Video Article

An *In Vitro* Model of the Blood-brain Barrier Using Impedance Spectroscopy: A Focus on T Cell-endothelial Cell Interaction

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Abstract

Breakdown of the blood-brain barrier (BBB) is a critical step in the development of autoimmune diseases such as multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). This process is characterized by the transmigration of activated T cells across brain endothelial cells (ECs), the main constituents of the BBB. However, the consequences on brain EC function upon interaction with such T cells are largely unknown. Here we describe an assay that allows for the evaluation of primary mouse brain microvascular EC (MBMEC) function and barrier integrity during the interaction with T cells over time. The assay makes use of impedance cell spectroscopy, a powerful tool for studying EC monolayer integrity and permeability, by measuring changes in transendothelial electrical resistance (TEER) and cell layer capacitance (Ccl). In direct contact with ECs, stimulated but not naïve T cells are capable of inducing EC monolayer dysfunction, as visualized by a decrease in TEER and an increase in Ccl. The assay records changes in EC monolayer integrity in a continuous and automated fashion. It is sensitive enough to distinguish between different strengths of stimuli and levels of T cell activation and it enables the investigation of the consequences of a targeted modulation of T cell-EC interaction using a wide range of substances such as antibodies, pharmacological reagents and cytokines. The technique can also be used as a quality control for EC integrity in *in vitro* T-cell transmigration assays. These applications make it a versatile tool for studying BBB properties under physiological and pathophysiological conditions.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54592/>

Introduction

The blood-brain barrier separates the systemic circulation from the central nervous system (CNS)¹⁻³. It provides a physical barrier that inhibits the free movement of cells and the diffusion of water-soluble molecules and protects the brain from pathogens and potentially harmful substances. In addition to its barrier function, the BBB enables the delivery of oxygen and nutrients to the brain parenchyma, which ensures proper functioning of the neuronal tissue. Functional properties of the BBB are highly regulated by its cellular and acellular components, with highly specialized ECs being its main structural element. ECs of the BBB are characterized by the presence of tight junction (TJ) complexes, the lack of fenestrations, extremely low pinocytotic activity, and permanently active transport mechanisms. Other components of the BBB the EC basement membrane, pericytes embedding the endothelium, astrocytic end feet and = associated parenchymal basement membrane also contribute to the development, maintenance and function of the BBB^{2,4-6} and, together with neurons and microglia, form the neurovascular unit (NVU), which enables proper functioning of the CNS⁷⁻⁹.

In a variety of neurological diseases, such as neurodegenerative, inflammatory or infectious diseases, the function of the BBB is compromised^{2,5,10}. Dysregulation of TJ complexes and molecular transport mechanisms leads to increased BBB permeability, leukocyte extravasation, inflammation and neuronal damage. In order to study BBB properties under such pathophysiological conditions, various *in vitro* BBB models have been established^{9,11,12}. Together they have provided valuable insights into the changes of barrier integrity, permeability as well as transport mechanisms. These models employ endothelial cells of human, mouse, rat, porcine or bovine origin¹³⁻¹⁸; primary endothelial cells or cell lines are cultured either as a monoculture or together with pericytes and/or astrocytes in order to mimic more closely the BBB *in vivo*¹⁹⁻²⁵. In recent years, measurement of transendothelial electrical resistance (TEER) has become a widely accepted tool to assess endothelial barrier properties^{26,27}.

TEER reflects the impedance to the ion flux across the cell monolayer and its decrease provides a sensitive measure of compromised endothelial barrier integrity and hence increased permeability. Various TEER measurement systems have been developed, including Epithelial Voltammeter (EVOM), Electric Cell-substrate Impedance Sensing (ECIS), and real-time cell analysis^{15,28-30}. TEER reflects the resistance to the ion flux between adjacent ECs (paracellular route) and is directly proportional to the barrier integrity. In impedance spectroscopy^{27,31}, complex

total impedance (Z) is measured, which provides additional information about the barrier integrity by measuring Ccl. Ccl relates to the capacitive current through the cell membrane (transcellular route): the cell layer acts like a capacitor in the equivalent electric circuit, separating the charges on both sides of the membrane and is inversely proportional to the barrier integrity. When grown on permeable inserts, ECs adhere, proliferate and spread over the microporous membrane. This resists the background capacitive current of the insert (which itself acts like a capacitor) and leads to a decrease in the capacitance until it reaches its minimal level. This is followed by the establishment of TJ complexes that seal off the space between adjacent ECs. This restricts the ion flux through the paracellular route, and TEER increases until it reaches its plateau. Under inflammatory conditions, however, the endothelial barrier is compromised: TEER decreases as TJ complexes get disrupted and Ccl increases as the capacitive component of the insert rises again.

