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## Co-culture model consisting of human brain microvascular endothelial and peripheral blood mononuclear cells

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### Abstract

**Background**—Numerous systems exist to model the blood-brain barrier (BBB) with the goal of understanding the regulation of passage into the central nervous system (CNS) and the potential impact of selected insults on BBB function. These models typically focus on the intrinsic cellular properties of the BBB, yet studies of peripheral cell migration are often excluded due to technical restraints.

**New Method**—This method allows for the study of in vitro cellular transmigration following exposure to any treatment of interest through optimization of co-culture conditions for the human brain microvascular endothelial cells (BMEC) cell line, hCMEC/D3, and primary human peripheral blood mononuclear cells (PBMCs).

**Results**—hCMEC/D3 cells form functionally confluent monolayers on collagen coated polytetrafluoroethylene (PTFE) transwell inserts, as assessed by microscopy and tracer molecule (FITC-dextran (FITC-D)) exclusion. Two components of complete hCMEC/D3 media, EBM-2 base-media and hydrocortisone (HC), were determined to be cytotoxic to PBMCs. By combining the remaining components of complete hCMEC/D3 media with complete PBMC media a resulting co-culture media was established for use in hCMEC/D3 – PBMC co-culture functional assays.

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### 7. Conflict of interest

The authors declare that they have no conflicts of interest.

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**Comparison with existing methods**—Through this method, issues of extensive differences in culture media conditions are resolved allowing for treatments and functional assays to be conducted on the two cell populations co-cultured simultaneously.

**Conclusion**—Described here is an in vitro co-culture model of the BBB, consisting of the hCMEC/D3 cell line and primary human PBMCs. The co-culture media will now allow for the study of exposure to potential insults to BBB function over prolonged time courses.

### Keywords

Blood-brain barrier; peripheral blood mononuclear cells; brain microvascular endothelial cells; hCMEC/D3 cell line

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## 1. Introduction

The blood-brain barrier (BBB) regulates passage between peripheral circulation and the central nervous system (CNS). Aside from limiting permeability to circulating proteins, ions, and therapeutic compounds, the BBB also controls cellular transmigration and immune surveillance (Loeffler et al., 2011; Strazza et al., 2011). In comparison to other tissues and organs throughout the body, immune surveillance of the CNS is more limited and tightly regulated in order to control the neuroinflammatory response and to minimize the risk of deleterious neuroinflammation within the brain (Miner and Diamond, 2016; Ousman and Kubes, 2012; Ransohoff and Engelhardt, 2012). In this regard, accelerated immune cell extravasation has been linked to a number of disease pathologies (Katsetos et al., 1999; Ryan et al., 2005; Song et al., 2015). Understanding the impact of compounds or proteins that alter proper BBB function is a critical first step in developing appropriate interventions to specific neuropathologic conditions.

In order to study BBB integrity following exposure to experimental conditions, model systems must be utilized. These model systems, either in vivo or in vitro, incorporate, at minimum, brain microvascular endothelial cells (BMEC), which comprise the BBB endothelium into selected co-culture combinations with astrocytes, pericytes, and under certain circumstances, neurons. In vitro models using human cells are generally preferred to animal models when addressing many types of experimental questions, and the culture of primary human BMECs is generally accepted to require or be greatly enhanced by co-culture with primary human astrocytes (Eugenin and Berman, 2003; Hayashi et al., 1997). The in vitro primary co-culture model maintains the BBB characteristics for a window of one to two days beyond establishing confluence, which can limit the duration of experimental exposures. The recent development of the immortalized human BMEC cell line, hCMEC/D3 cells, has led to the development of another human in vitro BBB model; the hCMEC/D3 cell line was established through the in vitro immortalization of BMECs isolated from the temporal lobe (Weksler et al., 2005). Critical to defining the hCMEC/D3 cell line as a BBB model, these cells were shown to express BBB-specific markers, including ZO-1, JAM-A, occludin, and claudin-5 (Eigenmann et al., 2013; Helms et al., 2016; Huang et al., 2009; Pu et al., 2005; Weksler et al., 2005). In addition, hCMEC/D3 cells maintain the functional characteristics of the BBB in monoculture for several days beyond confluence, allowing for extended duration exposures that are critical to drug studies

