BBB ON CHIP: microfluidic platform to mechanically and biochemically modulate blood-brain barrier function

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Abstract The blood-brain barrier (BBB) is a unique feature of the human body, preserving brain homeostasis and preventing toxic substances to enter the brain. However, in various neurodegenerative diseases, the function of the BBB is disturbed. Mechanisms of the breakdown of the BBB are incompletely understood and therefore a realistic model of the BBB is essential. We present here the smallest model of the BBB yet, using a microfluidic chip, and the immortalized human brain endothelial cell line hCMEC/D3. Barrier function is modulated both mechanically, by exposure to fluid shear stress, and biochemically, by stimulation with tumor necrosis factor alpha (TNF-α), in one single device. The device has integrated electrodes to analyze barrier tightness by measuring the transendothelial electrical resistance (TEER). We demonstrate that hCMEC/D3 cells could be cultured in the microfluidic device up to 7 days, and that these cultures showed comparable TEER values with the well-established Transwell assay, with an average ($\pm$ SEM) of 36.9 $\Omega.cm^2$ ($\pm$ 0.9 $\Omega.cm^2$) and 28.2 $\Omega.cm^2$ ($\pm$ 1.3 $\Omega.cm^2$) respectively. Moreover, hCMEC/D3 cells on chip expressed the tight junction protein Zonula Occludens-1 (ZO-1) at day 4. Furthermore, shear stress positively influenced barrier tightness and increased TEER values with a factor 3, up to 120 $\Omega.cm^2$. Subsequent addition of TNF-α decreased the TEER with a factor of 10, down to 12 $\Omega.cm^2$. This realistic microfluidic platform of the BBB is very well suited to study barrier function in detail and evaluate drug passage to finally gain more insight into the treatment of neurodegenerative diseases.

Keywords Blood-brain barrier · hCMEC/D3 · Microfluidics · Shear stress · TNF-α · Transwell

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1 Introduction

The blood-brain barrier (BBB) is a distinctive tissue structure that separates the peripheral blood from the central nervous system, thereby maintaining brain homeostasis (Cardoso et al. 2010; Paolinelli et al. 2011). Disruption of the BBB is implicated in many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis (Grammas et al. 2011; Palmer 2011). The permeability of the BBB is controlled by the brain endothelial cells, as these cells regulate the selective transport from the blood to the brain and vice versa (Cardoso et al. 2010). The endothelial lining is crucial in preventing the entrance of toxic substances to the brain (Paolinelli et al. 2011). Interaction with other cells of the neurovascular unit, such as astrocytes, enhances barrier tightness (Cardoso et al. 2010). Due to this tight barrier, the majority of drugs fail to cross the BBB. Therefore, it is essential to have a realistic model of the BBB, not only to evaluate drug passage, but also to analyze the impairment of

