothelial interaction assays time-lapse confocal image z-stacks of fibrosarcoma and endothelial cells were acquired simultaneously with the dextran (10 kDa dextran with a similar molecular weight to EGF) fluorescent intensity data. In the tumor-endothelial interaction, intravasation, and macrophage polarization studies, devices were fixed with 4% Paraformaldehyde and stained for DAPI (Invitrogen), Alexa647 phalloidin (Invitrogen), or PE/Cy5 anti-mouse CD11b (Clone M1/70; BioLegend) and rabbit anti-human VE-cadherin (polyclonal; Alexis Biochemical) detected with a secondary Alex568 anti-rabbit antibody (Invitrogen). For the macrophage characterization experiments in the devices we used the rat anti-mouse CD206 (Clone MR5D3; Santa Cruz Biotech) and rat anti-IL12 (Clone C17.8; eBiosciences) antibodies, detected with a secondary goat anti-rat FITC (Santa Cruz Biotech). We used a 63 \times (N.A. = 1.2) objective to acquire confocal image stacks (slice thickness of 2.5 μ m) for at least 10 hydrogel ECM regions per device.

Quantifying endothelial monolayer barrier function: Experiments and simulations. Fluorescent dextrans [10 kDa Cascade Blue and 70 kDa Texas Red (Invitrogen)] were mixed with culture medium at a concentration of 12.5 μ g/mL. The quantification framework included a computational model of growth-factor transport in the device for predicting the concentration profiles within the device and confirming the method used to analyze experimental data. Imaging of the concentration profiles was performed in real-time and analysis of dextran transport was performed in MATLAB (MathWorks). The diffusive permeability coefficient, P_D of the endothelial barrier was computed using

$$P_D = \beta \cdot D \frac{dC/dx}{\Delta C},$$

where C is the dextran concentration (proportional to fluorescence intensity), ΔC the step drop in concentration across the monolayer, β is an area correction factor, dC/dx the slope of the concentration profile, and D the dextran diffusion coefficient inside the ECM.

The equation for P_D was derived by considering mass conservation inside the ECM gel region at steady state, where the total

dextran flux $P_D \cdot \Delta C \cdot A_{\text{MONOLAYER}}$ across the endothelial monolayer into the gel region is equal to the dextran outflux $D \cdot dC/dx \cdot A_{\text{GEL}}$. The area correction factor mentioned in the main text is given by $\beta = A_{\text{GEL}}/A_{\text{MONOLAYER}}$ and accounts for the linear changes in cross-sectional area along the direction of dextran transport. The diffusion coefficients of the 10 kDa and 70 kDa dextrans were assumed to be 9×10^{-11} m²/s and 4.5×10^{-11} m²/s, based on a scaling law for soluble factor diffusion in buffer solutions (D_{BUFFER}) (2). These values are very similar ($D_{\text{GEL}}/D_{\text{BUFFER}} \sim 0.94$) to the diffusion coefficients (D_{GEL}) in 2.5 mg/mL collagen gels, that have a very large pore size compared to the hydrodynamic radius of the dextrans (3).

The raw fluorescent intensity images were analyzed in MATLAB to compute the concentration gradient (dC/dx) and step drop in concentration (ΔC) across the endothelium. To obtain a single value of dC/dx and ΔC for each hydrogel region, we performed averaging across 30 pixel lines over the y-axis. A finite element model was developed in COMSOL (Burlington) for analyzing transport across the endothelial monolayer within the microfluidic device and validating the evaluation framework. Constant concentration boundary conditions were defined at the inlet (C_{SOURCE}) and sink conditions at the outlet (C_{SINK}) of the control channel and at the gel filling port. The numerical grid for performing the simulations consisted of approximately 70,000 finite elements.

