Supplementary Information

Supplementary Materials and Methods

Media exposure assay

To compare exposure of endothelial and immune cell media, HUVECs were cultured for 48 hours on the chip as described earlier. Then, EGM-2 (endothelial cell media) or RPMI (immune cell media) with or without 200.000 PBMCs was added to the endothelial/immune cell channel. Phosphate buffered saline (PBS, Sigma, USA) or 100ng/ml N-formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma) was added to the chemoattractant channel. Chips were incubated in their respective media for 4 hours on a rocking platform (Mimetas, Netherlands) at a 7° angle with rocking intervals of 8 minutes. After incubation, permeability was measured, and chips were fixed with 4% PFA for immunohistochemistry.

Immunohistochemistry

All steps were carried out at room temperature, except when indicated otherwise. Chips were washed 3 times with PBS, fixed for 12 minutes with 4% PFA (Invitrogen) and washed again 3 times with PBS. Cell permeabilization was performed by incubating 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes. After washing 3 times with PBS, blocking was done for 1 hour with a 2% BSA in PBS solution. Then, primary antibody (anti PECAM-1, Cell Signalling) diluted 1:100 in blocking solution was added and incubated at 4°C overnight. After washing 3 times with PBS, secondary antibody (1:500, anti-mouse 488 or anti-mouse 546, Invitrogen), Hoechst (1:1000, Sigma) and phalloidin (1:150, Cytoskeleton) in blocking buffer were added and incubated for 2 hours. The chips were washed 4 times with PBS and imaged on a widefield microscope (Nikon Ti-E) or a confocal microscope (LSM 710).

Automated analysis – detailed methods description

In the configuration of the infection-on-chip, cells migrate from the endothelial vessel into the hydrogel space. The migration distance is measured as the vertical distance between the cell position and the endothelial barrier. This quantification is achieved via the following procedure. First we detect the hydrogel area. The horizontal boundary between the endothelial channel and the hydrogel appears as a bright line in the phase contrast image. As the channel can be slightly tilted, this bright line is detected by splitting the image into successive vertical stripes, projecting these stripes horizontally, and looking for the coordinates of local maxima in these projections. These coordinates are then fitted with a line using a RANSAC regressor to ignore potential failed boundary detections. Cells are then only detected starting from this boundary plus a margin of 100 pixels. This margin is necessary as non-migrating cells can accumulate close to the boundary making accurate cell detection impossible close to it. Cells are detected using template matching in the phase contrast image. As cells can be slightly out-of-focus, a series of templates is used to detect both in-focus (disk shape) and out-of-focus (disk with a hole shape) cells. All detected cells are aggregated in a mask where each cell is represented by a disk of radius 5 pixels. For each detected cell, the mean intensity in the fluorescence channel is also measured. The template matching procedure can generate false positives by locating defects and dusts on the microfluidics chip. Such false positives have no fluorescence signal and can be discarded by setting a threshold on that signal. This threshold is set as the location of the half-maximum of the background intensity peak. For each detected cell, the vertical distance from the boundary is then measured. These distances are used to generate 2D histograms showing the distributions of cells that have migrated a certain distance in a certain time and the histograms showing the distribution of migration distances at the last frame. The Python analysis pipeline can be found at https://github.com/guiwitz/mfdiffusion and uses

numerous open-source packages including jupyter[1], numpy[2], pandas[3], scikit-image[4], scikit-learn[5], matplotlib[6] and seaborn[7].

List of supplementary videos

Video 1-4: PBMCs perfusion without other cells

- PBS Hyd^{Low}
- fMLP Hyd^{Low}
- PBS Hyd^{High}
- fMLP Hyd^{High}

Video 5: HUVEC endothelial vessel

Video 6-11: PBMCs and HUVECs

- PBS Hyd^{Low}
- fMLP Hyd^{Low}
- PBS Hyd^{Mid}
- fMLP Hyd^{Mid}
- PBS Hyd^{High}
- fMLP Hyd^{High}

Video 12: lung-on-chip 3D image

Video 13-16: PBMCs with hPMECs and 16HBE

- PBS hPMEC
- fMLP hPMEC
- PBS hPMEC + 16HBE
- fMLP hPMEC + 16HBE

Supplementary table

Supplementary table 1: calculation of flow rate inside chip on tilter.

Time (min)	Flow rate (µl/min)
0	8.48
0.5	6.15
1	4.79
1.5	3.93
2	3.32
2.5	2.88
3	2.54
3.5	2.28
4	2.06
4.5	1.88
5	1.73
5.5	1.60
6	1.49
6.5	1.40
7	1.31
7.5	1.24
8	1.17

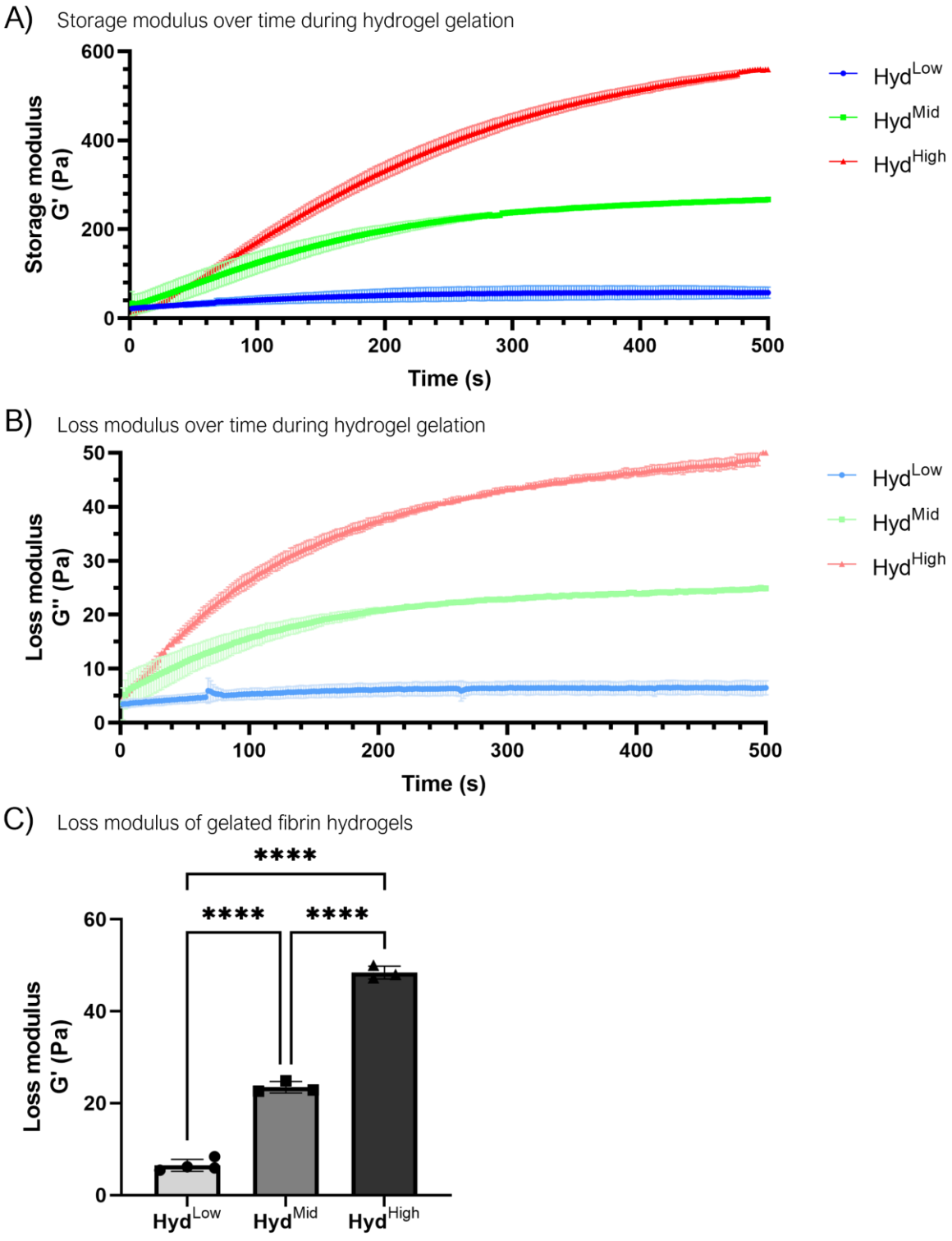
Median flow rate: 2.06 µl/min

Formula used:

