Supplementary Material:

Rapid Communication: A silicon nanomembrane platform for the visualization of immune cell trafficking across the human blood-brain barrier under flow

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Supplementary Methods

Induction of BBB characteristic in $CD34^+$ -derived endothelial cells

Induction of BBB like characteristics in $CD34^+$ -derived endothelial cells was achieved by co-culture with bovine pericytes as described before (15). In brief, pericytes were initially seeded on 100-mm gelatin-coated petri dishes (Corning) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 20% (v/v) FBS (Life Technologies), 2 mM L- glutamine, 50 mg/mL gentamycin. Upon reaching confluency pericytes ($2.25x10^4$ /well) were seeded into 24-well plates (Costar). On the following day, $CD34^+$-derived ECs grown on gelatin-coated 100 mm petri dishes in ECM (Sciencell) were trypsinized and seeded ($4x10^4$/filter insert) onto Matrigel-coated (Corning, 354230) Transwell® filter inserts (Costar cat n.3495) and placed above the pericyte cultures. At the end of this culture procedures the $CD34^+$ -derived ECs will acquire the features of brain like endothelial cells (BLECs) as revealed by the expression of BBB marker and functional properties revealed by LY permeability assay evaluated from 2 to 20 days of co-culture (15). The isolation of $CD34^+$ cells required the collection of human umbilical cord blood (UCB) from infants. Parents signed an informed consent form in compliance with French legislation. The protocol was approved by the French Ministry of Higher Education and Research (CODECOH Number DC2011-1321).

Endothelial Permeability (Pe) Measurements

Endothelial permeability was investigated by measuring the clearance of Lucifer yellow (LY; 20 nM; Life technologies) across the BLECs monolayer exactly as described before (15, 30, 31, 32). In brief, prior to experiments, LY (MW 457.3 g/mol) was diluted in assay medium (DMEM (Gibco, Paisley, UK), 5 % FCS (Gibco, Paisley, UK), 25 mM Hepes (Gibco, Paisley, UK), 2 % l-glutamine Gibco, Paisley, UK) as previously published in (15) and 100 µl of the LY solution (20 nM) was added to the upper side of the filter inserts, which were subsequently placed into wells containing 600 µl of assay medium. The plate was incubated at 37 °C and the inserts were transferred into wells containing fresh assay medium every 10 min, avoiding long exposure to light and room temperature. 200 µl samples from all lower compartments were transferred to a 96-well plate. For the "Transwell mimetic" and the µSiM-CVB, the membrane

compartment was filled with 75 µL of a LY solution (20 nM), and in the basal compartment with 15 µL of permeability assay medium. After 60 min incubation, the assay medium was collected from the basal compartment and the fluorescence was evaluated with the TECAN reader infinite M1000 PRO. A standard dilution curve (20–0.05 µM) and empty filters or "Transwell mimetic" were used to obtain the reference permeability of the empty filter inserts. The permeability coefficient was calculated as follows: the slope of the average tracer volume cleared was plotted versus time in order to obtain the linear regression designated as PSt. The slope of the tracer clearance curve of the coated empty filters was indicated as PSf. The permeability surface area product of the endothelial cell monolayer (PSe) was calculated from PSt and PSf: $1/PSe = 1/PSt - 1/PSf$. The PSe was divided by the filter surface area, in order to generate the epithelial Pe in cm/min. The fluorescence detection was performed using the Tecan Infinite M1000 device and the Tecan i-control software (Tecan Trading AG, Männerdorf, Switzerland).

Immunofluorescence stainings of BLECs

For staining in the Transwell® filter inserts, BLECs were fixed in 1% (w/v) formaldehyde diluted in calcium and magnesium-free phosphate- buffered saline (PBS) for 10 min at room temperature (RT) and permeabilized with Triton X-100 (0.1% (w/v) in PBS for 10min at RT. After three rinses with PBS, the filter membranes were carefully cut out with a scalpel and blocked for 30 min with skimmed milk 5% (w/v) in PBS. BLEC monolayers were incubated for 1h at RT with primary antibody against zonula occludens-1 at 5 µg/mL (ZO-1, Thermo Fisher Scientific, 617300) or VE-Cadherin at 7.5 µg/mL (Santa Cruz Biotechnology, sc-9989). For the staining of claudin-5, BLECs were fixed in methanol at -20°C for 30s before a permeabilization step of 20 min at RT in Triton X-100 0.3% in PBS, followed by a blocking step of 20 min at RT in in skimmed milk 5% in PBS. Next BLECs were incubated with primary antibody against claudin-5 at 2.5 µg/mL (Invitrogen, 34-1600) diluted in skimmed milk 5% (w/v) in PBS. After three washes in PBS cells were incubated with secondary antibody for 30 min at RT with goat anti rabbit/Alexa fluor 488 at 5 µg/mL (life technologies, A11034) for ZO-1 and claudin-5 and goat anti mouse-Cy3 at 7.5 µg/mL (Jackson ImmunoResearch, 115-166-06) for VE-Cadherin. After Triton permeabilization, staining of F-actin was obtained with rhodamine-labeled phalloidin (Invitrogen, R415) 1:50 in PBS for 1h at RT. Live cells were