To determine the tumor necrosis factor alpha (TNF- α) concentration for impairing endothelial barrier function during tumor cell intravasation, we seeded MVEC on collagen type I ECM and observed the monolayer morphology (Fig. S3), 24 h after stimulation with different concentrations of TNF- α (2 and 20 ng/mL). To minimize the formation of gaps, while still observing a significant change in permeability, we choose to stimulate the monolayers in the microfluidic experiments with 2 ng/mL. A dose-response to TNF- α was also performed (Fig. S14). The endothelial permeability was measured with the method described above, where different doses of TNF- α (0.2, 2, and 20 ng/mL) were applied for 48 h, after a confluent monolayer had formed (48 h after endothelial cell seeding) in the absence of any additional cell type. The reported permeability values were calculated as averages from twelve ($n = 12$) gel regions (treating each gel region as an independent experiment and including data from two devices with six gel regions analyzed per device).

Macrophage cytokine secretion, macrophage polarization, blocking antibody experiments and permeability measurements.

To investigate whether the macrophage-mediated permeability changes are specific to this cell type and whether other cell types could also induce changes in endothelial permeability we performed coculture of tumor (breast carcinoma) and epithelial cells (MCF10A) with the endothelial cells and measured permeability. In these experiments, macrophages, tumor, and epithelial cells were seeded at 0.4×10^6 cells/mL within the 3D hydrogel, similar to the intravasation experiments and allowed interactions for 48 h after the endothelium had formed before measuring endothelial permeability (Fig. S2D). The reported permeability values were calculated as averages from twelve ($n = 12$) gel regions (treating each gel region as an independent experiment and including data from two devices with six gel regions analyzed per device).

We performed immunofluorescence staining in the devices to investigate whether macrophage interactions with tumor cells, endothelial cells and the 3D matrix induce a macrophage polar-

ization to an M1 or M2 state. M1 and M2 states were assessed by staining against IL12 and CD206, respectively (Fig. S4C). We also included positive controls of M1 or M2 phenotype, by treating devices for 48 h with 10 ng/mL LPS (Sigma) and 10 ng/mL IL4 (Peprotech), respectively. To quantify the expression of IL12 and CD206 markers (Fig. S4D) we used ImageJ and measured the mean fluorescent intensity per cell for at least 10 cells per condition using the method described in ref. 4.

Macrophage TNF- α (M1 marker) and IL10 (M2 marker) cytokine secretion measurements (Fig. S4C) were performed in 24-well plates. One hundred thousand RAW264.7 cells were plated in each well and stimulated with 10 ng/mL IL4 or 10 ng/mL LPS to induce polarization to M1 or M2 phenotypes, respectively. Culture medium supernatant was collected 24 h after cell seeding and was centrifuged and stored at -80°C prior to cytokine measurement. To detect the absolute concentrations of TNF- α and IL10 we used the Bio-Plex bead-based cytokine array (Bio-Rad) and performed experiments in duplicate. To establish whether the macrophage secreted TNF- α facilitates intravasation, we performed blocking antibody experiments against soluble TNF- α (polyclonal; R&D Systems) and an IgG control antibody (R&D Systems). The blocking antibody concentration was selected to be 2 $\mu\text{g}/\text{mL}$ (10 higher than the ND_{50} neutralization dose).

Tumor cell intravasation assay. We formed a 3D ECM (2.5 mg/mL collagen type I) seeded with breast carcinoma cells (0.8×10^6 cells/mL) in the presence or absence of macrophages (0.4×10^6 cells/mL). Twenty-four hours after the cell-ECM mixture was injected in the devices we seeded MVEC or HUVEC (density 2×10^6 cells/mL), that were allowed to form an endothelial monolayer for 48 h. A 20 ng/mL per mm EGF gradient was established in all experiments, and in the devices with macrophage embedded in the ECM we also seeded macrophages (0.25×10^6 cells/mL) in the endothelial channel. The cells were allowed to interact for 48 h, after which fixation, staining, and imaging were performed. Image stacks were visualized using Imaris, and we quantified the number of tumor cells in contact (distance $< 10 \mu\text{m}$) with the endothelium and the cells that were located on the apical endothelial side. Intravasated tumor cell metrics represent the total number of tumor cells scored for $n = 3$ independent devices (approximately 100 cells/device). Live cell imaging was also performed to confirm the occurrence of intravasation in real time. To quantify macrophage localization, we counted the total

number of macrophages in subluminal and luminal endothelial spaces, per hydrogel region ($300 \times 300 \times 120 \mu\text{m}^3$).

