

SUPPLEMENTARY MATERIALS AND METHODS

Numerical simulations for the validation of the platform: evaluation of wall shear stress distribution on 3D reconstructions of the porous filter

The wall shear stress caused by the flow on the endothelial cell layer, laying on the porous filter at the inner side of the flow chamber, was estimated by an *in silico* model. The model was set in ANSYS.14 Workbench (ANSYS, Inc., Canonsburg, PA, USA) and the CFD simulations were run with Fluent, which allows the solution of the Navier–Stokes equations using a finite volume method. Steady-state momentum and continuity equations were solved for the fluid flowing in the flow channel. In order to avoid prescribing boundary conditions too close to the area of interest, an extension of the flow channel ($L = 10$ mm) was created normal to the inlet cross-section. Both the opposite inlet/outlet cross-sections were alternatively extended to account for different flow directions. A uniform, normal velocity profile was prescribed at the inlet of the extension. The velocity profile along the flow channel developed into a parabolic-shape velocity profile. A no-slip boundary condition was applied at both the walls of the channel and the filter surface while a uniform pressure condition ($p = 0$) was set at the outlet of the flow channel.

The computational grid of the channel was validated by a sensitivity study to guarantee the independence of the solution from the grid configuration. It was made of about 7×10^5 hexahedral cells for the flow channel and 3×10^5 cells for the extension. The height of the channel was discretized by 12 cells.

The regime was strictly laminar, characterized by a calculated Reynolds number of approximately 3 (160 $\mu\text{L}/\text{min}$), 6 (320 $\mu\text{L}/\text{min}$) and 20 (1060 $\mu\text{L}/\text{min}$). Therefore, no substantial differences were expected between the wall shear stress generated on the membrane by the flow from opposite inlets.

The low Reynolds regime provided a linear correlation between the volume flow rate and the local wall shear stress on the filter. The wall shear stress was then normalized with reference to the value of the wall shear stress τ_w in a planar parallel plate flow chamber for a given flow rate (**Eq. 1a**).

$$\tau_w = \frac{6 \mu v}{s} \quad \text{Eq. 1a}$$

where μ is the fluid viscosity, v is the average velocity, and s is the chamber height. In a standard parallel-plate chamber such a value is uniform along the horizontal surfaces, except for a narrow region in the proximity of the lateral walls, where the fluid velocity decreases to zero. It is worth noting that in the case of non-planar filter surface the actual value of the wall shear stress depends on the global configuration of the filter, that affects not only the local height but also the distribution of the fluid along the flow chamber section.

The test area is defined as a region of the filter surface where the flow is not affected by the presence of lateral gasket or inlet/outlet section and it includes the biological observation field. Based on results of prior simulations, the region shows an area of $\sim 13.9 \text{ mm}^2$, 0.35 mm from lateral gasket and 1.4 mm from inlet/outlet sections.

Numerical simulations for the validation of the platform: estimation of chemokine gradient in the multilayer chamber.

Steady-state, Navier-Stokes momentum and continuity equations for incompressible fluids (**Eq. 2a,b**) were solved for the fluid domain. Subsequently, the time-dependent diffusion-advection equation (**Eq. 2c**) was solved with an uncoupled approach.

$$-\nabla P + \mu \nabla^2 v = \rho(v \cdot \nabla) v \quad \text{Eq. 2a}$$

$$\nabla \cdot v = 0 \quad \text{Eq. 2b}$$

$$\frac{\partial c}{\partial t} + (v \cdot \nabla) c = D \nabla^2 c \quad \text{Eq. 2c}$$

where P is the pressure, v the velocity of the fluid, c the concentration and D the diffusion coefficient of the chemokine. The model was set in ANSYS.14 Workbench (ANSYS, Inc., Canonsburg, PA, USA) and the CFD simulations were run with Fluent.

The Darcy law (**Eq. 2d**) was used to describe the flow in agarose ($k = 9,26 \times 10^{-16} \text{ m}^2$) and extracellular matrix ($k = 1,49 \times 10^{-14} \text{ m}^2$) compartments, which were modeled as porous media (porosity 95%)

$$\nabla P = -\frac{\mu \vec{v}}{k} \quad \text{Eq. 2d}$$

where P is the pressure, μ [Pa·s] is the viscosity of the fluid, k [m²] is the permeability of the porous medium and v [m/s] the velocity of the fluid.