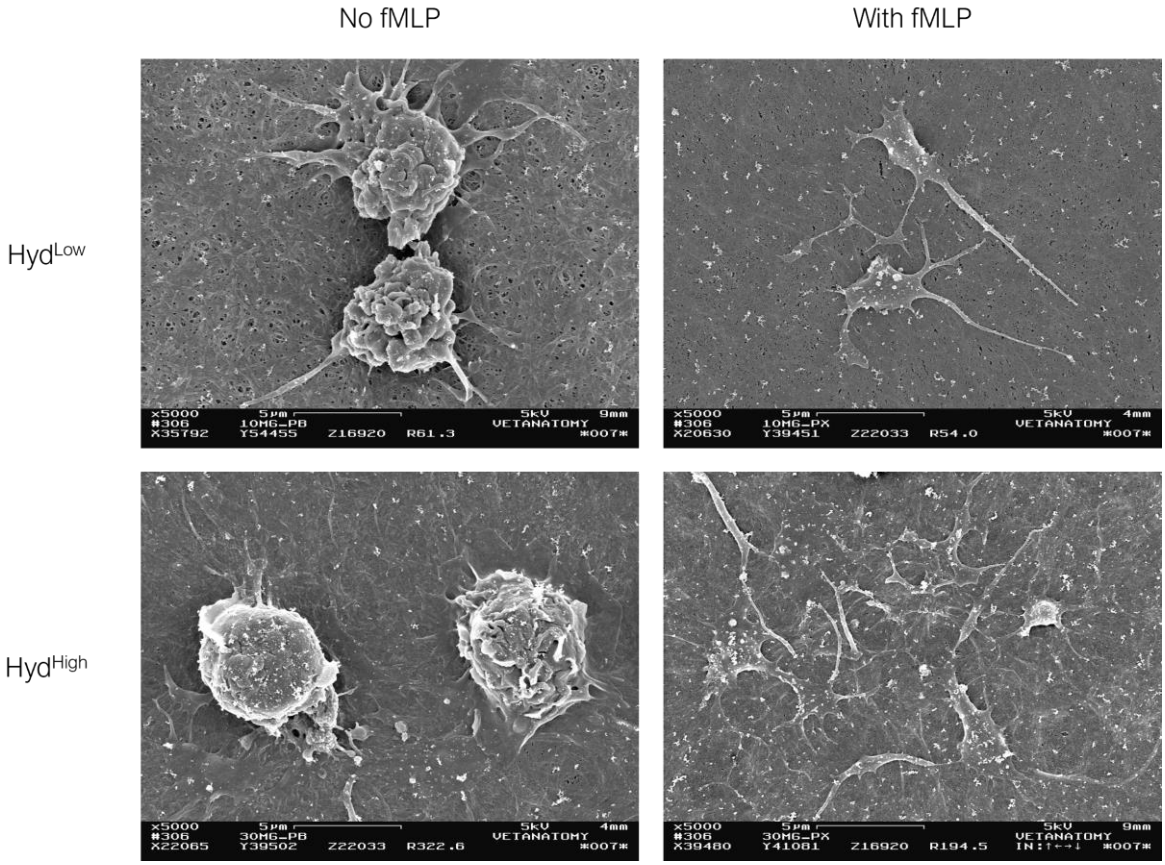
$$h\,(t) = \frac{1}{\frac{1}{\Delta h} + \frac{2*\rho*g*Rf*t}{A*\Delta h}} \qquad Q = \frac{\rho*g*h(t)}{Rf}$$

Variable	Abbreviation	Unit
Height difference between reservoirs at time (t)	h(t)	s
Initial height difference between reservoirs	Δh	m
Fluid density	ρ	kg/m^3
Gravity acceleration	g	m/s^2
Fluidic resistance	Rf	kg/m^4 s
Time	t	s
Channel area	A	m^2