Tumor-endothelial cell interactions assay. HT1080 cells were seeded in the tumor channel by flowing 60 μL of a 3×10^6 cells/mL cell suspension and were allowed to invade for 3 d into the 3D ECM (2.5 mg/mL collagen type I). Subsequently, a confluent endothelial monolayer was formed on the 3D ECM-endothelial channel interface by flowing 60 μL of a 2×10^6 cells/mL cell suspension through the endothelial channel and allowing 48 h for adhesion and growth. Next, we performed live cell imaging and established a 20 ng/mL per mm EGF gradient across the 3D ECM, while the endothelium was stimulated with 2 ng/mL TNF- α (when noted), by adding the EGF and TNF- α solution to the endothelial channel only. Images were analyzed using Imaris (Bitplane) to identify the ECM-endothelial channel interface and track tumor cell centroids, as described in the next section.

Quantifying intravasation metrics. We used the cell tracking algorithm in Imaris to capture the x, y coordinates of the endothelial monolayer interface and the tumor cell centroids. These coordinates were in turn analyzed with a custom-written MATLAB script to automatically identify the distance of each tumor cell from the monolayer. To characterize the tumor-endothelial interactions during intravasation, we computed the number of tumor cells that have migrated beyond the ECM-endothelial channel interface over 10 h. To analyze the dynamics of tumor-endothelial interactions we computed the time required for each tumor cell to migrate across a total distance of 60 μm across the ECM-endothelial channel interface. We also characterized the migration dynamics of tumor cells inside the 3D ECM hydrogel by calculating the average migration speed of each tumor cell (ratio of total distance migrated to total time observed).

Data analysis and quantification. All intravasation metrics were calculated by averaging the mean values of at least three microfluidic devices, with each device representing one independent experiment. This mean value for each device was calculated as an average of at least 10 hydrogel ECM regions, which was equivalent to scoring at least 50 tumor cells per device. Student's t tests (two-sample, $P < 0.05$) and correlation analysis were performed in MATLAB.

1. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS (2001) ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol* 3:785–792.
2. Gribbon P, Hardingham TE (1998) Macromolecular diffusion of biological polymers measured by confocal fluorescence recovery after photobleaching. *Biophys J* 75:1032–1039.
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4. Burgess A, et al. (2010) Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proc Natl Acad Sci USA* 107:12564–12569.