With regard to the laminar steady-state flow model, a parabolic velocity profile was set at the inlet cross-section, providing a total flow rate of 320 $\mu\text{L}/\text{min}$. No-slip conditions ($v = 0$) were set at the top of the flow channel, at the upper and lower surfaces of the filter and at the bottom of the agarose compartment. A uniform pressure condition ($P = 0$) was set at the outlet. All lateral surfaces were set as symmetries, i.e. with normal velocity components and their first derivative set to zero.

In the case of absence of flow the fluid velocity was set to zero everywhere and the simulation involved time-dependent diffusion only.

With regard to the advection-diffusion time-dependent model, the concentration of chemokine in both the agarose and the extracellular matrix was set to the equilibrium condition of $c(t_0) = 1 \times 10^{-6}$ g/mL, while that in the flow channel were set as null. This condition mimics that reached by diffusion during the polymerization of the extracellular matrix ($c = 0$) on the agarose layer ($c = 2 \times 10^{-6}$ g/mL) and the device set up (flow rate = 0 $\mu\text{L}/\text{min}$, 45 min). Indeed a further simulation (not shown) indicates that the time to reach this condition (the average concentration of chemokine in the collagen layer equal to $0,999 \times 10^{-6}$ g/ml) is about 14 min. The top of the flow channel, the upper and lower surfaces of the filter, the bottom of the agarose compartment and all the lateral surfaces were set as zero-gradient (i.e. zero-flux). The solute reaches the top channel by advection and diffusion across the cylindrical channels, inserted to reproduce the presence of the through porosity. The diffusion and convection of chemokine in the multilayer chamber were simulated for 45 min, under a steady-state flow of 320 $\mu\text{L}/\text{min}$, in the presence and absence of flow. All these conditions were pre-set in order to reproduce the most critical experimental scenario, which corresponds to the fastest depletion of chemokine in time.

The geometry was discretized with a tetrahedral mesh, which turns into a hexahedral one in the volume correspondent to the filter pores.

The gradient $\Delta C/\Delta Z$ was calculated through interpolation of concentration values available at the grid points.

The difference in concentration across filter and collagen matrix is calculated as the difference between the concentration at the bottom and the top of the filter, and between the bottom and the top of the collagen matrix (in contact with the filter).

Cell treatment with inhibitors

To inhibit Gi protein signals neutrophils were treated with 100 ng/mL pertussis toxin (PTX, Sigma-Aldrich) for 16 hours.

Neutrophil Romanowski staining

Cells were stained with the “Diff-Quick” stain kit following manufacturer’s instructions (“Diff-Quick” kit; IHC World).

Flow cytometry

For flow cytometric analysis, murine neutrophils or endothelial cells were labeled on ice with primary specific antibodies for 20 min (anti-mouse Gr-1, F4/80, CD117 antibodies are from AbD Serotec; anti-mouse CD11b, E-selectin antibodies are from BD Pharmingen™ - BD Biosciences; anti-mouse CXCR2 and VCAM are from R&D Systems, anti-mouse PECAM1 antibody (MEC13.3) was produced in the lab) followed by incubation with FITC-conjugated secondary antibodies for 30 min (Sigma-Aldrich). Cells were then washed and analyzed on a BD FACSCanto™ flow cytometer (BD Biosciences). Data analysis was performed with FlowJo Software (Tree Star, Inc).

Neutrophil staining with fluorescent dyes

Murine neutrophils were harvested by centrifugation, resuspended in prewarmed CellTracker™ Green CMFDA or Orange CMRA (Molecular Probes - Invitrogen) solution (final concentration 0.5 μ M in HBSS) and incubated for 20 minutes at 37 °C in a water bath. The dye solution was replaced by complete culture medium and cells were incubated for additional 30 minutes at 37 °C. Upon harvesting

and washing by centrifugation, cells were resuspended in working medium at a concentration of 3×10^6 cells/mL.

Preparation of collagen I solution

Rat tail collagen I solution (1.6 mg/mL final concentration; BD Biosciences) was prepared following manufacturer's instructions using reagents kept at 4°C.

Culture of endothelial cells onto porous filters

bEnd.3 endothelial cells were seeded onto polycarbonate filters (Millipore®) pre-coated with 1.2 - 1.8 mg/mL Reduced Growth Factor Membrane Basement Matrix (Geltrex™; GIBCO – Invitrogen) and were cultured for two days in order to form confluent monolayers. Cells were then stimulated for 20 hours with 20 ng/mL Tumor Necrosis Factor alpha (TNF alpha; home-made recombinant pro-inflammatory cytokine, a kind gift from Angelo Corti) and coated for 15 minutes at 37 °C with 2 µg/mL KC (R&D Systems) prior to the assay in order to facilitate leukocyte arrest and subsequent transmigration.