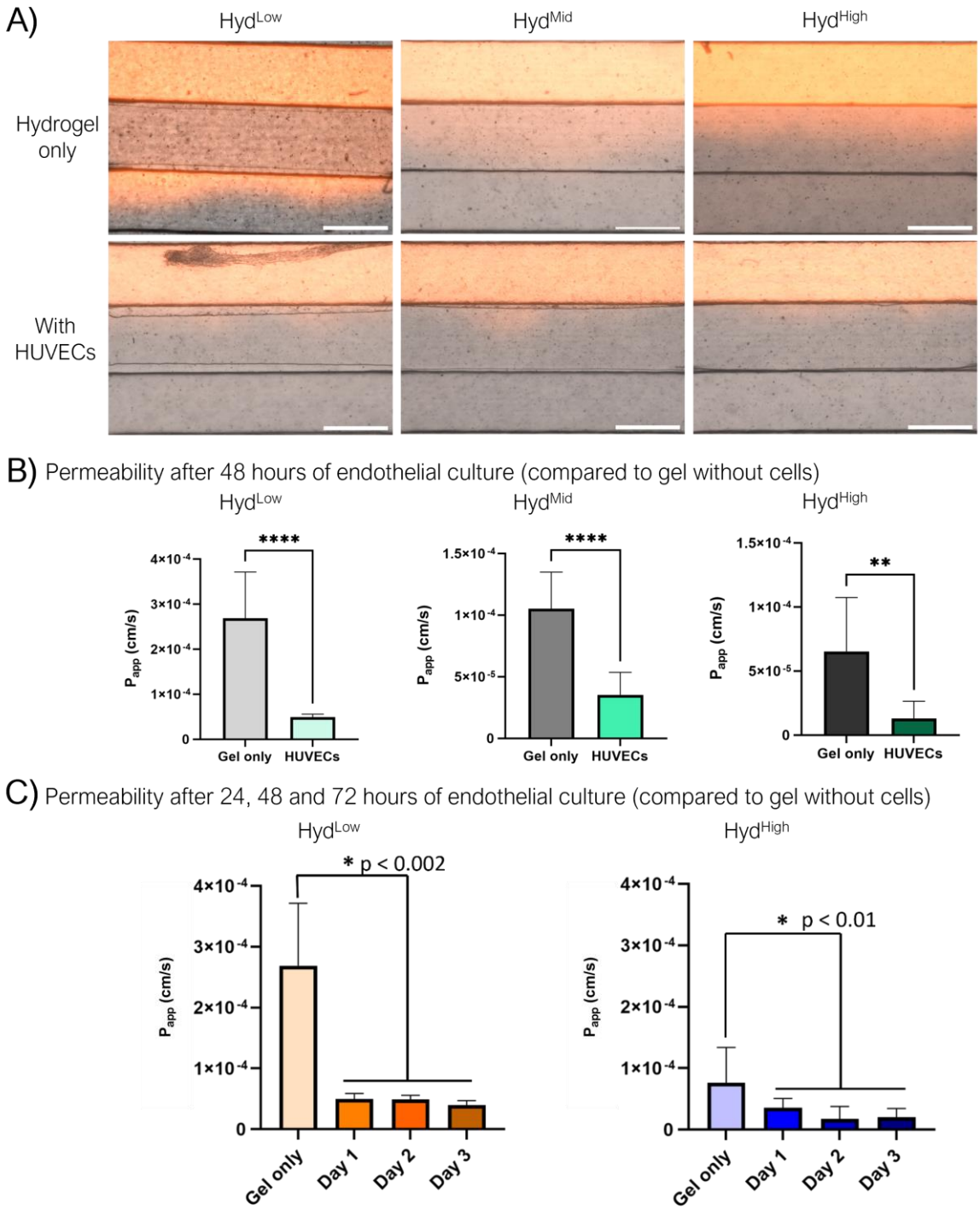
Supplementary Figures



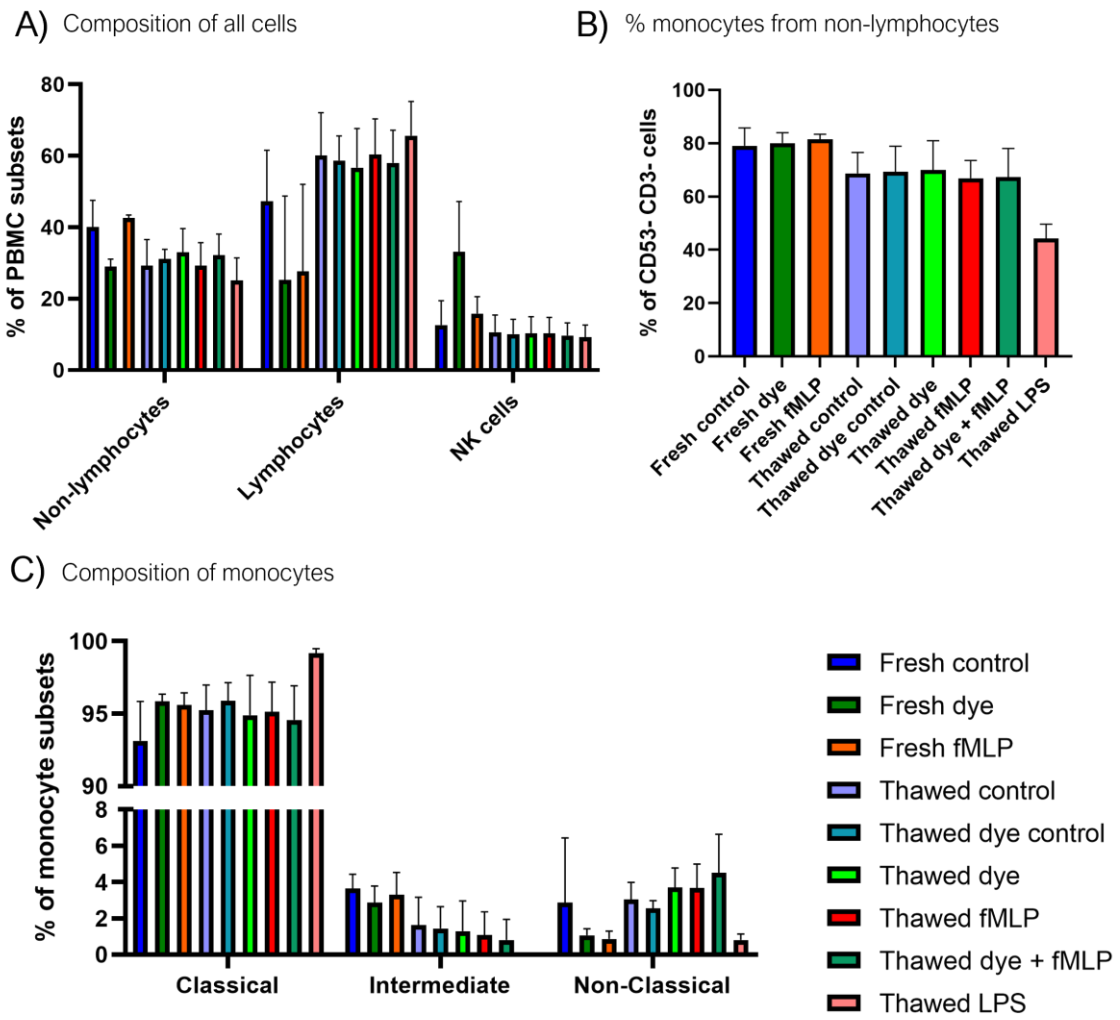
Supplementary figure 1. Rheological properties of fibrin hydrogels. Fibrin hydrogels were formed on the rheometer and rheological properties were measured during the gelation phase. **A)** Storage modulus of Hyd^{Low}, Hyd^{Mid} and Hyd^{High} hydrogels over time during hydrogel gelation shows a clear difference in mechanical properties and gelation time of the three hydrogels. **B)** Loss modulus of fibrin hydrogels over time. **C)** Loss modulus of fully gelated hydrogels. Mean with SD is displayed in all graphs. Statistics: ANOVA with Tukey's multiple comparison test. **** $p < 0.0001$.



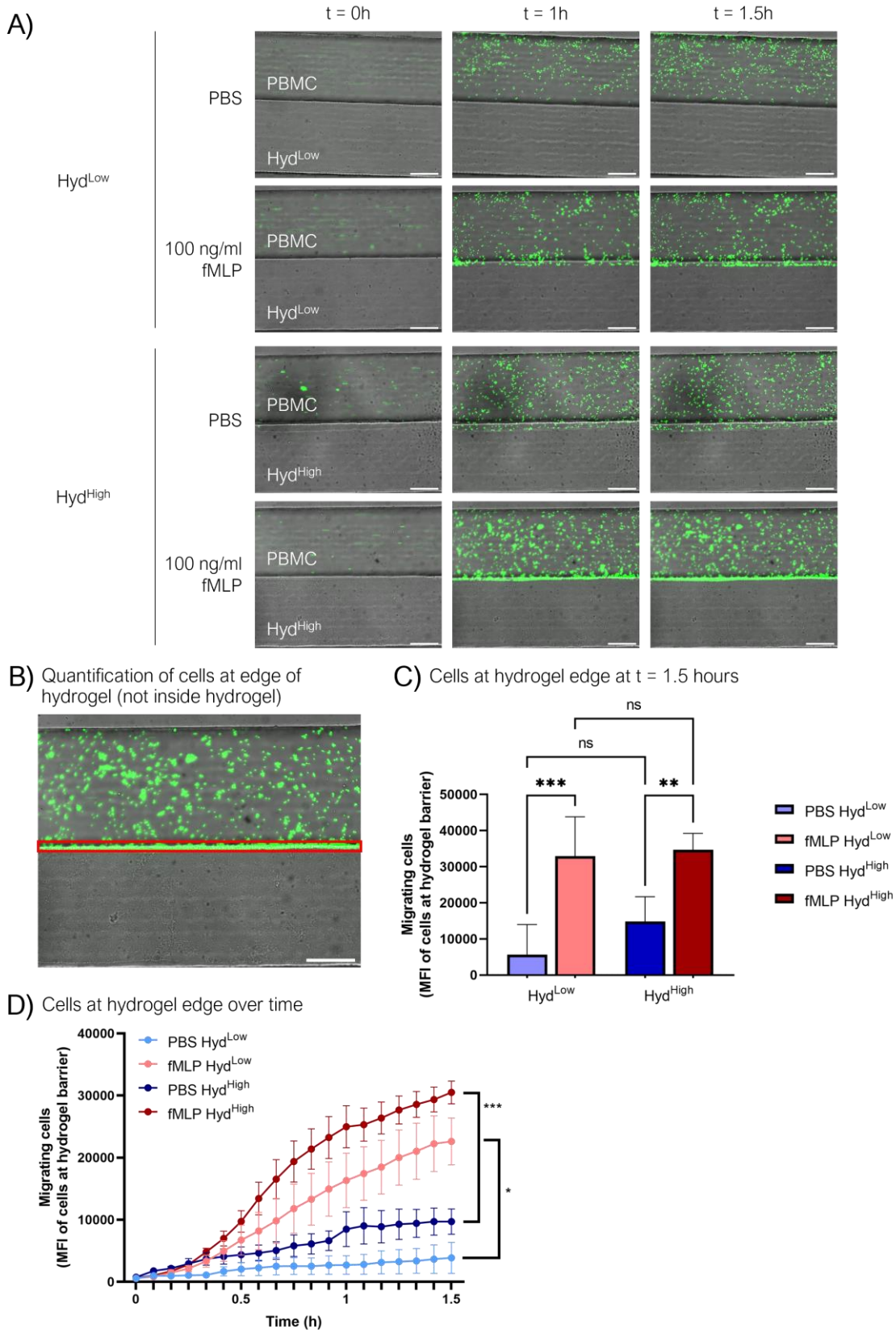
Supplementary figure 2. SEM images of PBMCs on hydrogels with and without fMLP. Fibrin hydrogels were formed on coated SEM coverslips and PBMCs were seeded on top. PBMCs were incubated with RPMI media or RPMI + 100ng/ml fMLP for 4 hours. Figure shows representative images of immune cells on the hydrogels. Cells seem more spread when fMLP was added into the media.