Our TEER measurement uses the automated cell monitoring³² system: it follows the principle of impedance spectroscopy and extends its previous applications. Here, we describe an *in vitro* BBB model that enables the study of the barrier properties, including the interaction of brain endothelium with immune cells; in particular activated T cells. Such pathophysiological conditions are observed in autoimmune diseases of the CNS, such as multiple sclerosis and its animal model experimental autoimmune encephalomyelitis³³⁻³⁷. Here, a crucial step is the transmigration of encephalitogenic, myelin-specific T cells across the BBB. This is followed by their reactivation in the perivascular space and entry into the brain parenchyma, where they recruit other immune cells and mediate inflammation and subsequent demyelination^{1,35,38}. However, molecular mechanisms of the interaction between such T cells and endothelial cells, the main constituents of the BBB, are not well understood. Our protocol aims to fill this gap and give new insights into the consequences on endothelial cells (*i.e.*, barrier integrity and permeability) upon their direct contact and complex interplay with activated T cells.

The protocol described here makes use of primary mouse brain microvascular endothelial cells, grown as a monolayer on permeable inserts with microporous membranes. Endothelial cells are co-cultured with CD4⁺ T cells, which can be pre-activated either polyclonally or in an antigen-specific fashion. Co-culture of MBMECs with pre-activated, but not naïve T cells induces a decrease in TEER and an increase in Ccl, which provides a quantitative measure of the MBMEC dysfunction and barrier disruption. The technique is non-invasive: it uses built-in instead of chopstick electrodes, which prevent major disturbance of the EC monolayer; it can be used to monitor barrier function without the use of cell markers. It makes continuous measurements in an automated fashion and enables an independent assessment of the two barrier parameters (TEER and Ccl) simultaneously over time. The method is also sensitive enough to distinguish between different levels of T cell activation and effects of such T cells on ECs.

It can be used in a wide range of functional assays: different cytokines and/or chemokines implicated in inflammatory processes can be added to the co-culture of MBMECs and T cells; blocking antibodies against cell adhesion molecules on either the EC or T-cell side can be used; and inhibitors of T cell activation markers or of their cytolytic properties can be added during the T-cell priming or their co-culture with ECs. The assay is also useful for T-cell transmigration assays, as it can serve as a quality control of the MBMEC monolayer integrity prior to the addition of T cells. All this makes this method a versatile and reliable tool to study the BBB *in vitro*, with a focus on the effect of activated T cells on EC monolayer integrity. This is of particular importance for understanding the mechanisms of the BBB disruption in the pathogenesis of autoimmune diseases, such as MS and its animal model EAE, where self-reactive, encephalitogenic T cells cross the BBB and cause inflammation and neuronal damage.

Protocol

For all experiments, mice were bred and maintained under specific pathogen-free conditions in the central animal facility at the University of Münster, according to German guidelines for animal care. All experiments were performed according to the guidelines of the animal experimental ethics committee and approved by the local authorities of North Rhine-Westphalia, Germany (LANUV, AZ 84-02.05.20.12.217).

1. MBMEC Isolation and Culture

NOTE: Isolate MBMECs as previously described in detail¹⁴ with the following modifications:

1. Sacrifice 20 adult C57BL/6 mice (6-8 weeks old) by CO₂ inhalation and confirm their death by ascertaining cardiac and respiratory arrest.
2. Rinse the mouse with 70% ethanol and decapitate it using scissors; remove the scalp using forceps and scissors. Make incisions on the left and right side of the skull, starting from the foramen magnum. Lift the skull from its caudal side and take out the brain with forceps.
3. Under a laminar flow hood, use sterile forceps to place the brain in a Petri dish and remove the brain stem, cerebellum, and thalamus, keeping only the cortex.
NOTE: Use of a microscope is not necessary for any of these steps.
4. Place the cortex onto a piece of sterile Western blotting paper and roll it gently with forceps until meninges are no longer visible.
5. After mechanical and enzymatic digestion (following the protocol¹⁴), collect endothelial cells from the density gradient by using a long, sterile needle and a 5 ml plunger. Endothelial cells appear as a murky layer above the red ring with erythrocytes. Transfer 20-25 ml of this layer into a new 50 ml centrifugation tube; top up the tube with DMEM.
6. After two rounds of washing¹⁴, resuspend the cells in 6 ml of MBMEC medium containing Puromycin (4 µg/ml) and seed them onto six coated wells of a 24-well plate; leave them in a tissue culture incubator at 37 °C and 5% CO₂ (referred to as 'incubator' from here on) for three days.
NOTE: For details of MBMEC coating solution, see **Materials Table**.
7. Change the medium by adding 1 ml to each well of fresh MBMEC medium without Puromycin; put the plate back at 37 °C and 5% CO₂ for two more days.