Endothelial cell staining with fluorescent dyes

Endothelial cells plated onto filters and treated with TNF alpha were washed, prewarmed CellTracker™ Green CMFDA (Molecular Probes - Invitrogen) solution (final concentration 0.5 µM in HBSS) was added and cells were incubated for 25 minutes at 37 °C. The dye solution was replaced by complete culture medium and cells were incubated for additional 30 minutes at 37 °C. Finally, working medium was added to the stained cells.

***In vitro* assays to monitor the endothelial cell layer before and after flow application**

To assess the endothelial cell layer's morphology, phase contrast (PC) and fluorescence images of Cell Tracker Green-labeled endothelial cells grown to confluency were taken both on the plate and the filter. Image acquisition was performed by using an Inverted Laboratory Microscope with LED Illumination - Leica DM IL LED - (objective 20X).

The polycarbonate filter coated with green-labeled endothelial cells was then assembled into the chamber. Time-lapse images of endothelial cells onto the

filter were acquired in the absence of flow (pre-flow) and flow was then initiated. Myeloid precursors stained with CellTracker™ Orange, indeed, were perfused through the flow chamber at increasing flow rates: 160 $\mu\text{L}/\text{min}$ (0.12 Pa expected shear stress) for 2 minutes, 320 $\mu\text{L}/\text{min}$ (0.24 Pa expected shear stress) for 2 minutes and 1060 $\mu\text{L}/\text{min}$ (0.8 Pa expected shear stress) till the end of the acquisition. Time-lapse imaging of the same observation field as in the absence of flow was performed (objective 20X).

For further analysis of the endothelial layer, fixation and permeabilization solutions followed by TRITC-Phalloidin and Hoechst were perfused through the flow chamber to detect (F)-actin (red) and nuclei (blue), respectively. The endothelial cell layer in the absence of flow (pre-flow) was similarly stained and imaged (objective 40X).

Movie analysis software

The cell movement analysis is made up of three steps: I) cell recognition, II) track reconstruction and III) track clustering.

I) Cell recognition

Each frame of the acquired video is first correlated with a Gaussian kernel and then binarized in order to identify only those spots that can be identified as cells moving in front of the camera, with respect either to the spot shape or its intensity. Each cell/spot is localized by means of the x- and y- coordinates of its centroid. The output of this step is a disordered list of two-dimensional (2D) points for each frame.

II) Track reconstruction

Once all the frames are analyzed, each cell movement path is reconstructed by “following” the point movement frame by frame. Starting from frame i , an initial estimate of the position of the cell in frame $i+1$ is made by selecting the nearest calculated point. In case of uncertainties, like when two cells “collapse” into one spot and then “divide” again or when a cell “disappears” in a frame and then “reappears” in the same position after a few frames, simple assumptions are used:

- the paths followed by the cells cannot make sharp bends, as the movement is determined by the shear flow: if two tracks intersect each other, the corresponding cells are considered moving along straight paths.
- if a cell is moving quickly and within two consecutive frames traverses the image, the two identified points are considered belonging to the same track if the second one is localized along the main direction of the cell movement, otherwise two different tracks are registered.
- if a cell “disappears” in the frame i and then appears again after two or more frames, the two points are considered belonging to two different cells and, consequently, two different tracks.

III) Track clustering

Once all the tracks are identified as a list of 2D points each, the classification of the paths completes the analysis procedure. The cells are divided into categories as described above with respect to the cell speed modulus along the main direction of the cell movement:

$$v = \frac{\vec{x}_{i+1} - \vec{x}_i}{\Delta t} \cdot \vec{n}$$

where v is the instantaneous speed, \vec{x} is the absolute position of the spot centroid in the frame, Δt is the time step between two consecutive frames and \vec{n} is the unit vector defining the main direction of the cell movement. The direction unit vector is considered in order to exclude transverse or backwards movements that may be registered between two frames due to small variations in the spot area identification that may generate errors in the cell centroid calculation. Once the cell speed is calculated, this is classified in one of the aforementioned classes by simply comparing its values frame by frame. Moreover, the cells considered as “arrested” may be further clustered with respect to the duration of the connection with the substrate.