the BBB, as present in neurodegenerative diseases. *In vivo* models are the best candidates to study the BBB, however, these resources are scarce, expensive and are difficult to study both in detail and real-time. *Ex vivo* and *in vitro* models are good alternatives, as they are credited for their simplicity and controlled environment (Cardoso et al. 2010; Cucullo et al. 2005). Nowadays, most BBB experiments are performed in static Transwell assays (Avdeef 2011; Hatherell et al. 2011; Patabendige et al. 2012). However, for a realistic BBB model, the addition of shear stress to mimic the blood flow, is essential (Huh et al. 2011; Cucullo et al. 2008). Shear stress positively affects endothelial cell physiology and tight junction formation (van der Meer et al. 2010). Till now, there are only a few papers published of a dynamic BBB model (Yeon et al. 2012; Booth and Kim 2012; Cucullo et al. 2008; Neuhaus et al. 2006). Recently, the use of microtechnologies to study organ physiology has received rapidly growing attention (Huh et al. 2011). These organs-on-chips have the potential to be used as human-relevant disease models, and additionally provide detailed insight into drug effects (van der Meer et al. 2012; Huh et al. 2011). Two microfluidic models of the BBB have recently been reported, demonstrating the feasibility of an organ-on-chip approach (Booth and Kim 2012; Yeon et al. 2012). However, the reported microfluidic models lack a number of physiologically relevant factors, such as shear stress and human brain endothelial cells. We present here, for the first time, a realistic microfluidic model of the BBB, using immortalized human brain endothelial cells (hCMEC/ D3, Weksler et al. 2005) and including dynamic flow conditions. The transendothelial electrical resistance (TEER) is measured and the tight junction protein Zonula Occludens-1 (ZO-1) is stained to analyze barrier tightness. Moreover, the barrier function is modulated both mechanically (shear stress, 5.8×10^{-1} Pa) and biochemically (tumor necrosis factor alpha (TNF-α), 1 ng/mL). The pro-inflammatory cytokine TNF-α negatively affects barrier integrity and overexpression of this cytokine causes chronic neuroinflammation, which is a common feature in several neurodegenerative diseases (Mc Guire et al. 2011; Frankola et al. 2011; Park and Bowers 2010). The BBB chip is a two-layer device made of PDMS, with a membrane in between to separate the top and bottom channel, based on the work of Douville et al. (2010). Specific key improvements in the chip design were realized in this work. Firstly, inert platinum (Pt) electrodes were integrated, to replace the oxidation sensitive Ag/AgCl electrodes, for TEER measurements. Secondly, the cross-sectional area is 4 times smaller (even 40 times smaller compared to the work of Booth and Kim (2012)). This reduces the number of cells, enabling the use of primary cells, and less medium and drugs are required in dynamic experiments. Besides, the BBB chip is a low-cost and easy to fabricate device of gas permeable PDMS, without the critical need for cleanroom facilities to produce glass devices. Furthermore, in our set-up, the shear stress and

subsequent TNF-α experiment is following the static culture experiment, which in our opinion is of vital importance for relevant comparison. Hence, one single device is used, avoiding possible variability in experimental set-up. Overall, we demonstrate a realistic microfluidic platform, comprising the smallest BBB on chip, and capable of mechanically and biochemically modulating the barrier function.

2 Materials and methods

2.1 Cell culture

The immortalized human brain endothelial cell line hCMEC/ D3 was cultured in EBM-2 basal medium (Lonza, Basel, Switzerland), containing 5 % Fetal Bovine Serum (FBS, PAA Laboratories GmbH, Pasching, Austria), 1 % Penicillin-Streptomycin (Life Technologies, Paisley, UK), 1.4 µM hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 5 µg/ml L-acid ascorbic (Sigma-Aldrich), 1 % CD Lipid Concentrate (Life Technologies), 10 mM HEPES (Sigma-Aldrich) and 1 ng/ml basic fibroblast growth factor (bFGF, Sigma-Aldrich) (= D3 medium). Medium was refreshed every 3–4 days. Confluent cell cultures were detached using 0.05 % trypsin and subcultured in culture flasks coated with 150 µg/ml rat collagen I (R&D Systems, Abingdon, UK). hCMEC/D3 was maintained in a humidified incubator at 37 °C and 5 % CO_2.

2.2 BBB chip

2.2.1 Fabrication

The BBB chip (Fig. 1) is a two-layer membrane-based device made of poly(dimethylsiloxane) (PDMS, Sylgard 184 Silicone elastomer kit, Dow Corning, Midland, MI, USA). Both the top and bottom channel have a length of 1 cm, a width of 500 µm and a depth of 100 µm. Moreover, the top and bottom part of the BBB chip contains a groove (l=1 cm, w=200 µm, d=300 µm) for the platinum (Pt) electrodes. The BBB chip was fabricated by pouring PDMS pre-polymer (degassed mixture of the silicone elastomer and the curing agent at a weight ratio of 10:1), on a 4-inch silicon wafer, containing the photolithographically patterned, micrometer-sized SU-8 structures. The second layer of the SU-8 structure was spin-coated on the wafer after an additional step of aluminum deposition, which was removed by etching, after the final photolithographic step. The PDMS pre-polymer was baked at 60 °C overnight to cure and thereafter the inlet and outlet were punctured. At the cross-section, a Transwell polycarbonate membrane with a thickness of 10 µm and a pore size of 0.4 µm (Corning Inc, Corning, NY, USA), was used to separate the top channel from the bottom channel. Pt wires with a diameter of 200 µm (Heraeus Drijfhout BV, Amsterdam, The