incubated with 10 µg/ml of anti-ICAM-1 (R&D system, BBA3), anti-ICAM-2 (Fitzgerald, 10R-6458), or anti-VCAM-1-FITC (BD Biosciences, 551146) antibody for 20 min at 37°C in the culture medium. After a washing step with PBS, BLECs were fixed with 1% PFA in PBS and permeabilized with Triton X-100 (0.1% (w/v) in PBS) for 10min at RT. For ICAM-1 and ICAM-2 staining, cells were incubated with the secondary antibody donkey anti-mouse-Cy3 (Jackson ImmunoResearch, 715-165-151). Nuclei were stained using DAPI at 1 µg/mL. Each filter was mounted on a glass slide under a rounded coverslip, using a drop of Mowiol (Sigma-Aldrich) containing 1,4-diazabicyclo [2.2.2] octane (Sigma-Aldrich) as an antiquenching agent.

For BLECs staining on NPN membranes, ICAM-1, ICAM-2 and VCAM-1 are labelled as described above with the Transwell® filter inserts. For F-Actin staining, after Triton permeabilization, cells were incubated with rhodamine- labeled phalloidin (Invitrogen, R415) 1:50 in PBS for 1h at RT. Nuclei were stained with DAPI at 1 µg/mL. For the staining of ZO-1 and VE-Cadherin, after incubation with primary antibody, BLECs were washed in PBS and incubated with secondary antibodies, goat anti-rabbit-Alexa fluor 488 (Life Technologies, A11034) and goat anti mouse-Alexa fluor 488 (Jackson ImmunoResearch, 115-546-072), respectively, for 30 min at RT. For the claudin-5 staining, the same protocol as for BLECs staining on Transwell® filters was applied. Stainings were analyzed with a fluorescence microscope (Axio Observer, Zeiss) and pictures were acquired at the day of the staining and processed with the ZEN software.

Supplementary Discussion

The use of a nanoporous membrane scaffold supporting the BLEC monolayer does prevent the clearance of T cells from below the endothelial monolayer as would eventually occur with T cells migrating across the BBB into the brain parenchyma *in vivo*. In theory this could result in a build-up of T cells between the BLEC monolayer and the nanoporous membrane, which may have consequences for the outcomes we observe. An artificially dense subluminal T-cell population for example, might encourage frequent 'reverse transmigration' events, where T cells return to the luminal side of the BLEC monolayer after some time crawling beneath them. Reverse T cell migration was however a very rare occurrence in all our experiments and only seen under inflammatory conditions. The chemical and mechanical

microenvironment of the vascular mimetic might also be altered by a high number of trapped T cells beneath the endothelium, particularly if a chemokine gradient were used to compel large numbers of T cells to traverse the BLEC monolayer. These experimental issues can easily be addressed by modifying the T-cell numbers superfused over the BLEC monolayer and the time of recording. At this point the µSiM-CVB flow system thus allows for studying the molecular mechanisms involved in each step of the multi-step T cell extravasation cascade. It does however not allow for easy collection of T cells that have successfully passed the BLEC monolayer for further characterization of the T cell subset. We are in the process of introducing a derivative of NPN that will eventually allow for clearance of T cells from the mimetic without compromising the imaging and adhesion benefits of the native material.

Supplementary Table 1. List of commercially available cell culture filter inserts tested to adapt the human *in vitro* BBB model established from $CD34^+$EC co-cultured with bovine pericytes as described (15) to our established custom-made flow chamber system exactly as described (35). The table lists the technical problems encountered for each cell culture filter insert and gives reference to Supplementary Videos provided allowing to judge the image quality of BLECs on the respective filter systems.