2. Harvesting MBMECs

1. On day five after MBMEC isolation, pre-coat permeable inserts with MBMEC coating solution: add 80 µl of solution per insert and leave them at 37 °C and 5% CO₂ for 3 hr.

2. Carefully pipette out the coating solution and let the inserts air-dry for 30 min. Add 2 ml of room temperature (RT) PBS to each well of the plate, using MBMECs to wash the medium; repeat this step. Add 0.05% Trypsin-EDTA (300 μ l per well) and leave the plate in the incubator for 5-10 min.
 3. Add 2 ml of MBMEC medium to each well to stop trypsinization. Transfer the collected cells to a 15 ml centrifugation tube and centrifuge them at 700 x g for 8 min at 4 °C. Discard the supernatant and resuspend the cells in 1 ml of MBMEC medium without Puromycin.
 4. Count the cells; mix 10 μ l of cell suspension with 90 μ l of 0.04% Trypan Blue (10x dilution of cells). Pipette 10 μ l of this mix between the glass cover and the cell counting chamber (hemocytometer). Count Trypan Blue-free cells in all four quadrants of the chamber (N) and determine the average number of counted cells (N') as follows: $N' = N/4$.
 5. Calculate the cell concentration (in 10^6 cells/ml) using the following formula: $C = N' \times 10^4 \times 10$, where 10^4 is given by the dimensions of the hemocytometer and 10 is the dilution factor from step 2.4.
 6. Seed 2×10^4 cells in a volume of 260 μ l per insert. Add 810 μ l of MBMEC medium to the lower compartment of each well. Place the plate with inserts in the incubator until ready to proceed with section 4.
- NOTE: The volumes for the upper and lower compartment are insert-specific and do not work for all 24-well formats.

3. CD4⁺ T Cell Isolation and Stimulation

1. CD4⁺ T Cell Isolation
 1. Sacrifice one adult mouse (6-8 weeks old) by CO₂ inhalation and confirm its death by ascertaining cardiac and respiratory arrest.
 2. Place the mouse on its back on a clean dissection board and rinse it with 70% ethanol; use sterile scissors and forceps to open the peritoneum and remove the spleen and lymph nodes (inguinal, axillary, brachial and cervical); finally, transfer the tissue into a 15 ml centrifugation tube containing 5 ml of PBS on ice.
 3. Under the laminar flow hood, transfer the tissue by decanting PBS with the tissue into a 50 ml centrifugation tube with a 70 μ m cell strainer on top; homogenize the tissue by pressing it with a 1 ml plunger through the strainer.
 4. Add 30 ml of FACS buffer and centrifuge it at 500 x g for 5 min at 4 °C. Discard supernatant and resuspend the cells in 10 ml of FACS buffer. Filter the suspension through a 40 μ m cell strainer, add another 20 ml of FACS buffer to the tube.
 5. Centrifuge it at 500 x g for 5 min at 4 °C. Discard the supernatant and resuspend the cells in 500 μ l of FACS buffer.
 6. Add 20 μ l of mouse CD4 magnetic microbeads, mix well and incubate for 15 min at 4 °C. Add 25 ml of FACS buffer and centrifuge it at 500 x g for 5 min at 4 °C.
 7. In the meantime, place an LS separation column into the magnet and rinse it with 3 ml of FACS buffer. Discard the supernatant and resuspend the cells in 3 ml of FACS buffer.
 8. Add cells to the column. Wash the column with 3 ml of FACS buffer three times. Remove the column from the magnet and place it onto a new 15 ml centrifugation tube. Add 5 ml of FACS buffer, flush out the labelled cells with the column plunger and centrifuge it at 500 x g for 5 min 4 °C.
 9. Discard the supernatant and resuspend the cells in 3 ml of T cell medium. Count the cells as described in 2.4 and seed 1×10^5 cells in 100 μ l of medium per well.
2. CD4⁺ T Cell Stimulation
 1. Polyclonal CD4⁺ T cell stimulation
 1. Pre-coat a round-bottom 96-well plate with purified anti-mouse CD3 antibody (clone 145-2C11) in PBS, at a desired final concentration. For example, to pre-coat the full plate with α -CD3 at 1 μ g/ml, mix 10 μ l of the antibody (stock concentration = 0.5 mg/ml) with 5 ml of PBS, vortex and add 50 μ l of the mix to each well with a multichannel pipette.
 2. Leave the plate in the incubator for 3 hr. After isolating the T cells, wash the pre-coated plate twice with PBS. Add purified anti-CD28 antibody (clone 37.51) to isolated T cells at a desired final concentration (e.g., at 1 μ g/ml); mix well. Seed the T cells and leave them in the incubator for two to three days.
 2. Antigen-specific CD4⁺ T cell stimulation with dendritic cells (DCs)