Supplementary figure 3. Addition of a HUVEC endothelial barrier decreases barrier permeability compared to gel only. HUVEC endothelial cells were grown to confluency on chip and permeability was measured after 24, 48 and 72 hours of culture. **A)** Representative images of barrier permeability of the three hydrogels with and without endothelial cells. **B)** Permeability of HUVEC endothelial barrier after 48-hour culture time compared to the permeability of the hydrogel the cells were grown alongside. N = 8-12 chips per condition, at least 3 independent experiments. Graph shows mean with SD. Statistics: student's T test. *0.05 < p < 0.01. **0.01 < p < 0.001. **** p < 0.0001. **C)** Permeability of HUVEC endothelial barrier after different culture timepoints. N = 8-12 chips per condition, at least 3 independent experiments. Graph shows mean with SD. Statistics: ANOVA with Tukey's multiple comparison test.

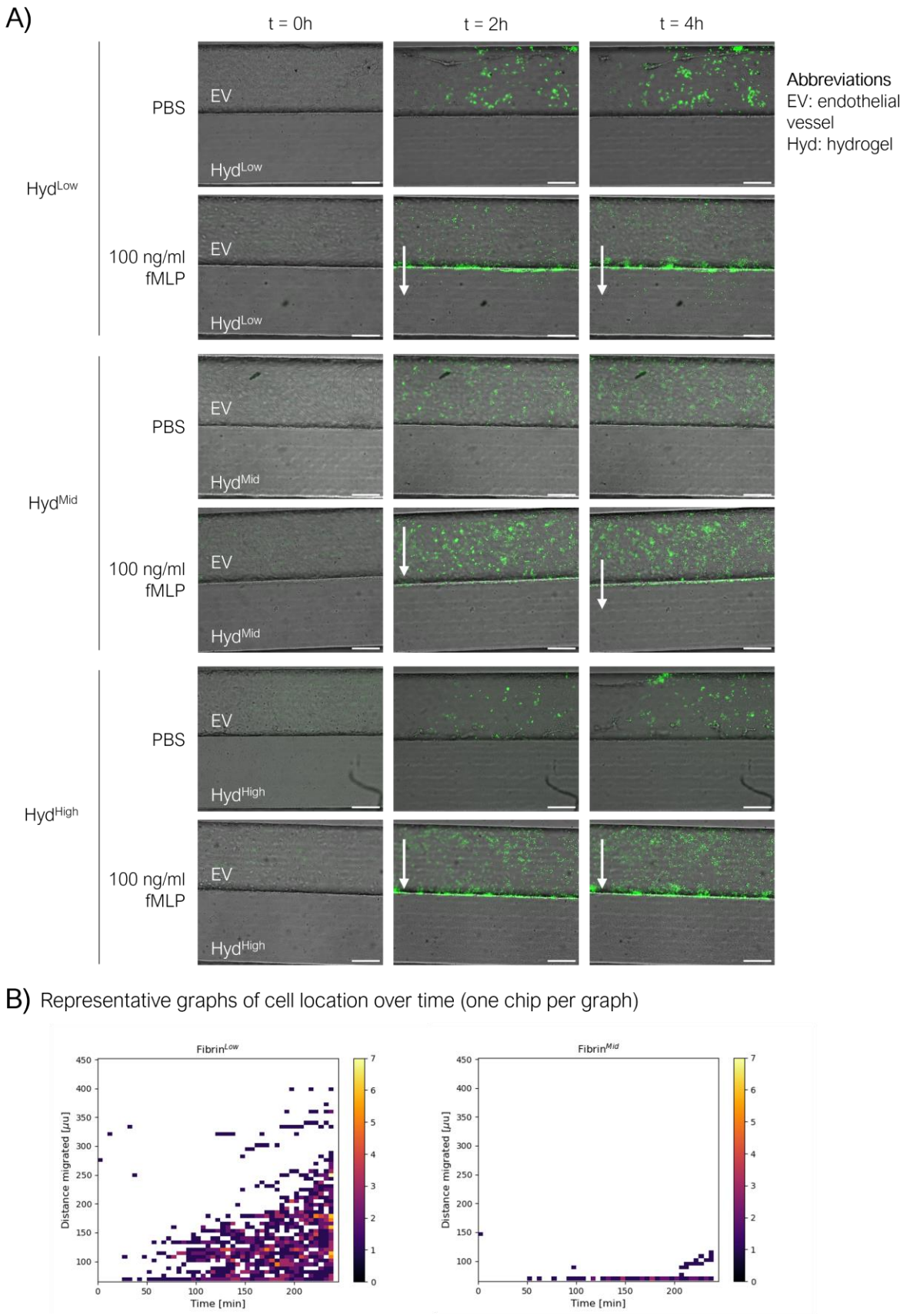


Supplementary figure 4. FACS of PBMCs: additional conditions. PBMCs (fresh and frozen) were stained with a FACS marker panel and FACS was carried out. List of conditions: 1. Fresh control: freshly isolated cells kept in RPMI media for 4 hours. 2. Fresh dye: freshly isolated cells dyed with PkH67 dye and kept in RPMI media for 4 hours. 3. Fresh fMLP: freshly isolated cells kept in RPMI with 100ng/ml fMLP for 4 hours. 4. Thawed control: frozen cells thawed and kept for 4 hours in RPMI media. 5. Thawed dye control: frozen cells were thawed and went through the dyeing process without adding dye. Cells were kept for 4 hours in RPMI media. 6. Thawed dye: frozen cells were thawed, dyed with PkH67 and kept for 4 hours in RPMI media. 7. Thawed fMLP: frozen cells thawed and kept for 4 hours in RPMI media with 100ng/ml fMLP. 8. Thawed dye + fMLP: frozen cells were thawed, dyed with PkH67 and kept for 4 hours in RPMI media with 100ng/ml fMLP. 9. Thawed LPS: frozen cells thawed and kept for 4 hours in RPMI media with 1000ng/ml LPS. **A)** Percentage of lymphocytes, NK cells and non-lymphocytes in the total cell population. **B)** Percentage of monocytes in the non-lymphocyte population. (**C**) Percentage of classical, intermediate and non-classical monocytes within the monocyte population.