Fig 1 Schematic representation of (**a**) (1) the top part, (2) Transwell membrane and (3) the bottom part of the BBB chip, with side-channels to incorporate the (4) Pt electrodes and (**b**) the assembled device. (**c**) Picture of the BBB chip made in PDMS. The opaque rectangle represents the Transwell membrane
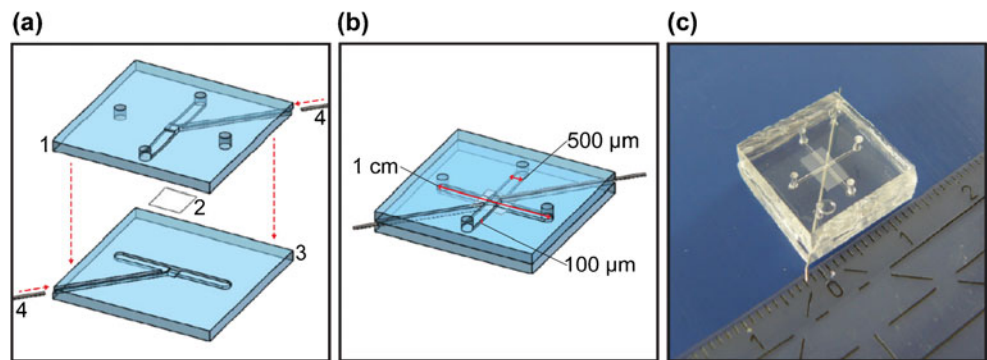
Netherlands) were gently slided in the top and bottom groove. The top and bottom of the chip, with the membrane in the middle, were assembled together using a PDMS mortar (Chueh et al. 2007) to ensure a tight and leakage free bonding. Finally, the Pt electrodes were fixed with norland optical adhesive 81 (NOA 81, Fisher Scientific, Hampton, NH, USA) and the assembled BBB chip was left at 60 °C overnight for curing.

2.2.2 Experimental set-up

The BBB chip was coated with 150 µg/ml rat collagen type 1, before hCMEC/D3 cell seeding (10×10^6 cells/ml). Non-adhered cells were removed 1 h after seeding. D3 medium was refreshed every 12 h in the chip (= static culture).

2.2.3 Mechanical and biochemical modulation of the barrier

After 3 days of static culture in the BBB chip, the hCMEC/D3 cells were exposed to shear stress by flowing medium through the top and bottom channel at a flow rate of 2.5 ml/h for 18 h, generating an average wall shear stress of 5.8×10^{-1} Pa. Thereafter, the TEER was measured. Then, barrier function was modulated biochemically, by exposing the hCMEC/D3 cells to 1 ng/ml TNF-α (Sigma-Aldrich) for 2 h at a flow rate of 1 µl/min. After 2 h, the TEER was measured, as described in Section 2.2.4.

2.2.4 TEER analysis using impedance spectroscopy

To measure the TEER, the BBB chip was connected to a HP4194A impedance/gain phase analyzer (Hewlett-Packard, Palo Alto, CA, USA). Every collagen coated chip was analyzed prior to cell seeding. Impedance spectra were recorded using an alternating current (AC) with an amplitude of 0.2 V ranging from 100 Hz to 10 MHz over the two embedded Pt electrodes. For each measurement, five readings and 400 data points per reading were recorded. The impedance analyzer was controlled with LabVIEW 7.1 software (National Instruments, Austin, TX, USA).

The least-square optimization method was used to fit the measured impedance data on the BBB chip to an electrical equivalent circuit model, adapted from Wegener et al. (1999), in EC-lab V10.02 (Fig. 2 insert). In this model, the constant phase element represents the double layer capacitance of the Pt electrodes (CPE_{dl}), which is in series with the resistance of the medium (R_{med}) and the cell monolayer, which is denoted as the resistance of the cell (R_{cel}) and the capacitance of the cell membrane (C_{cm}) in parallel. First, the impedance spectrum (Fig. 2a) and phase shift (Fig. 2b) recorded at day 0 were both used to fit values for CPE_{dl} in the low frequency range (150–300 Hz). Second, in the high frequency range (2 MHz) R_{med} is calculated. This was done in each experiment for every single device. Finally, these parameter values were put in the equivalent circuit model. For every following day, the equivalent model was loaded, 160 data points from 2 kHz to 200 kHz were selected and R_{cel} and C_{cm} were fitted to this data by least-square optimization. R_{cel} was normalized for the cross sectional surface area of the chip (2.5×10^{-3} cm^2) to calculate the TEER value in $\Omega \cdot$cm^2.