NOTE: If DCs are used as antigen-presenting cells (APCs), follow the Protocol for T cell isolation, with these exceptions:

 1. Before homogenizing the spleen, inject it with 1 ml of Collagenase type IA in PBS at 0.5 mg/ml and transfer it to a 15 ml centrifugation tube.
 2. Incubate in the water bath at 37 °C for 15 min. After washing with PBS, resuspend the pellet in FACS buffer and add 20 μ l of mouse CD11c magnetic microbeads, instead of CD4 microbeads.
 3. Use an MS separation column and the appropriate volumes: rinse the column with 1 ml of FACS buffer; resuspend cells in 1 ml of FACS buffer and wash the column with 1 ml of FACS buffer three times.
 4. Add antigen of choice to DCs (e.g., myelin oligodendrocyte glycoprotein (MOG) a.a. 35-55 at 20 μ g/ml), mix well and seed 5×10^4 cells in 100 μ l of T cell culture medium per 96-round-bottom-well.
 5. Add 1×10^5 T cells in 100 μ l of T cell culture medium per 96-round-bottom-well at the end, as described in the Protocol for T cell isolation.
 3. Antigen-specific CD4⁺ T cell stimulation with B cells

NOTE: If B cells are used as APCs, follow the Protocol for T cell isolation, with these exceptions:

 1. Use spleens from transgenic mice whose B cells are antigen-specific (e.g., IgH^{MOG} (Th) mice, whose B cells specifically recognize MOG₃₅₋₅₅).
 2. Use antigen-specific T cells (e.g., from TCR^{MOG} (2D2) mice). Add 20 μ l of mouse CD19 magnetic microbeads instead of CD4 microbeads.
 3. Add the antigen of choice to the B cells (e.g., MOG₃₅₋₅₅ at 20 μ g/ml), mix well and seed 5×10^4 cells in 100 μ l of B cell culture medium per 96-round-bottom-well.
 4. Add 1×10^5 T cells in 100 μ l of T cell culture medium per 96-round-bottom-well, as described in the Protocol for T cell isolation.

4. Setting Up and Performing TEER Measurement

1. Place the 24-well module of the TEER instrument under the laminar flow hood. Remove the lids and place inserts with MBMECs in the instrument using forceps.
2. Pipette 810 μ l of fresh medium to the lower compartment of the module wells: add it carefully between the insert and the wall of the module well.
3. Close the lids and place the instrument in the incubator. Connect the instrument to its computer; turn on the instrument controller and open the software.
4. Select 'new measurement' in the pop-up window. Check 'show TEER' and 'show Ccl' boxes; then press 'start'. After completing the first measurement, select 'check all wells' in the 'results' tab to see all TEER and Ccl values.
5. Save the file: File>Save as>'the name of your file'.
NOTE: As TEER and Ccl are continuously measured in an automated fashion, monitor their values over a period of three to five days. Changing the medium is not necessary, unless cell viability is suboptimal, as visualized by a lack of increase in TEER.
6. Choose the time point to co-culture MBMECs with T cells when Ccl is stable and lower than 1 μ F/cm² and TEER has reached its maximum level.
7. Carefully inspect absolute TEER and Ccl values and exclude the wells in which MBMECs have not developed confluent enough monolayers by unchecking such wells.
8. Group the rest of the wells: right-click on a well and select 'add well to new average well'. Name it in the pop-up window; do the same for all individual wells to be grouped.
9. Check all average wells to confirm that all of them have the same initial conditions before the co-culture. If some have significantly different absolute TEER values or TEER slopes, or the standard errors are too large, redo the grouping.
NOTE: The optimal grouping of wells provides minimal variation in TEER values, both within and between experimental groups.
10. In the 'experiment' tab, press 'pause', disconnect the instrument and take it out of the incubator. Remove the lids under the laminar flow hood.
11. Prepare pre-activated and/or naïve T cells, with or without specific cytokines, antibodies or other substances, as desired: For example: mix purified NA/LE rat anti-mouse IFN- γ antibody (clone XGM1.2) with prepared T cells, at 20 μ g/ml per well of the TEER instrument.
NOTE: If substances such as granzyme B inhibitor are used, they are added to T cell culture at the beginning of T cell stimulation: e.g., Granzyme B Inhibitor II (Calbiochem) is mixed with isolated T cells at a final concentration of 10 μ M in DMSO. See Materials and Equipment for more details.
12. Remove some of the medium from the insert (the upper well compartment): e.g., carefully pipette out 150 μ l (removing all the medium should be avoided as it could disturb the MBMEC monolayer).
13. Add T cells to MBMECs by carefully pipetting 150 μ l of medium containing 2×10^5 cells/insert.
NOTE: When harvesting pre-activated T cells, count only blasting, Trypan Blue-free cells.
14. Close the lids and place the instrument back to the incubator. Reconnect the instrument and press 'resume measurement'. After 24 hr, press 'stop' in the 'experiment' tab and save the file (File>Save).