Insert description	**Experiments Performed**	**Limitations of inserts**
1. 6 well standing inserts, low wall, 0.4 µm pore size, PTFE Millipore, Sevelen, Switzerland (Enzmann et al. 2013)	Cell growth on coated filter Membranes: Matrigel, laminin, gelatine and rat tail collagen	BLECs do not adhere on this filters
2. 6 well standing inserts, high wall, 0.4 µm pore size, polycarbonate Millipore, Sevelen, Switzerland	Cell growth Light Microscopy	Filter membrane is not transparent
3. 6 well standing inserts high wall 0.4 µm pore size mixed cellulose esters Millipore, Sevelen, Switzerland	Cell growth Light Microscopy	Filter membrane is not transparent
4. 6 well hanging inserts, 0.4 µm pore size, polycarbonate Corning Incorporated, NY, USA	Cell growth Light Microscopy	Filter membrane is not transparent
5. 6 well hanging inserts, 0.4 µm pore size, PTFE coated Corning Incorporated, NY, USA	Cell growth Light Microscopy In vitro flow assay	Filter membrane is insufficiently transparent
6. 6 well hanging inserts, 0.4 µm pore size, PET Corning Incorporated, NY, USA	Cell growth Light Microscopy In vitro flow assay	Filter membrane is insufficiently transparent
7. 6 well hanging inserts, 0.4 µm pore size, PET Greiner, Huber & Co. Ag Reinach Switzerland	Cell growth Light Microscopy In vitro flow assay	Filter membrane is insufficiently transparent

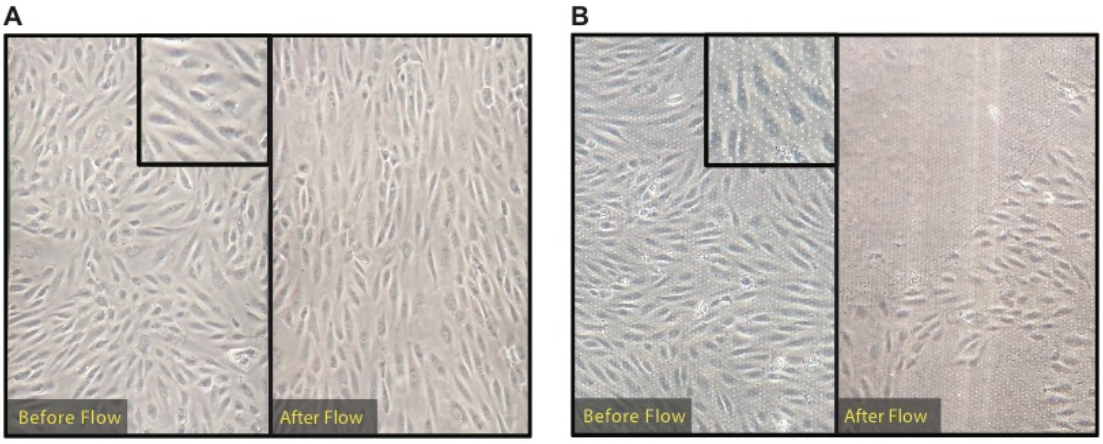

Figure S1. Comparison of the NPN and SiO_2 membranes endothelial cell attachment properties under flow

(A) Human umbilical vein endothelial cells (HUVECs) are grown on nanoporous silicon nitride (NPN) for 24 hours under static condition (left panel), and were later exposed to ~9-10 dynes/cm^2 of shear stress of fluid flow for another 24 hours (right panel). Inset represents a zoomed in image of the cell monolayer without any noticeable pore distribution on the membrane substrate. (B) HUVECs were grown under similar static conditions on 3 µm pores silicon dioxide membranes (left panel) followed with identical shear stress exposure. Notice the visible presence of 3 µm pores during phase imaging as observed in the inset (magnified view). Within 2 hours of shear introduction, there is over 60% cell loss from the membrane substrate as shown in the right panel.

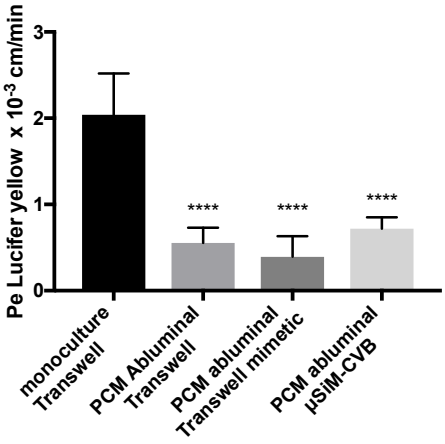

Figure S2. Barrier characteristics of BLECs in the µSiM-CVB flow system compared to the Transwell mimetic

The permeability for Lucifer Yellow (PeLY) was measured across monolayers of $CD34^+$-derived ECs grown in the presence of PCM in the abluminal compartment of the µSiM-CVB and compared to the values obtained in the normal Transwell (Figure 2) and the Transwell mimetic (Figure 3). The values show mean ± SD of 14 customized devices for the $CD34^+$ ECs cultured in the presence of abluminal PCM in the µSiM-CVB. Statistical analysis: one-way ANOVA followed by Dunnett's multiple comparison test ****$p < 0.0001$ vs monoculture Transwell.