Considering the variability of the cell behavior among the test conditions, the parameters that control the described steps may be tuned through a simple user interface that shows as well the original and the elaborated movies. In particular, the tunable parameters are divided into:

1. Track parameters

- Threshold: upper and lower bounds for the binarization of the frame after the correlation with the Gaussian kernel
- Moving tolerance: search radius to select nearby points for track reconstruction
- Cell radius: minimum cell radius; spots with smaller radius are not considered for the analysis
- Lenses: drop-down menu to select acquisition lens type (10x or 20x); used for setting the right $\mu\text{m}/\text{pixel}$ ratio

2. Speed parameters

- Adhesion speed: maximum speed for cell adhesion on the substrate
- Rolling speed: maximum speed for cell rolling on the substrate

3. Time parameters:

- Transient adhesion: upper and lower time limit for cell transient adhesion on the substrate
- Arrest 1/2/3: minimum time limits for the three arrest classes

Moreover, it is possible to select if a “slow rolling” (i.e. cell rolling at speeds lower than the adhesion one) must be considered in the analysis or not.

Tracks generated by varying the flow rates during the acquisition (e.g. when assessing resistance to detachment at increasing flow rates) can be analyzed independently by the software and shown cumulatively at the end of the analysis in a spreadsheet.

Statistical analysis

Paired or unpaired two-tailed Student t test and two-way ANOVA were calculated for comparison between groups and curves respectively. P values less than 0.05 were considered statistically significant.

Data were analyzed using Graphpad Prism (Graphpad Software, San Diego, CA).

SUPPLEMENTARY EXPERIMENTAL REFERENCES

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2. S. Ramanujan, A. Pluen, T. D. McKee, E. B. Brown, Y. Boucher and R. K. Jain, *Biophysical journal*, 2002, 83, 1650-1660.

SUPPLEMENTARY FIGURE LEGENDS

Fig. S1: Cellular model: FACS analysis of neutrophil and macrophage surface markers expressed by myeloid precursor and differentiated neutrophils

Fig. S2: Characterization of endothelial cells: (a) FACS analysis of surface markers expressed by bEnd.3 endothelial cells pre-treated or not with TNF alpha. (b) Phase contrast (PC) and fluorescence images (Cell Tracker Green) of endothelial cells grown to confluency both on the plate and the filter. Arrows indicate some filter pores. Scale bar, 50 μm . (c) Confocal images of endothelial cells grown onto the filter prior to or post increasing flow application (see Supplementary materials and methods for details). Endothelial cells labeled with Cell Tracker Green are incubated with Phalloidin and Hoechst to detect F actin (red) and nuclei (blue), respectively. Scale bar, 20 μm .

Fig. S3: Effect of pertussis toxin (PTX)-mediated inactivation of $G_{\alpha i}$ protein on neutrophil extravasation and interstitial migration. (a,b) Comparative analysis of control and PTX-treated neutrophils for the ability to resist shear-induced detachment from endothelial cells. Representative fluorescent images (a) of neutrophils settled onto endothelial cells under a defined shear stress (0.12 Pa expected shear stress, 0 s), which are subjected to increased shear stress (0.24 Pa expected shear stress, 30 s and 60 s). Arrows indicate settled cells detaching under increased shear stress (0.24 Pa). Scale bar, 50 μm . Quantitative analysis of cells detaching under increased flow rate (320 $\mu\text{L}/\text{min}$, 0.24 Pa expected shear stress) for 1 min (b). $*P < 0.05$ (Paired student t test). Data are expressed as mean \pm s.e.m. of three independent experiments. (c) Quantification of neutrophil interstitial migration: xz and xy track vectors.

SUPPLEMENTARY MOVIE LEGENDS

Movie 1: Imaging of endothelial cells prior to application of flow. Endothelial cells grown onto the filter, which is observable in the phase contrast image, were labeled with Cell Tracker Green and imaged prior to flow application (pre-flow).

Movie 2: Imaging of endothelial cells during flow application in the same observation field as in the absence of flow. Endothelial cells grown onto the filter, which is observable in the phase contrast image, were labeled with Cell Tracker Green and imaged during increasing flow application (160 $\mu\text{L}/\text{min}$ for 2 minutes, 320 $\mu\text{L}/\text{min}$ for 2 minutes and 1060 $\mu\text{L}/\text{min}$ till the end of the acquisition). At the end of the time-lapse acquisition, images of the same observation field were taken upon perfusion of the chamber with fixation and permeabilization solutions followed by phalloidin and Hoechst to stain F-actin (red) and nuclei (blue) respectively.