5. Data Export and Statistical Analysis

1. Export the results by choosing File>Export.
2. In the "settings" tab of the pop-up window, select ".dot" in the "decimal separator" option and "tabulator" in the "field delimiter" option.
3. In the "export results" tab, name the file, check all the wells to be exported, and check the "TEER" and "Ccl" boxes in the "choose data" option.
4. Press "export data," open the exported file, and copy the data to a spreadsheet.
5. Normalize the exported data (sorted by each replicate, with TEER and Ccl values given for each run (measurement)): Set TEER and Ccl values of the last time point before the co-culture to 100% and change accordingly the values for all other runs, relative to the '100%' run.
6. Copy normalized data to a software of choice to generate a graph, using individual wells and displaying the standard error of the mean for each treatment group.
7. Perform Two-way ANOVA statistical test with a Bonferroni correction for multiple comparisons, using the statistical software of choice.

Representative Results

Figure 1 provides a general overview of the *in vitro* BBB model used to study the interaction between T cells and endothelial cells. The experiment consists of three major steps. The first step is the isolation of primary MBMECs from brain cortices, and their culture for five days. When they reach confluence in the cell culture plate, MBMECs are trypsinized and reseeded onto permeable inserts, which are then placed in the TEER instrument. The TEER and Ccl of MBMECs are continuously measured and monitored over a period of three to five days. In the meantime, T cells are isolated, stimulated (step 2), and cultured for two to five days, type and strength of the stimulus. In step 3, T cells are added to and co-cultured with MBMECs when Ccl is at a stable low level and the TEER is at its maximum level. Choosing this time point for the co-culture when the TEER is still at its plateau is crucial for the success of the whole measurement. Thus, it is extremely important that the time point for T cell isolation and stimulation is carefully chosen, so that T cells reach the desired level of activation by the time of their addition to the ECs. After adding T cells, the measurement is resumed for one more day and the results are analyzed.

After the initial five-day culture, MBMECs readily show characteristic spindle-shaped morphology (**Figure 2A**, left panel) and express endothelial cell-specific markers such as PECAM-1 and Claudin-5 (**Figure 2A**, middle and right panels). However, this alone does not provide sufficient evidence of their complete confluence and proper barrier integrity. Impedance spectroscopy, on the other hand, gives a good estimate of the monolayer integrity and serves as a quality control of each well before the performance of a functional assay. In **Figure 2B**, most of the wells contained MBMECs whose Ccl values were stable and low, and their TEER values reached a plateau. These wells were used for subsequent co-culture experiments. They were grouped in such a way that variance within and between groups was minimal before adding T cells (**Figure 2C**). The wells whose TEER values differed significantly from the rest (such as the blue curve indicated in **Figure 2B**) were not used, as they would lead to ambiguous results or misinterpretation of the data.

Provided that initial requirements for MBMEC culture and T cell stimulation have been met, impedance spectroscopy can give valuable insights into the T cell-EC interaction. TEER measurement can serve as the primary readout for such an interaction and a measure of T cell-mediated EC dysfunction, as shown in **Figure 3A** and **3B**. In this case, MBMECs are grown on inserts with micropores 0.4 μm in diameter, which doesn't allow T cells to pass through the insert membrane. **Figure 3A** shows that only stimulated, but not naïve T cells are capable of inducing EC dysfunction, as determined by a decrease in TEER and increase in Ccl. Naïve T cells can thus serve as a negative control, since TEER and Ccl during the co-culture with naïve T cells stay at their initial levels. The exception is the peak at the very beginning of the co-culture, caused by handling the instrument (lifting the lids, adding new medium with cells that may have slightly different temperatures and pH values, and closing the lids). This artifact appears with all kinds of TEER measurements and is ignored during the analysis. The addition of the pro-inflammatory cytokines IFN- γ and TNF- α (at 100-500 U/ml), which are known to be capable of inducing EC inflammation, can be used as positive control. In **Figure 3A**, MOG₃₅₋₅₅-specific CD4⁺ T cells were stimulated in an antigen-specific fashion for five days. T cells can also be stimulated polyclonally (e.g., with purified anti-mouse CD3 and CD28 antibodies) (**Figure 3B**). Here, the length of the stimulus, and consequently the level of T cell activation, was varied. T cells were stimulated by the same amount of α -CD3 and α -CD28 antibodies, and the ones cultured for three days exhibited a greater propensity for barrier disruption compared to the T cells cultured for only two days.