Video 1. Th1 cell interactions with the unstimulated BBB under flow

The BLECs were induced by growing $CD34^+$ -derived ECs in the top channel of the flow chamber with the PCM in the bottom channel for 6 days before the assay. Th1 cells are allowed to accumulate on the BLECs monolayer at a low flow rate of 0.1 dyne/cm^2 for 4 min from the first frame after the first Th1 cells appear in the field of view. After the accumulation phase at precisely 4 min 55 sec, the flow rate was set to 1.5 dyne/cm^2 for 30 min (shear phase). The dynamic T cell interactions with the BLECs monolayer under a physiological flow were recorded with a 10x magnification (EC Plan-Neofluar 10X/0.30 Ph1) with a Zeiss Axiocam MRm camera. During the recording, 1 picture was acquired every 5s and the time laps video was exported with a framerate of 30 images/s. The video shown here is repeated 3 times, the 1^{st} run shows the full-scale video, the 2^{nd} run is a zoom in of one area highlighting a T cell crawling (green circle) on the BLECs monolayer, and the 3^{rd} run is a zoom in of one area highlighting T cell diapedesis (blue circle) and T cell probing (orange circle) events. The red arrow shows the direction of flow and the time is displayed on the top left of the video (min:sec format). Scale = 100 µm. The video is representative of 10 experiments and the statistical analysis of the T cell behavior is shown in the Figure 5B.

Video 2. Th1 cell interactions with the TNF stimulated BBB under flow

The $CD34^+$ ECs were cultured in the top channel of the flow chamber with the PCM in the bottom channel for 6 days and the $CD34^+$ ECs were stimulated with TNF (10 ng/mL) 16h to 20h before starting to record the video. Th1 cells are allowed to accumulate on the BLECs monolayer at a low flow rate of 0.1 dyne/cm^2 for 4 min from the first frame after the first Th1 cells appear in the field of view (accumulation phase until 4min55s). After the accumulation phase of precisely 4 min 55 sec, the flow rate was set to 1.5 dyne/cm^2 for 30 min (shear phase). The dynamic T cell interactions with the BLECs monolayer under a physiological flow were recorded at a 10x magnification (EC Plan-Neofluar 10X/0.30 Ph1) with a Zeiss Axiocam MRm camera. During the recording, 1 picture is acquired every 5s then the video is exported with a framerate of 30 images/s. The video is repeated 3 times, the first run shows the full-scale video, the second run is a zoom in of one area highlighting T cells crawling (green circles) and detaching (purple circles) and the last run is a zoom in of one area highlighting diapedesis (blue circles) and probing (orange circles) events. The

red arrow shows the direction of flow and the time is displayed on the top left of the video (min:sec format). Scale = 100 µm. The video is representative of 10 and the statistical analysis of the T cell behavior is showed in the Figure 5B.

Supplementary video 1. Flow chamber adaptation of the human BBB model using 6 well hanging inserts, 0.4 µm pore size, PTFE, coated, Millipore, Sevelen, Switzerland (1st device listed in the supplementary table 1). BLECs are detaching under the flow, and the lack of transparency of the filter makes the imaging of the cell difficult.

Supplementary video 2. Flow chamber adaptation of the human BBB model using 6 well hanging inserts, 0.4 µm pore size, PTFE, coated, Corning Incorporated, NY, USA (5th device listed in the supplementary table 1). T cell on top of BLECs not clearly visible under the flow.

Supplementary video 3. Flow chamber adaptation of the human BBB model using 6 well hanging inserts, 0.4 µm pore size, PET Corning Incorporated, NY, USA (6th device listed in the supplementary table 1). T cell on top of BLECs not visible under the flow. Imaging disturbed by the membrane pores brightness, respect to the membrane insufficiently transparent.

Supplementary video 4. Flow chamber adaptation of the human BBB model using 6 well hanging inserts, 0.4 µm pore size, PET Greiner, Huber & Co. Ag Reinach Switzerland, (7th device listed in the supplementary table 1). T cell on top of BLECs not visible under the flow. Imaging disturbed by the membrane pores brightness, respect to the membrane insufficiently transparent.