2.3 Conventional Transwell assay

Transwell polycarbonate membrane inserts (12 mm diameter, 0.4 µm pore size, Corning Inc.) were coated with 150 µg/ml rat collagen I for 1 h at 37 °C prior to seeding 2×10^5 hCMEC/D3 cells per insert. Non-adhered cells were removed after 2 h and D3 medium was refreshed at day 4 and 7.

2.3.1 TEER analysis

TEER values of hCMEC/D3 monolayers in the Transwell inserts were measured daily up to 7 days. Medium of the luminal compartment was refreshed prior to TEER measurements. Pt electrodes were placed at a fixed distance in the Transwell and were connected to a SP-300 potentiostat (BioLogic, Claix, France). Impedance spectra were recorded using an alternating current (AC) with an amplitude of 10 mV ranging from 1 Hz to 3 MHz over the two Pt electrodes. For each measurement, four readings and 51 data points per reading were recorded. The TEER of the cell
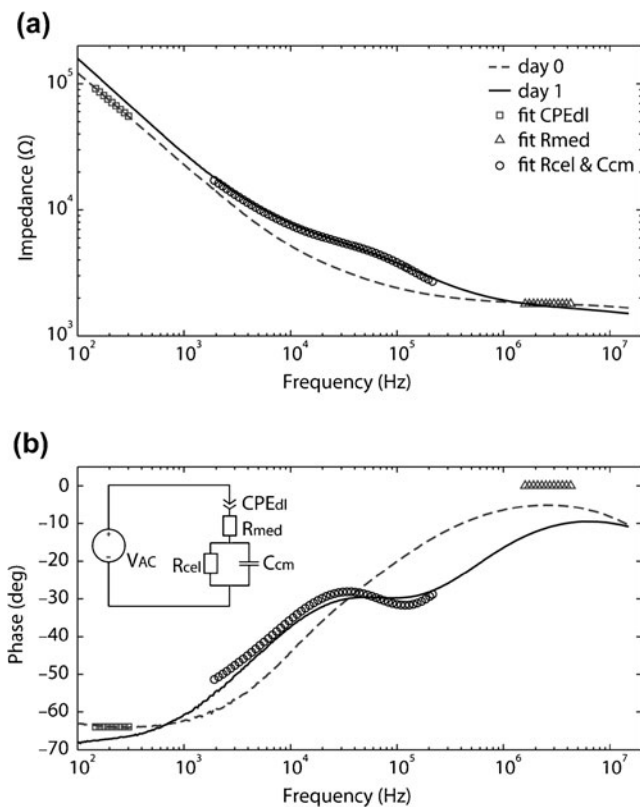
Fig 2 (a) Impedance plot and (b) phase plot of a collagen coated chip (blank, day 0, dashed line) and of hCMEC/D3 cells cultured in the BBB chip (day 1, black line), including the equivalent circuit model and fitted data points. For readability, every third consecutive point of the fitted data points is represented in the graph. The least-square optimization method was used to fit the measured impedance data on chip to an electrical equivalent circuit model, see insert phase plot. In the low frequency range (150–300 Hz), the phase shift was approximately −65 °, therefore this is represented as a constant phase element (CPE$_{dl}$) in the equivalent circuit model. In the high frequency range (2 MHz), the phase shift was equal to zero, hence a resistor (R$_{med}$). In the middle frequency range (2 kHz–200 kHz), a maximum was reached in the phase shift at day 1. This is depicted in the analogous impedance plot as the onset of a resistive plateau (R$_{cel}$) and is due to the presence of the tight monolayer. In this experiment R$_{cel}$ was 2374Ω, resulting in a TEER of 5.9 Ω.cm^2 at day 1, with χ^2=0.4. For detailed information, see materials and methods Section 2.2.4

monolayer measured at the frequency of 10 kHz was corrected for collagen coated blank inserts.