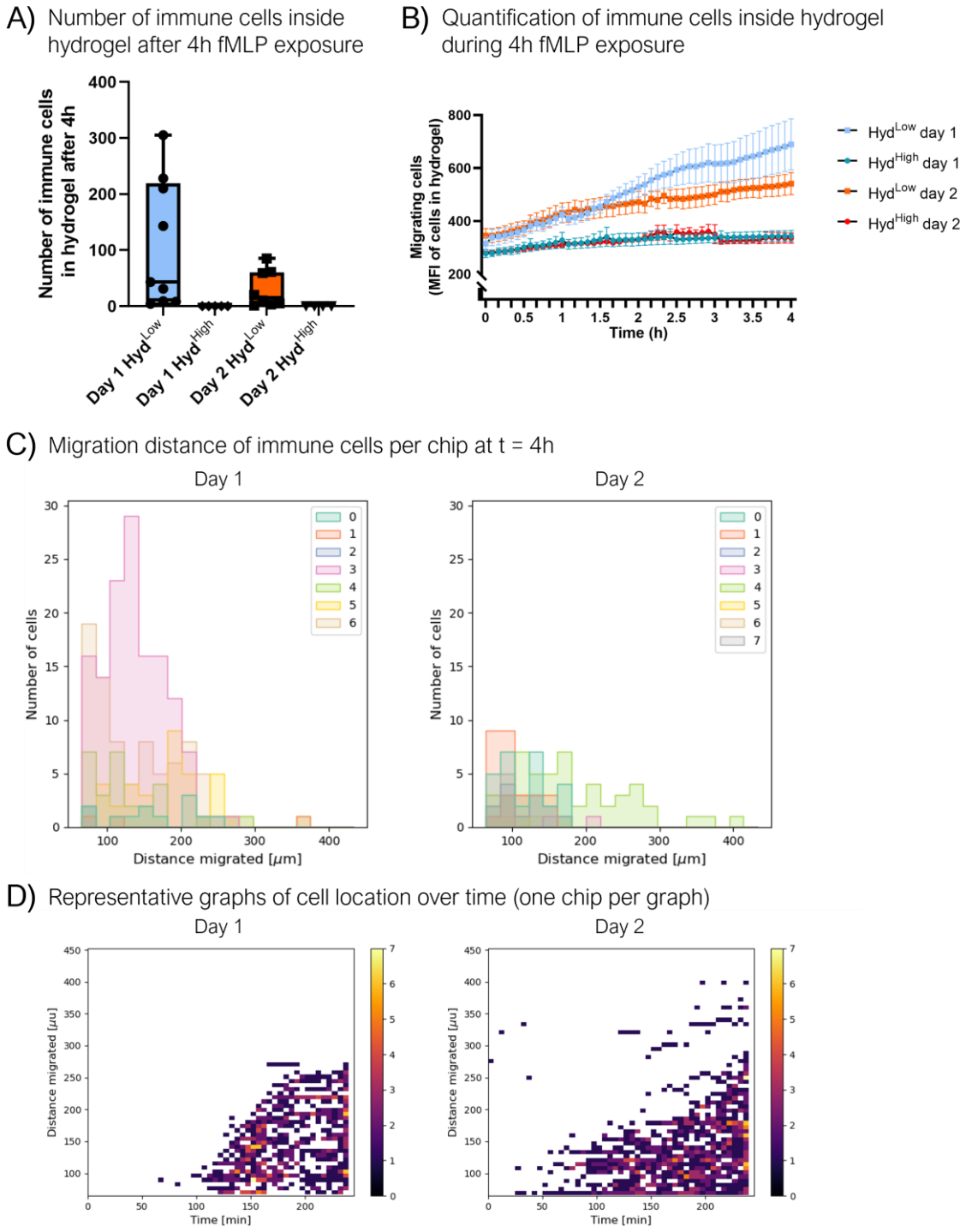
Supplementary figure 5. Immune cells on the chip sense chemotactic gradient through Hyd^{Low} and Hyd^{High} hydrogels. PBMCs were thawed and dyed with green fluorescent dye. PBMCs were perfused through the endothelial/immune cell channel, while PBS or fMLP was perfused through the opposite channel. Migration was followed for 90 minutes. **A)** Representative images of PBMC

attachment after 0, 60, and 90 minutes. Scale bar: 200µm. **B)** Mean fluorescent intensity in the marked rectangle was measured to quantify immune cell attachment. **C)** Quantification of mean fluorescent intensity at the hydrogel barrier at t=90 minutes to assess the amount of PBMCs attached to the hydrogel barrier. **D)** Quantification of mean fluorescent intensity at the hydrogel barrier shows an increase in PBMC attachment over time, specifically in the fMLP chemoattractant conditions. N = 5 chips per condition, three independent experiments. Graphs show mean with SEM. Statistics: ANOVA with Tukey's multiple comparison test. *$0.05 < p < 0.01$. **$0.01 < p < 0.001$. ***$0.001 < p < 0.0001$.

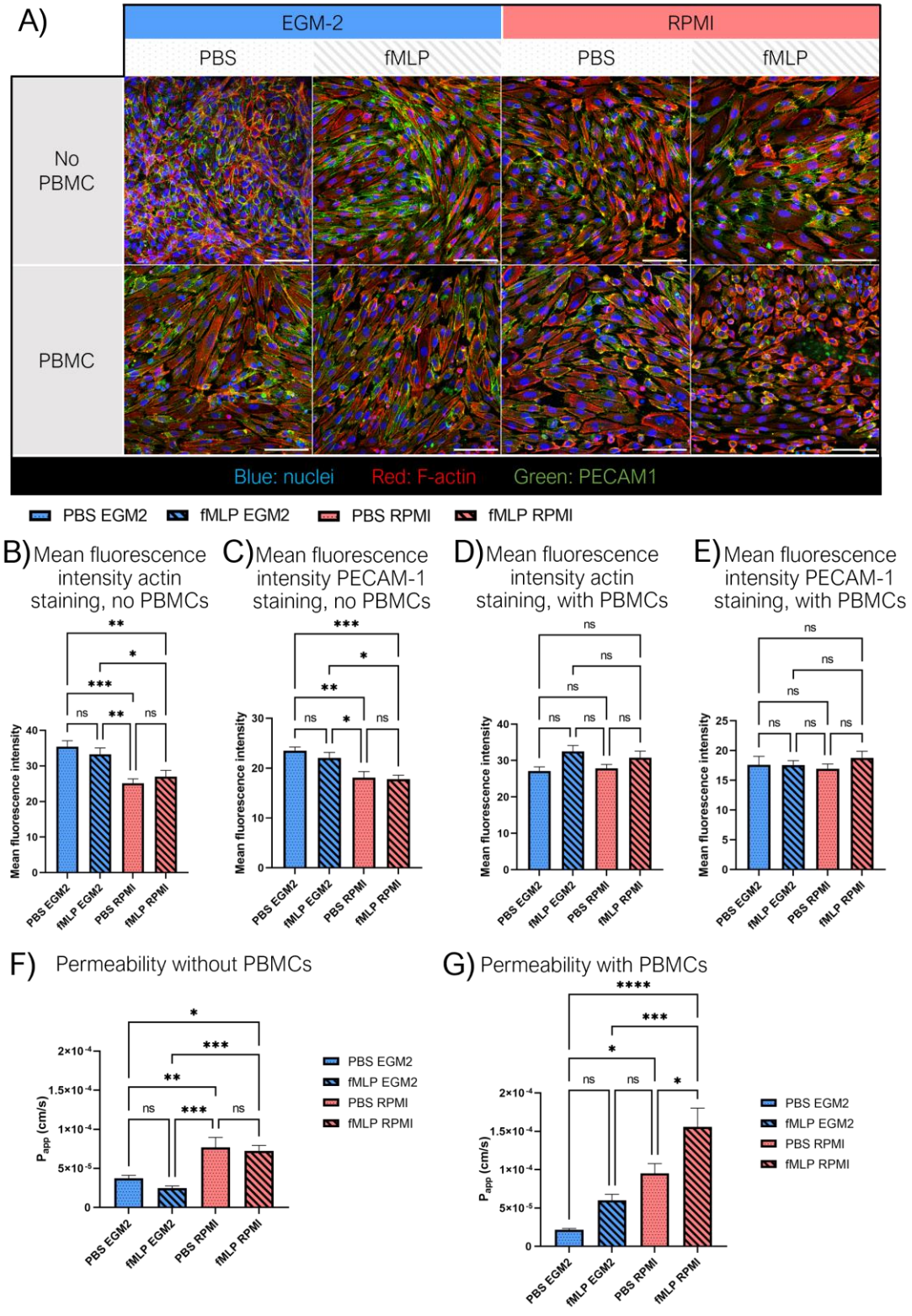


Supplementary figure 6. Immune cells extravasate depending on the hydrogel behind the endothelial barrier. PBMCs were perfused through the endothelial channel, and fMLP or PBS was perfused through the chemoattractant channel. **A)** Representative images at t = 0h, t = 2h and t =4h

show increased attachment of PBMCs over time and extravasation of immune cells in the Hyd^{Low} and Hyd^{Mid} conditions with fMLP. **B)** Representative graphs of immune cell location over time show an increase in immune cell extravasation over time. These graphs represent all immune cells in one chip.