In order to investigate the mechanisms of the described MBMEC monolayer disruption, various approaches can be used. Results in **Figure 3C** show that the stimulated T cells, but not their supernatants caused a substantial damage to the MBMECs, indicating that direct contact between T cells and MBMECs is critical for barrier disruption. Moreover, addition of a neutralizing α -IFN- γ antibody during the TEER measurement only slightly improved MBMEC barrier properties, suggesting that IFN- γ may not be the primary cause of this disruption. On the other hand, adding granzyme B Inhibitor II to T cell culture (**Figure 3D**) restored MBMEC integrity to a greater extent, pointing to this cytolytic molecule as an important player in causing MBMEC damage and subsequent decrease in TEER.

Besides its use as a primary readout for the effect of T cells on EC monolayer integrity, TEER measurement can also be used as a quality control of barrier integrity prior to other assays, such as T-cell transmigration assay. In this case, inserts with micropores of 3 μm in diameter are used. In **Figure 3E**, TEER measurement was performed in order to ensure that MBMEC monolayer was of the same level of integrity in all wells before the addition of T cells for subsequent transmigration assay. This was followed by harvesting of T cells from the lower compartment of the instrument wells, staining T cells with α -CD4 antibody and flow cytometric analysis, using cell counting beads to determine absolute numbers of transmigrated T cells; shown in **Figure 3E**, middle and right). Note that stimulated T cells used for transmigration did not cause a substantial decrease in TEER, although they were pre-activated as in **Figure 3A**. This could reflect the transcellular route immune cells may use during the transmigration, as has been observed previously³⁹. In **Figure 3F**, half of the wells with MBMECs were inflamed with IFN- γ and TNF- α (uninflamed vs. inflamed), and later on, naïve T cells were added for assessment of trans migratory activity. In this case, TEER measurement provided a clue as to how strong the inflammation with cytokines was and when T cells should be added for the transmigration.

Figure 4 shows some of the most common situations that may lead to misinterpretation of TEER results. In **Figure 4A**, for example, not all MBMECs developed a fully confluent monolayer at the same time. One of the groups ('naïve T cells - excluded') contained MBMECs whose TJ complexes were maturing at a different rate compared to the other groups just before the addition of T cells. This group consequently developed TEER values above 100% during the experiment and was excluded from further analysis. Thus, the slope of the TEER curve (rate of TJ maturation) before the experiment is as important as absolute TEER values for proper data interpretation. **Figure 4B** shows that starting the co-culture not only too early but also too late can lead to false results. Here, in both the group with stimulated T cells and the negative control group (medium change), TEER values have already passed their plateau and started to decrease independently from the experimental condition, hence indicating suboptimal culture conditions before and during the experiment. Such groups should not be included in the analysis. Finally, although TEER and Ccl usually change their values in such a way that a greater decrease in TEER is accompanied by a greater increase in Ccl, this may not always be the case. In **Figure 4C**, stimulated T cells B caused a bigger decrease in TEER, but a smaller increase in Ccl than stimulated T cells A did.

Figure 1: General overview of the technique. After the initial culture, MBMECs are reseeded onto permeable inserts and placed into the automated cell monitor; TEER and Ccl are measured every hour for 4-5 days. In the meantime, T cells are activated *in vitro* and are added to MBMECs when Ccl is stable and low, and TEER has reached a plateau. Measurement is then resumed for another day, and the results are exported and analyzed. The image of automated cell monitor: re-print with permission from reference⁴⁰. [Please click here to view a larger version of this figure.](#)

Figure 2: MBMECs develop a confluent monolayer suitable for an *in vitro* BBB model. (A) Spindle-shape morphology (left) and immunofluorescent staining of PECAM-1 (middle) and Claudin-5 (right) five days after MBMEC isolation. (B and C) TEER and Ccl values before (B) and after (C) grouping of individual wells, prior to addition of T cells. The blue curve in (B) is not used for the experiment, since MBMECs in this well have not developed TEER as high as in the other wells. Data in (C) show mean $\pm$ SEM. [Please click here to view a larger version of this figure.](#)