2.4 Fluorescent staining of cells

Barrier characteristics of the hCMEC/D3 monolayer in the BBB chip was analyzed with confocal microscopy. The presence of tight junctions was determined by staining for Zonula Occludens-1 (ZO-1) and actin filaments were stained with phalloidin. hCMEC/D3 cells were fixated with 3.7 % paraformaldehyde (Sigma-Aldrich) in PBS for 15 min and permeabilized for 30 min in blocking buffer (BB). BB consisted of PBS with 2 % goat serum (Life Technologies) and 0.1 % Triton X-100 (Sigma-Aldrich). hCMEC/D3 cells were stained

with phalloidin-FITC (1 μg/mL in BB; Bio-Connect, Huissen, The Netherlands) for 1 h or the primary antibody ZO-1 (1:50 in BB; BD Biosciences, Oxford, UK) for 2 h. Then, the secondary antibody labeled with Alexa488 (1:1000 in BB; Life Technologies) for ZO-1 and propidium iodide (PI, 1 μg/ml, Life Technologies) for nucleus staining, were added for 1 h. Images were taken using a ZEISS LSM 510 confocal microscope.

2.5 Statistical analysis

Results are expressed as the mean±standard error of the mean (SEM). In the static experiments, a complete series up to day 7 was performed 4 times on the Transwell (each individual experiment measured in quadruplicate) and at least 5 times on the BBB chip. For the BBB chip TEER analysis, the Pearson's chi-squared test was used, and fitted datasets with χ^2<10 were included. The shear stress and subsequent TNF-α experiment was performed once.

3 Results and discussion

3.1 BBB chip

The BBB chip consists of two microfluidic compartments, separated by a conventional Transwell polycarbonate membrane with 0.4 μm pore-size (Fig. 1a). Two Pt electrodes are used to analyze the electrical impedance over the membrane (Fig. 1b). The chips are disposable and can be fabricated rapidly (4 devices in one day) (Fig. 1c).

3.2 Confocal microscopy

hCMEC/D3 cells were seeded in the top channel and allowed to adhere for 1 h to the membrane. After 1 day, the cells had formed a monolayer in the top channel, as demonstrated by the actin filament staining (Fig. 3a). No cells were found in the bottom channel. In the following days, the monolayer developed as a typical blood-brain barrier and formed tight junctions, as evidenced by the clear, ZO-1-positive staining between the cells on day 4 (Fig. 3b).

3.3 TEER analysis BBB chip vs. Transwell

The tightness of the monolayer was analyzed by measuring the TEER every day. After 2 days in the BBB chip, the TEER reached a value (± SEM) of 33.3 Ω.cm^2 (± 10.3 Ω.cm^2), the onset of a plateau with an average of 36.9 Ω.cm^2 (±0.9 Ω.cm^2) (Fig. 4). These values were similar to TEER values (plateau average of 28.2 Ω.cm^2±1.3 Ω.cm^2) of monolayers of hCMEC/D3 cultured in conventional Transwell plates (Fig. 4), and comparable with reported Transwell data (Hatherell et al.
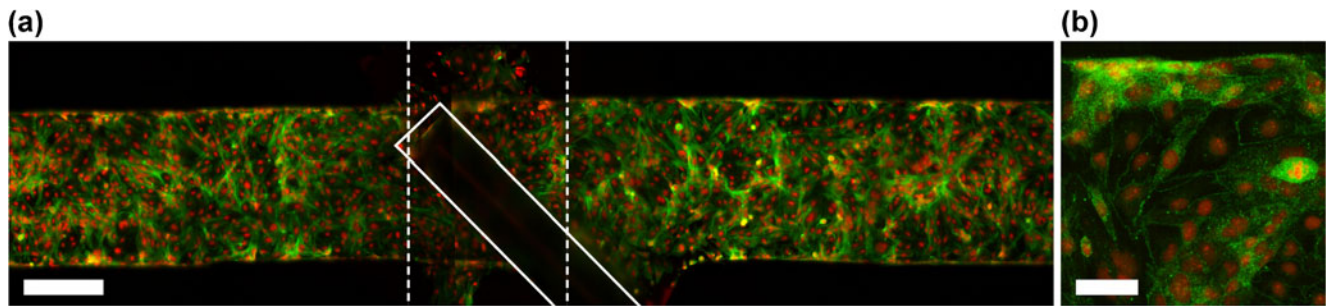
(a)