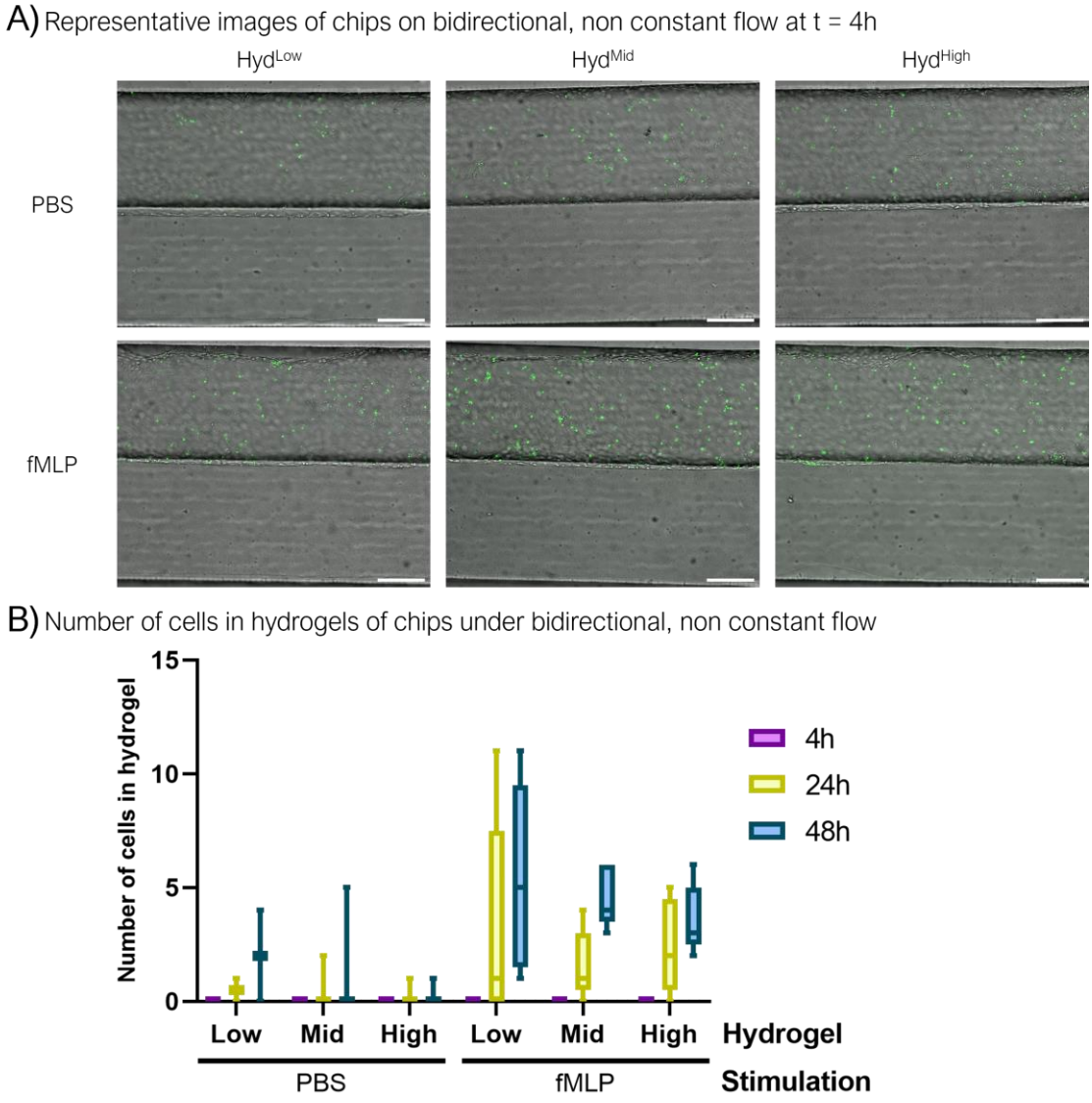


Supplementary figure 7. Culture time of HUVECs affects immune cell migration. HUVECs were seeded on chip and cultured for 24 or 48 hours until the migration experiment was carried out. fMLP and PBMCs were perfused for 4 hours. Migration was quantified using Fiji. **A)** Number of immune cells inside hydrogel after 4h fMLP exposure. Graph shows median with SD. **B)** Quantification of immune cells inside hydrogel over time. Graph shows mean with SEM. **C)** Migration distance of all immune cells at t = 4h, represented per chip sample. **D)** Representative graphs of immune cell location over time show an increase in immune cell extravasation over time. These graphs represent all immune cells in one chip, but do not average multiple samples.

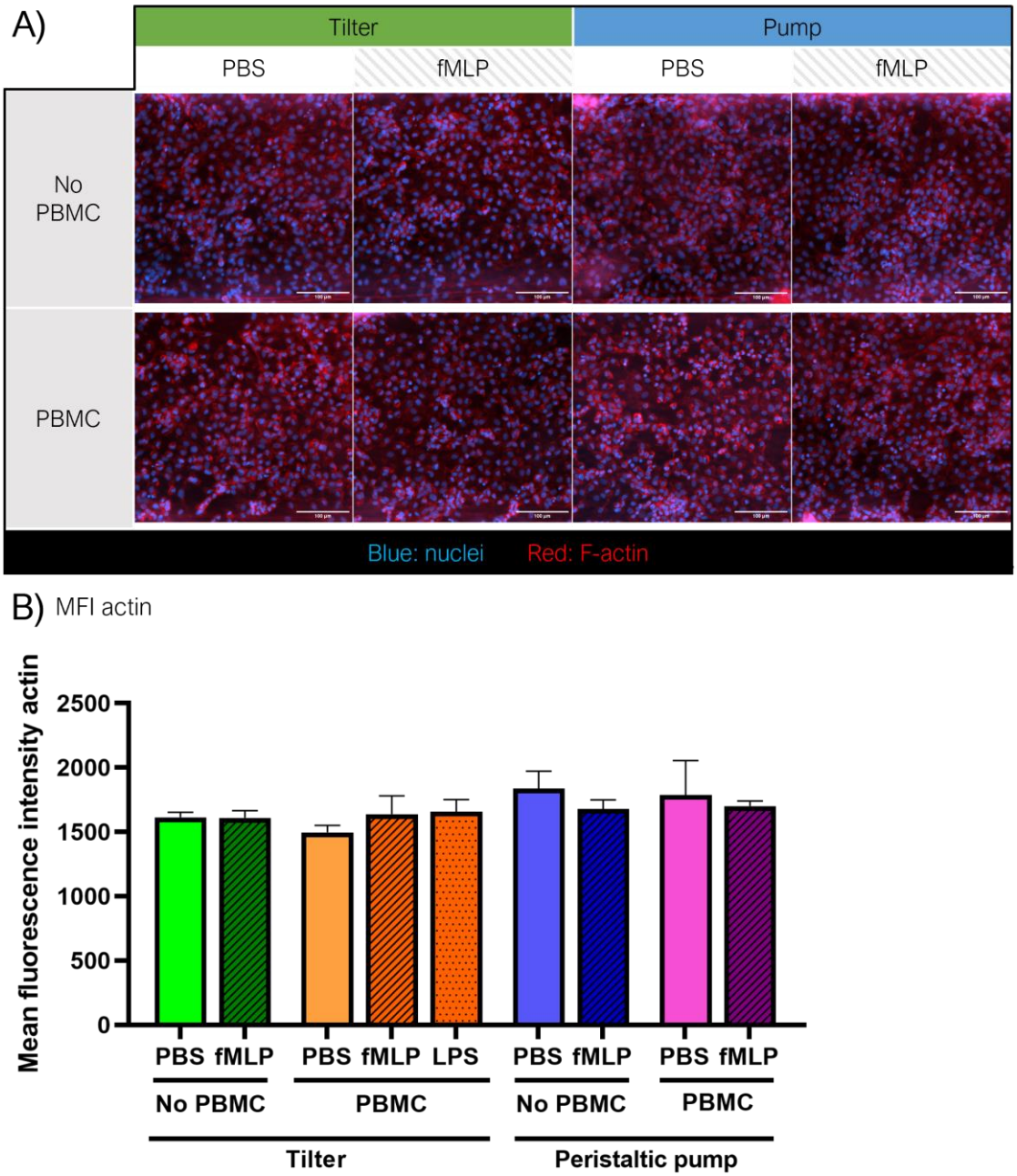


Supplementary figure 8. RPMI media increases endothelial barrier permeability. HUVEC endothelial cells were grown to confluency on the chip and exposed to different media conditions: EGM-2 or RPMI with/without PBMCs in the endothelial/immune cell channel and PBS or fMLP in the opposite channel. After 4 hours, apparent permeability was measured, and cells were fixed and stained for immunohistochemistry. **A)** Representative images of HUVEC cells after 4-hour exposure. Green: PECAM-1, red: actin, blue: nuclei. Scale bar: 100µm. **B-E)** Quantification of the mean fluorescent intensity of actin (B/D) and PECAM-1 (C/E) staining of HUVECs. Without PBMCs,