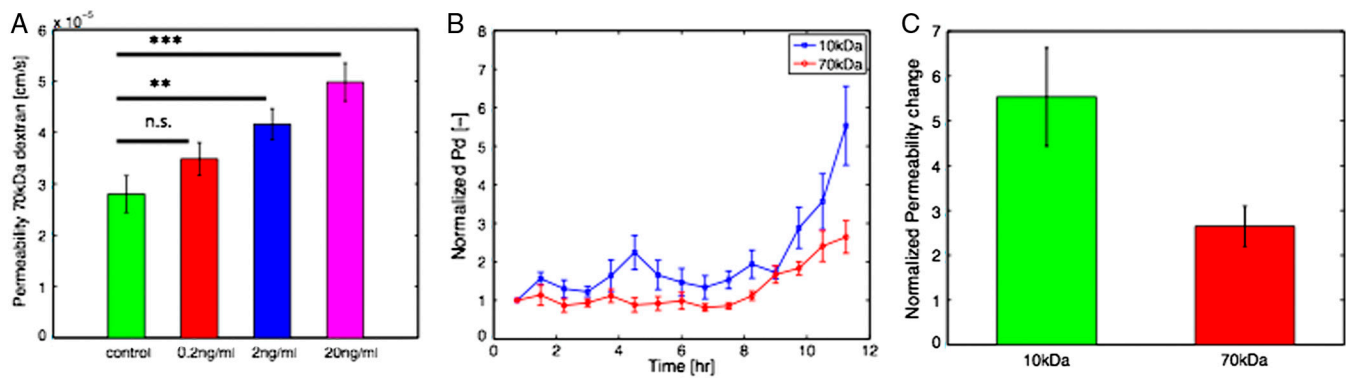


Fig. S1. Endothelial barrier function response to TNF- α stimulation. (A) Endothelial monolayers were formed in the device and stimulated for 48 h with different doses (0, 0.2, 2, and 20 ng/mL) of TNF- α , after which permeability was measured. Stimulation with 2 ng/mL and 20 ng/mL resulted in statistically significant changes compared with the control ($P = 0.008$ and $P = 3 \times 10^{-4}$), whereas stimulation with 0.2 ng/mL did not result in statistically significant changes ($P = 0.16$). Error bars represent SEM for 12 regions. (B) Real-time measurement of diffusive endothelial (MVEC) permeability for 10 (blue) and 70 kDa dextrans (red), after TNF- α (2 ng/mL) stimulation at $t = 0$ h. Measurements taken at 1 h, after steady-state concentration profiles were established. Permeability values were normalized with the initial value at $t = 1$ h. Error bars represent SEM for 10 hydrogel regions in a single device. (C) Quantification of endothelial permeability after 12 h of 2 ng/mL TNF- α stimulation for 10 and 70 kDa dextrans. Normalization method and error bars are the same as in C.