(b)

Fig 3 Confocal microscopy pictures of (**a**) the cross-section (scale bar 250 μm) and (**b**) a zoom-in of the top channel (scale bar 50 μm). hCMEC/D3 cells were stained (**a**) 1 day or (**b**) 4 days after seeding, with PI (*red*, nucleus) and (**a**) phalloidin (*green*, actin filaments) or (**b**) ZO-1 (*green*, tight junctions). White line marks the top electrode, and white dashed lines the bottom channel

2011; Markoutsa et al. 2011; Weksler et al. 2005). The TEER remained constant up to day 7, which illustrates the stability and biocompatibility of this realistic microfluidic platform. Moreover, the TEER data in the BBB chip matched the confocal microscopy pictures of ZO-1 staining. The SEM of the BBB chip is larger compared to the SEM of the Transwell data (Fig. 4). This is attributed to the difference in electrode design. The electrode surface used in Transwell is 28 times larger than in the BBB chip, which results in a higher double layer capacitance of the electrodes. As a consequence, the transition of the double layer capacitance to the resistive plateau occurs at higher frequencies for the BBB chip, therefore increasing the noise. In addition, on chip, the position of the electrodes varied slightly from batch to batch due to the fabrication method. To overcome these problems, sputtered electrodes with a higher surface area will be integrated in a future design.

3.4 Mechanical modulation of the barrier

Numerous reports have emphasized the importance of physiological levels of shear stress on endothelial cell function (van der Meer et al. 2010; Davies 2009; Li et al. 2005) in general and on cerebral microvascular endothelial physiology in particular (Huh et al. 2011; Cucullo et al 2008). As a result of the microfluidic origin of the BBB chip, shear stress could be applied easily to the endothelial monolayer. After culturing the cells in the BBB chip under static conditions for 3 days, the device was connected to a syringe pump and the cells were subjected to a flow of 2.5 ml/h. The theoretical value of the wall shear stress due to this flow is 5.8×10^{-1} Pa (= 5.8 dyn/cm^2), which is in the physiologically relevant range. The maximal shear stress in blood capillaries in the brain is about 5 dyn/cm^2 (Cucullo et al. 2005). After subjecting the endothelial monolayers to shear stress for 18 h, the TEER increased by a factor of 3, up to 120 Ω.cm^2 (Fig. 5). To compare our measured TEER values with the work of Cucullo et al. (2008) is difficult. Although Cucullo et al. (2008) used the same cell line and comparable shear stress (4 dyn/cm^2), their DIV-BBB is a macroscopically measurement set-up and the TEER value (400 Ω.cm^2) is measured after applying shear stress for 2 days. Neuhaus et al. (2006) also described a dynamic model (shear stress between 2.7 and 3.9 dyn/cm^2) of the BBB, using endothelial cells of porcine origin, however, no dynamic TEER values were described. Till now, only Booth and Kim (2012)
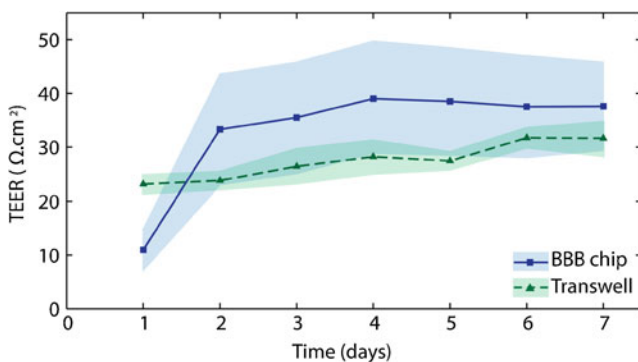