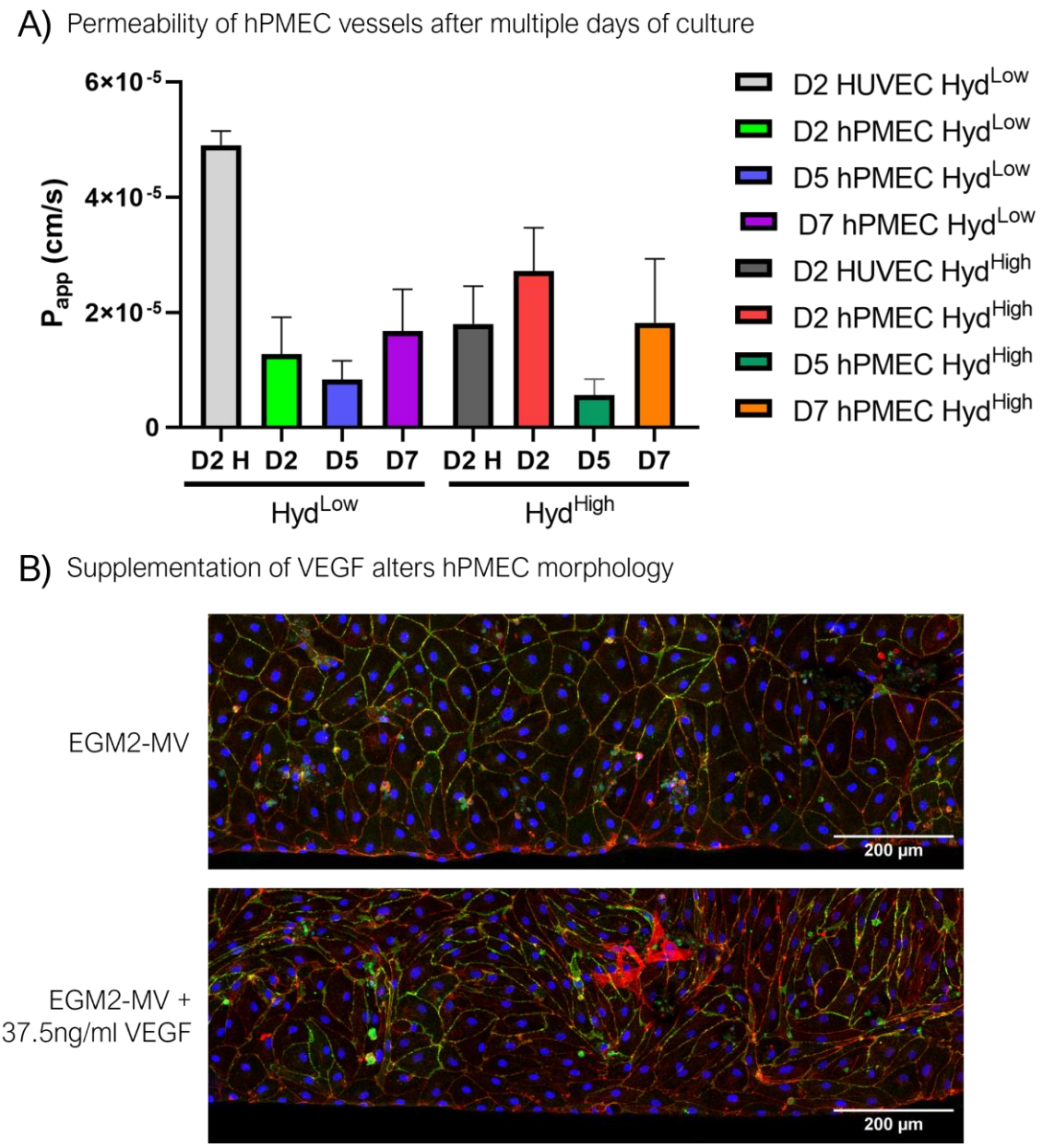
decreased actin and PECAM-1 levels after RPMI exposure compared to EGM-2 are observed (B/C). With PBMCs, the combination of HUVEC and PBMC staining prevents from observing any significant difference in barrier protein expression (D/E). **F)** HUVEC barrier permeability is increased following exposure to RPMI alone. **G)** Permeability assays of endothelial cells exposed to PBMCs and RPMI media show that RPMI exposure increases endothelial barrier permeability. Moreover, fMLP has a significant additive effect on barrier permeability. Immunohistochemistry: N = 9 chips per condition, three independent experiments. Permeability: N = 4 chips per condition, three independent experiments. Graphs show mean with SD. Statistics: ANOVA with Tukey's multiple comparison test. *0.05 < p < 0.01. **0.01 < p < 0.001. ***0.001 < p < 0.0001. ****p < 0.0001.



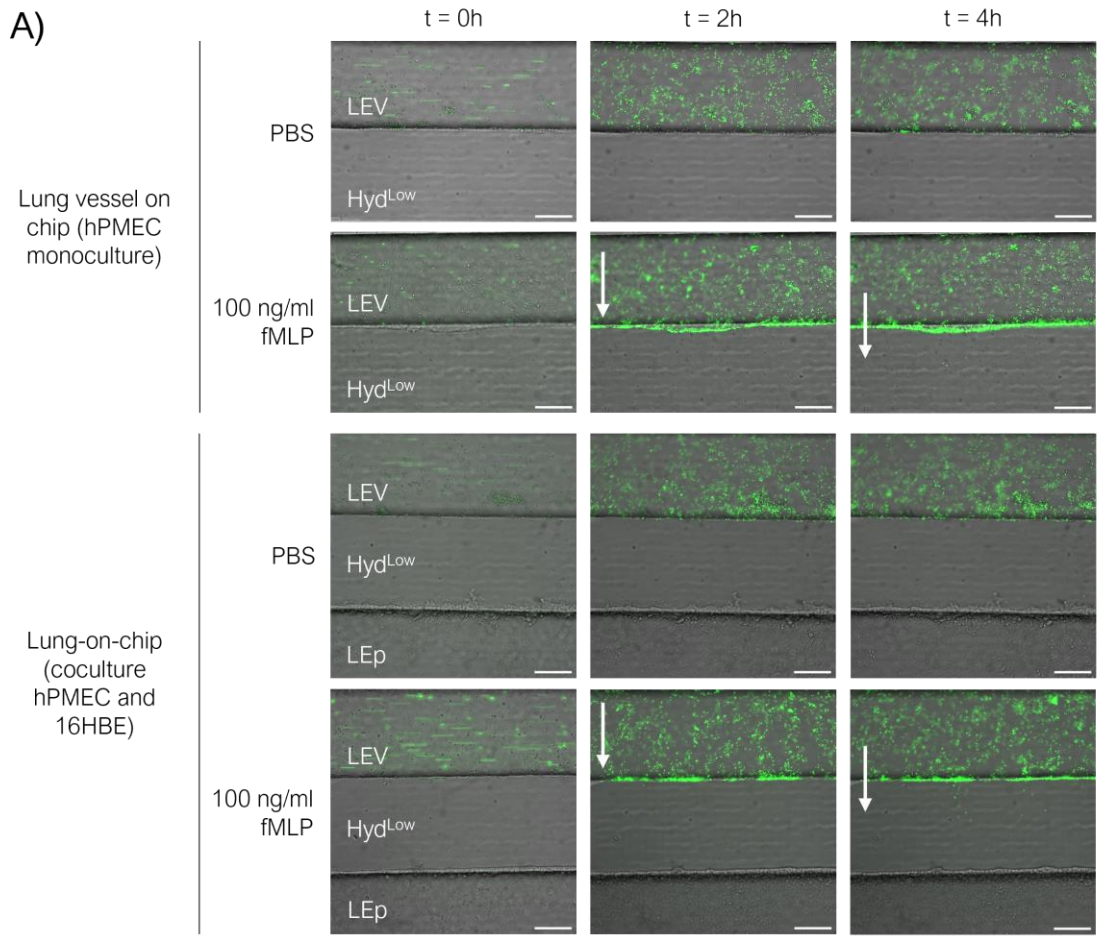
Supplementary figure 9. Immune cells extravasate in infection-on-chip on rocker platform after 24 hours. HUVECs were seeded on chip and cultured to confluency. PBMCs were dyed green and added to the endothelial channel, with simultaneous addition of PBS/fMLP to the chemoattractant channel. Chips were placed on a rocker platform for 48 hours. Imaging was carried out after 4, 24 and 48 hours. **A)** Representative images of PBMCs in the endothelial vessel on a rocker platform at t = 4 hours. No transmigration can be observed at this timepoint. **B)** Quantification of immune cells inside hydrogel at 4, 24 and 48 hours after PBMC addition. N = 3-5 chips per condition.