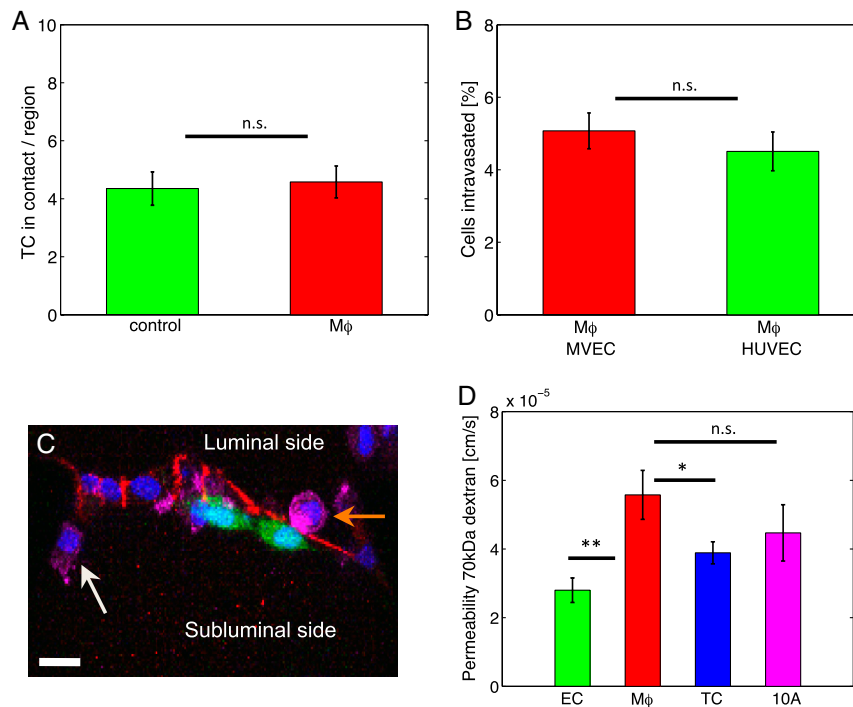


Fig. S2. Tumor cells in contact, effect of endothelial cell origin, and macrophage localization. (A) Number of tumor cells (TC) in contact with the endothelium (HUVEC) within each 3D ECM region for the intravasation assay. (B) Effect of endothelial cell origin (MVEC and HUVEC) on the number of TC that have intravasated. (C) Breast carcinoma cell (green) interacting with the endothelial monolayer (red, VE-cadherin), in the presence of macrophages (magenta) in the luminal (orange arrow) and subluminal (white arrow) endothelial sides. (Scale bar: 30 μ m.) Blue, DAPI. (D) Macrophage (M ϕ), tumor (TC), and epithelial (10A) cells were seeded in the 3D ECM and interacted with the endothelial cells (EC) for 48 h prior to permeability measurements. The presence of macrophages resulted in a 1.99-fold increase ($P = 0.002$), a 1.43-fold increase ($P = 0.049$), and a 1.25-fold increase ($P = 0.36$) compared with EC, TC, and 10A conditions, respectively. Error bars represent SEM.

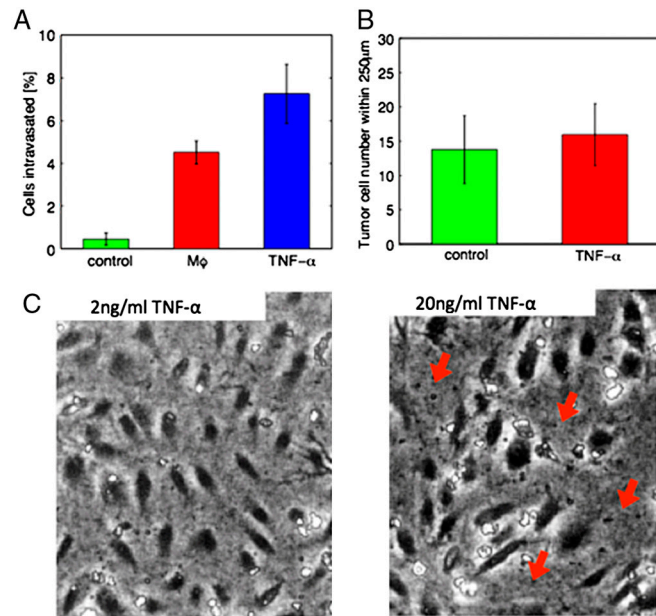
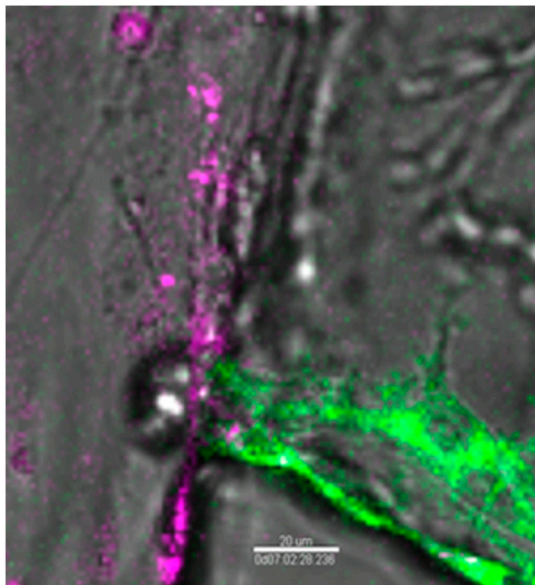


Fig. S3. TNF- α effects on intravasation and endothelial monolayer confluency. (A) Effect of TNF- α on tumor cell intravasation across HUVEC monolayers. (B) Number of tumor cells within 250 μ m from endothelial (MVEC) monolayer for the tumor-endothelial interaction assay. (C) Titration of TNF- α concentration for ensuring confluent endothelial (MVEC) monolayer (red arrows show gaps in endothelial monolayer).



Movie S1. Real-time movie of breast carcinoma cell intravasation. Human breast carcinoma cells (green) migrating (from the *Right* to *Left* of the image) toward an endothelial (HUVEC) barrier and intravasating in the presence of RAW264.7 macrophages. Each cell type is labeled in the movie. Total movie duration: 12 h, time-step: 1 h. (Scale bar: 20 μm.)

[Movie S1 \(MOV\)](#)