Figure 3: Representative results. (A-D) Changes in TEER as a primary measure of MBMEC dysfunction upon interaction with T cells. (A) MOG-specific T cells were activated by MOG-specific B cells in the presence of MOG₃₅₋₅₅ peptide for five days. Naïve T cells and pro-inflammatory cytokines IFN- γ and TNF- α (both at 100 U/ml) served as a negative and positive control, respectively. (B) T cells were stimulated with α -CD3 and α -CD28 antibodies (1 μ g/ml each) for two or three days, as indicated, before adding them to MBMECs. (C) T cells (pre-activated as in (A)) or their supernatants, in the presence of α -IFN- γ antibody or the corresponding isotype control. (D) T cells (pre-activated as in (B)) for two days, in the presence of granzyme B inhibitor II or its diluent DMSO. (E and F) TEER measurement used only as a quality control for barrier integrity prior to T-cell transmigration. MBMECs were grown on inserts with pores of 3 μ m in diameter. (E) T cells, stimulated as described in (A), were let to transmigrate for 18 hr (left). Next, they were harvested, stained for α -CD4 antibody (clone RM4-4) and analyzed by flow cytometry, using cell counting beads (middle and right). (F) Monitoring of MBMEC monolayer integrity upon stimulation with IFN- γ and TNF- α , and choosing the appropriate time point for subsequent addition of T cells for transmigration assay. (A-F) Results show technical triplicates and are representative of three independent experiments each. Data show mean $\pm$ SEM. (E, right) Unpaired, two-tailed Student's t test. **, P < 0.01. [Please click here to view a larger version of this figure.](#)

Figure 4: Practical considerations and data interpretation. (A) MBMECs from one experimental group (red curve) were excluded from analysis, since the rate of maturation of their junctional complexes was different compared to other groups. (B) An example of adding T cells too late, which prevented proper assessment of MBMEC dysfunction. (C) Upon disruption by stimulated T cells 'A' (black curve), Ccl values of MBMECs showed a more dramatic increase than would be expected from the concomitant decrease in TEER caused by the same T cells. (A-C) T cells were stimulated with α -CD3 and α -CD28 antibodies (1 μ g/ml each) for two days. Results show technical triplicates and are representative of three independent experiments each. Data show mean $\pm$ SEM. [Please click here to view a larger version of this figure.](#)

Discussion

Several steps of the described protocol are essential for a successful experiment. During the initial MBMEC isolation and culture, it is crucial that work is performed under sterile conditions as much as possible, to prevent the contamination of the cell culture with fungal spores or bacteria. In order to obtain a pure culture of ECs, it is recommended to use a medium containing Puromycin for the first three days, which enables survival of ECs, but not other cells types (especially pericytes)^{41,42}. Another critical step that deserves special attention is the identification of the correct time point for adding pre-activated T cells. During the first 48 hr of TEER measurement MBMECs proliferate on permeable inserts and close the gaps between each other. Hence, Ccl decreases until it reaches a stable and low level (around 0.6 μ F/cm²). This is the first sign of a confluent EC monolayer, but is not yet a sign of a tight barrier. Only after the Ccl has reached a stable level does the TEER start to increase. When TJ complexes are fully formed and the EC barrier is completely sealed off, TEER reaches its maximum level. This plateau is usually reached three to five days after reseeding the ECs and is maintained for another 24 to 36 hr. The best time point for adding the T cells is at the beginning of the plateau, which ensures that the ECs are still viable enough to be co-cultured with the T cells. As the T cell activation needs to be initiated before the optimal time point for co-culture can be safely predicted, it can be advantageous to stimulate two batches of T cells on two different days in order to increase the flexibility of this timing. Finally, grouping of individual replicate wells before adding T cells to the MBMECs needs to be done in such a way that variability within and between the groups is minimal. This ensures that initial conditions are equal among all groups during the co-culture of T cells and MBMECs.

Our protocol describes TEER measurement during the co-culture of primary mouse brain ECs with pre-activated CD4⁺ T cells. Its simple form allows for multiple modifications, according to the scientific needs. For instance, the type, strength, and duration of the stimulus can be varied. Antigen-specific CD4⁺ T cells can be activated by their cognate antigen, in the presence of suitable antigen-presenting cells (APCs); alternatively, T cells can be polyclonally activated by α -CD3 and α -CD28 antibodies. The T-cell activation status can be modulated by various cytokines, blocking antibodies, inhibitors. Replacing primary brain ECs with endothelial cell lines is not recommended, however, as it is known that the latter show less restrictive TJ complexes and higher permeability compared to the primary cells⁴³. In addition to measuring TEER as the primary readout, this protocol provides a good quality control of the EC monolayer integrity prior to the T-cell transmigration assay (Figure 3E, F). Of note, TEER values may not necessarily decrease during the transmigration of T cells across the MBMECs (Figure 3E): this could reflect the transcellular route immune cells may use during transmigration, as has been observed previously^{39,44}.