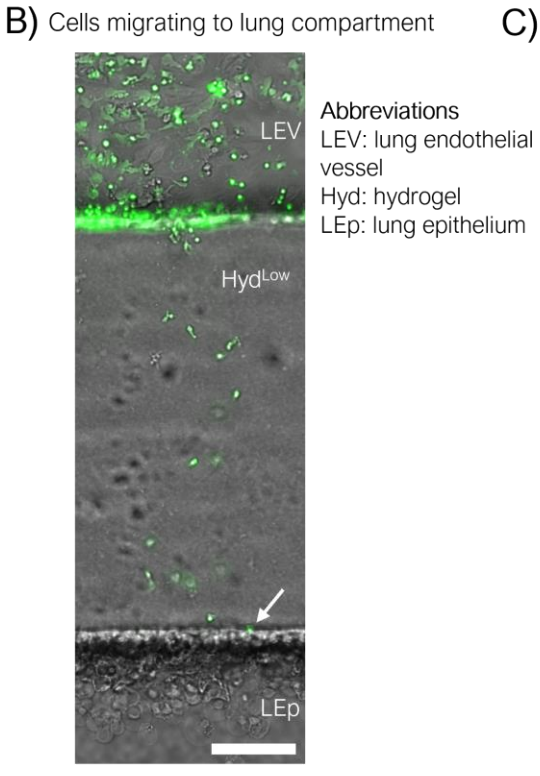


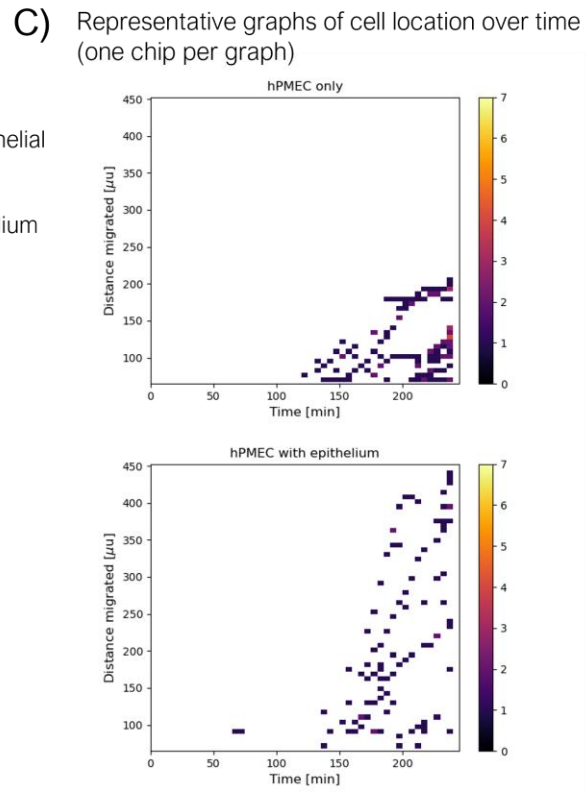
Supplementary figure 10. Pump-based flow and tilter-based flow of RPMI media have a similar effect on HUVEC morphology. HUVECs were grown to confluency on chip and exposed to RPMI media for 4 hours. During this 4-hour exposure, chips were either put on a tilting platform or attached to a peristaltic pump. After incubation, chips were fixed and immunohistochemistry was performed. Cells were stained for F-actin (red) and nuclei (blue). Imaging was carried out on a Nikon widefield microscope. N=2-3 chips per condition. **A)** Representative images of HUVECs exposed to fMLP and PBMCs under tilting or pump-based flow. Scale bar: 100µm. **B)** Quantification of F-actin expression in all conditions shows a similar effect of RPMI in all conditions.



Supplementary figure 11. hPMECs can be cultured on chip and are affected by VEGF supplementation. hPMECs were seeded on chip and cultured to confluency. Chips were cultured either in EGM2-MV or EGM2-MV supplemented with 37.5ng/ml VEGF. Fixed cells were stained for PECAM-1 (green), nuclei (blue) and F-actin (red). **A)** Permeability of microfluidic vessel over time. **B)** Representative images of hPMECs grown for 5 days in EGM2-MV (top) or EGM2-MV + 37.5ng/ml VEGF (bottom). Mesenchymal-like cells with a high amount of actin fibers can be observed when VEGF is supplemented (bottom). Graph shows mean with SEM.







Supplementary figure 12. Immune cell migration in lung infection. PBMCs were perfused through the hPMEC-coated endothelial channel, while PBS/fMLP was perfused through the opposite epithelial

channel to model infection. **A)** Representative images of the infection assay at t = 0h, t = 2h and t = 4h show an increase in extravasation over time in the fMLP conditions, and no extravasation in the PBS conditions. **B)** Representative image of a chip where immune cells reached the epithelial barrier (occurred in 2/6 chips tested). **C)** Representative graphs of immune cell location over time show an increase in immune cell extravasation over time. These graphs represent all immune cells in one chip, but do not average multiple chip samples.

References

1. Kluyver T, Ragan-Kelley B, Pérez F, et al. Jupyter Notebooks – a publishing format for reproducible computational workflows. *Position Power Acad Publ Play Agents Agendas - Proc 20th Int Conf Electron Publ ELPUB 2016*. 2016:87-90. doi:10.3233/978-1-61499-649-1-87
2. Harris CR, Millman KJ, van der Walt SJ, et al. Array programming with NumPy. *Nat 2020 5857825*. 2020;585(7825):357-362. doi:10.1038/s41586-020-2649-2
3. Pandas Development Team T. pandas-dev/pandas: Pandas 1.1.3. January 2020. doi:10.5281/ZENODO.7549438
4. Van Der Walt S, Schönberger JL, Nunez-Iglesias J, et al. scikit-image: image processing in Python. *PeerJ*. 2014;2014(1). doi:10.7717/PEERJ.453
5. Pedregosa F, Varoquaux G, Gramfort A, et al. Scikit-learn: Machine Learning in Python. *J Mach Learn Res*. 2012;12:2825-2830. doi:10.48550/arxiv.1201.0490
6. Hunter JD. Matplotlib: A 2D graphics environment. *Comput Sci Eng*. 2007;9(3):90-95. doi:10.1109/MCSE.2007.55
7. Waskom M, Botvinnik O, Ostblom J, et al. mwaskom/seaborn: v0.10.1 (April 2020). April 2020. doi:10.5281/ZENODO.3767070