Movie 3: Bi-dimensional imaging of neutrophil interactions with endothelial cells during the multistep extravasation process. Green- and red-labeled primary mouse neutrophils were compared for the ability to roll and adhere onto endothelial cells under a 160 $\mu\text{L}/\text{min}$ flow rate. Total movie duration is 3 minutes. Individual frames were acquired every 1.442 seconds.

Movie 4: Imaging of neutrophil transendothelial migration under flow. Green- and red-labeled neutrophils migrating across endothelial cells under 320 $\mu\text{L}/\text{min}$ flow rate are indicated by arrows. Fluorescence of transmigrating cells noticeably moved out of focus and faded due to their movement underneath the endothelium. Total movie duration is 20 minutes. Individual frames were acquired every 30.286 seconds.

Movie 5: Three-dimensional imaging of neutrophil interstitial migration in the presence of a chemokine gradient (keratinocyte-derived chemokine, KC) through the collagen matrix. Green- and red-labeled neutrophils were compared for the ability to migrate under a 320 $\mu\text{L}/\text{min}$ flow rate. 25 planes (from Top - $z = 0$ -, proximal to the endothelium, to bottom - $z = 24$ -, 100 μm far from the top) along

the z-axis of the collagen matrix were acquired. Z step = 4.17 μm . Total movie duration is 30 minutes. Individual frames were acquired every 34.636 seconds.

Movie 6: Three-dimensional imaging of neutrophil interstitial migration in the absence of a chemokine gradient through the collagen matrix. Green- and red-labeled neutrophils were compared for the ability to migrate under a 320 $\mu\text{L}/\text{min}$ flow rate. 25 planes (from Top - $z = 0$ -, proximal to the endothelium, to bottom - $z = 24$ -, 100 μm far from the top) along the z-axis of the collagen matrix were acquired. Z step = 4.17 μm . Total movie duration is 30 minutes. Individual frames were acquired every 35.541 seconds.

Movie 7: Enlargement of a representative neutrophil migrating interstitially towards a KC gradient within the collagen matrix. 25 planes (from Top - $z = 0$ -, proximal to the endothelium, to bottom - $z = 24$ -, 100 μm far from the top) along the z-axis of the collagen matrix were acquired. Z step = 4.17 μm . Phase contrast and fluorescence acquisitions are shown. Arrows at each time point indicate the migrating neutrophil in the z plane where the cell is in focus, as revealed by its highest fluorescence. Total movie duration is 45 minutes. Individual frames were acquired every 30.286 seconds.

Movie 8: Enlargement of another representative neutrophil migrating interstitially towards a KC gradient within the collagen matrix. 25 planes (from Top - $z = 0$ -, proximal to the endothelium, to bottom - $z = 24$ -, 100 μm far from the top) along the z-axis of the collagen matrix were acquired. Z step = 4.17 μm . Phase contrast and fluorescence acquisitions are shown. Arrows at each time point indicate the migrating neutrophil in the z plane where the cell is in focus, as revealed by its highest fluorescence. Total movie duration is 45 minutes. Individual frames were acquired every 30.286 seconds.

Movie 9: Bi-dimensional imaging of control and pertussis toxin (PTX)-treated neutrophils interacting with endothelial cells during the multistep extravasation process. Control (Green) and PTX-treated (Red) cells were compared for the ability to roll and adhere onto endothelial cells under a 160 $\mu\text{L}/\text{min}$ flow rate.

Total movie duration is 3 minutes. Individual frames were acquired every 1.614 seconds.

Movie 10: Imaging of control and PTX-treated neutrophils migrating across endothelial cells under flow. Control (Green) and PTX-treated (Red) cells arrested to and, in case, migrating across endothelial cells under 320 $\mu\text{L}/\text{min}$ flow rate are indicated by arrows. Fluorescence of transmigrating cells noticeably moved out of focus and faded due to their movement underneath the endothelium. Total movie duration is 6 minutes. Individual frames were acquired every 1.614 seconds.

Movie 11: Three-dimensional imaging of control (Green) and PTX-treated (Red) neutrophils migrating interstitially in the presence of KC. 25 planes (from Top - $z = 0$ -, proximal to the endothelium, to bottom - $z = 24$ -, 100 μm far from the top) along the z -axis of the collagen matrix were acquired. Z step = 4.17 μm . Total movie duration is 31 minutes. Individual frames were acquired every 38.936 seconds.