As activated T cells induce EC dysfunction during the co-culture, TEER shows a steady, predictable decrease, comparable across experiments with the same conditions. A greater decrease in TEER is usually accompanied by a greater concomitant increase in Ccl. On rare occasions, however, an increase in Ccl can be more pronounced than would be expected from the corresponding decrease in TEER (Figure 4C). This discrepancy may reflect different aspects of EC monolayer disruption. As ECs try to close the gaps formed during the barrier disruption, they can undergo dynamic changes in morphology and motility such as forming protrusions and increasing their total surface area, all of which can

contribute to a dramatic and rapid increase in Ccl. This makes changes in Ccl less predictable, compared to changes in TEER. Thus, the level of decrease in TEER remains the most reliable and representative measure of compromised barrier integrity.

This assay can be complemented by other techniques to investigate endothelial barrier properties. It can be followed by a permeability assay, which measures the amount of molecules of different sizes that diffuse through the disrupted EC monolayer⁴⁴. For this purpose, fluorescently labeled dextran conjugates, such as Fluorescein and Texas Red can be used; other molecular tracers are also available (e.g., sucrose, mannitol, albumin, Evans Blue and horseradish peroxidase)²⁶. Furthermore, immunofluorescence microscopy can be used to investigate the changes in protein expression and the cellular localization of TJ and cell adhesion molecules. Although the presented BBB model is very simple, the results obtained with it can provide valuable orientation for further assays under more physiological conditions. Such assays include (but are not limited to) *in vitro* shear flow assay to complement TEER measurement obtained under static conditions and *in vivo* permeability assay with Evans Blue injection into living mice to account for the full complexity of the BBB.

Although sensitive and reliable, the method described here has certain limitations that deserve special attention. The maximum TEER values measured in our experimental setup reaches values of 35–40 Ωcm^2 . These TEER values are much lower than *in vivo*, where different cell types and acellular components contribute to the integrity of the BBB, but they are also not as high as TEER values achieved in some other *in vitro* BBB models^{26,45,46}. This could be improved by the addition of hydrocortisone to the EC medium, which has been shown to markedly enhance barrier properties^{27,47–49}. However, usage of such substances - known to have anti-inflammatory and immunosuppressive effects – is not suitable for all functional assays. As the focus of this particular protocol is on the consequences of EC-T cell interactions on barrier integrity, interpretation would be hampered by the addition of hydrocortisone. Using the protocol presented here thus enables the assessment of the true inflammatory potential of stimulated T cells to cause EC barrier dysfunction. It is also worth noting that direct comparison of different TEER values reported in the literature should be made with caution. Such values are highly dependent upon the experimental setup, they are obtained with different cell types, seeding cell densities, media, cell growth areas, and TEER measuring systems (e.g., the design and size of the electrodes).

In order to mimic the *in vivo* situation more closely, complex *in vitro* BBB models have been established, where ECs are co-cultured with pericytes on the opposing side of the insert membrane, or where pericytes and astrocytes are grown on the bottom of the wells^{12,25,50–52}. In such co-culture systems, cross-talk between different cell types enables stronger tightening of the barrier, with triple co-cultures being the best in resembling the *in vivo* situation and thus providing the highest TEER values. The complexity of these systems, on the other hand, poses a limitation to their wider use as *in vitro* BBB models.

Another limitation of this model is the lack of shear stress, which has been shown to improve barrier integrity^{53,54}. To overcome this problem, new dynamic BBB models have recently been introduced: ECs are cultured in hollow fibers and subjected to pulsatile flow conditions, mimicking more closely microvasculature *in vivo*^{55,56}.

In summary, this protocol describes an *in vitro* model of the BBB based on impedance spectroscopy. Focusing on the interaction of primary mouse brain endothelial cells with activated T cells, the method allows for studying the barrier properties during such direct contact. This is of particular importance for understanding the crucial steps in the development of inflammatory CNS diseases such as multiple sclerosis and its animal model EAE, wherein the BBB is compromised during an interaction of ECs with encephalitogenic T cells. The described assay enables the investigation of the consequences of a targeted modulation of such an interaction by using a wide range of substances such as blocking antibodies, cytokines and inhibitors of T cell activation. The method is sensitive, reliable and non-invasive and measurements of TEER and Ccl are performed in an automated fashion. All this makes it a useful and versatile tool that adds a new layer to the rich body of BBB models. It may specifically expand our knowledge about BBB properties under pathophysiological conditions observed in autoimmune diseases such as MS.

Disclosures

I. Kuzmanov, A. M. Herrmann, S.G. Meuth, H. Wiendl and L. Klotz have nothing to disclose. H.-J. Galla is a scientific advisor of nanoAnalytics GmbH, the manufacturer of the automated cell monitor.

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