Fig 4 TEER measurements of hCMEC/D3 cells cultured in the Transwell and the BBB chip, both under static conditions. TEER was measured daily up to 7 days. In the Transwell a complete series up to day 7 was performed 4 times. In the BBB chip $n=6$ for day 2, 3, 4 and 7; and $n=5$ for day 1, 5 and 6. TEER of hCMEC/D3 reached a steady state at day 2, which persisted up to day 7 in both Transwell and the BBB chip. The average±SEM of this plateau is 28.2 Ω.cm^2±1.3 in Transwell and 36.9 Ω.cm^2±0.9 in the BBB chip. Mean values±SEM are shown
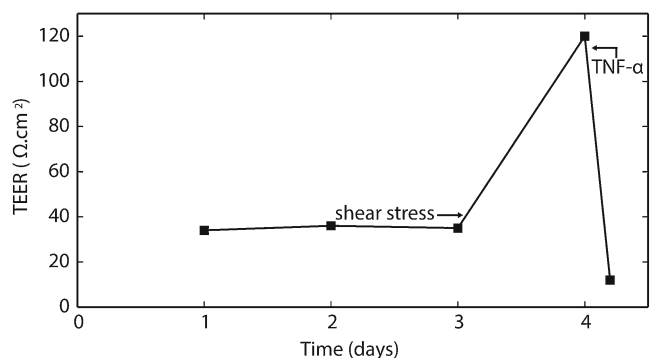

Fig 5 Mechanical and biochemical modulation of the BBB on chip. hCMECnn cells were statically cultured for 3 days with the TEER measured every day. Then, shear stress (5.8×10^{-1} Pa) was applied for 18 h, followed by a 2 h flow of TNF-α (1 ng/ml), with accompanied TEER values plotted. Graph represents data from one experiment performed in a single device

developed a dynamic model of the BBB on chip, though a shear stress of only 2.3×10^{-3} Pa (2.3×10^{-2} dyn/cm^2) was applied, which is not physiologically relevant. Therefore, the TEER value (250 Ω.cm^2) measured in their μBBB model demonstrates a discrepancy with TEER data (25 Ω.cm^2) acquired in the conventional Transwell model. Opposite to Cucullo et al. (2008) and Booth and Kim (2012), we are the first to compare static TEER values with dynamic TEER values, in the same microfluidic device, avoiding experimental variability.

3.5 Biochemical modulation of the barrier

In addition to shear stress-induced enhancement of the endothelial barrier function, barrier properties can also be affected by biochemical stimulation, both *in vivo* and *in vitro* (Neuwelt et al. 2008; Forster et al. 2008; Bellavance et al. 2008). In order to establish the utilization of the BBB chip, to determine the effects of biochemical stimulation, hCMEC/D3 cells were grown in the BBB chip for 3 days, stimulated with shear stress for 18 h and then stimulated with 1 ng/ml TNF-α for 2 h. This led to a 10-fold decrease in the TEER, down to 12 Ω.cm^2 (Fig. 5). We are the first to demonstrate the effect of TNF-α on shear-stress treated hCMEC/D3 cells, hence no comparison with other literature is possible. Forster et al. (2008) showed that under static conditions in Transwell, 10 nM TNF-α (17.4 ng/ml) decreased the TEER with a factor of 2 after 8 h incubation. This suggests that the effect of TNF-α after mechanical stimulation has more impact and occurs more rapidly.

4 Conclusion

In this work, we developed a microfluidic model of the BBB, comprising of a top and bottom channel, with a polycarbonate filter in between. Platinum electrodes were integrated to measure the transendothelial electrical resistance (TEER) over time. Human brain endothelial cells (hCMEC/D3) could be cultured for up to 7 days in the BBB chip, and expressed blood-brain barrier characteristics over time, as confirmed by clear Zona Occludens-1 staining at day 4. For BBB chip TEER analysis, an equivalent circuit was defined to fit the measured impedance data. Maximum TEER values were reached at day 2 in the chip, and remained constant till day 7. Blood-brain barrier function was modulated straightforward by stimulation of shear stress, followed by exposure to the inflammation cytokine TNF-α. In conclusion, these experiments demonstrate the versatile potential of this microfluidic platform as a human-relevant disease model and facilitates detailed barrier (dys)function studies.

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