as well as models of chronic inflammatory diseases (Daniels et al., 2013; Jacob et al., 2015; Urich et al., 2012; Weksler et al., 2013a; Weksler et al., 2005). Recent studies have been aimed at defining the specific attributes of hCMEC/D3 cells, as well as other endothelial cell lines that are emerging in popularity as BBB models (Helms et al., 2016). These studies confirmed tight junction protein expression and low transendothelial permeability that was not enhanced through the co-culture with astrocytes or pericytes, and conclude that the mono-culture of the immortalized human cell line will provide another valuable tool in the in vitro studies of the BBB (Cucullo et al., 2008; Eigenmann et al., 2013; Weksler et al., 2005).

Beyond studying characteristics of the BBB such as transendothelial electrical resistance (TEER), tight junction protein expression and localization, cell adhesion molecule expression, and small molecule permeability, the interaction between immune cells and the BBB during the process of transmigration must also be assessed following an experimental exposure in order to fully understand the nature of the dysfunction induced. Transmigration functional assays require the co-culture of BMECs with immune cell populations, in the absence or presence of astrocytes, pericytes, and cells relevant to a functional BBB. The merging of these cell types into a common culture setup creates the obstacle of also bringing together the culture conditions that were previously defined and optimized for monoculture of each unique cell type. In the case of the hCMEC/D3 cell line, the defined media for cell growth contains a complex combination of media components, each serving distinct roles while collectively promoting the in vitro growth and maintenance of the cells. In contrast, primary human PBMCs are often maintained in a simple media formulation consisting of a few essential supplements to the base media. Presented here is a unique media formulation that maintains viability and function of primary human PBMCs and hCMEC/D3 endothelial cells.

## 2. Methods

### 2.1 Materials

Mannitol and FITC-dextran (70 kDa) (FITC-D) were purchased from Sigma Aldrich. SDF-1 $\alpha$  was purchased from eBioscience. IL-2 was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Maurice Gately, Hoffmann - La Roche Inc (Lahm and Stein, 1985).

### 2.2 Cell culture and treatment

The human BMEC line, hCMEC/D3, was obtained from Dr. Babette Weksler (Weill Cornell Medical College) and cultured in EBM-2 (Lonza) supplemented with heat inactivated fetal bovine serum (FBS; 5%, GemBio), penicillin-streptomycin (1%, CellGro), hydrocortisone (1.4  $\mu$ M, CellGro), ascorbic acid (5  $\mu$ g/mL, Sigma Aldrich), chemically defined lipid concentrate (1%, Invitrogen), HEPES (10 mM, CellGro), and basic fibroblast growth factor (1 ng/mL, Sigma Aldrich) (designated herein as “CMEC media”). All experiments were conducted between cell passages 27–32. Cells were grown on petri dishes or 12-well plates coated with Cultrex rat collagen I (150  $\mu$ g/mL, Trevigen) in H<sub>2</sub>O. hCMEC/D3 cells were seeded on petri dishes and 12-well plates at a density of 37,000 cells/cm<sup>2</sup> or on transwell

inserts (details below). At confluence on plates or in transwells, hCMEC/D3 cells were treated where indicated with mannitol (1.4 M, Sigma Aldrich) for 30 minutes.

Primary human PBMCs were obtained from the Comprehensive NeuroAIDS Center (Basic Science Core 1, Temple University School of Medicine). Cells were cultured overnight under unstimulated, non-adherent conditions in polypropylene 50 mL conical tubes at a density of  $1 \times 10^6$  cells/mL in RPMI media supplemented with heat-inactivated FBS (10%), penicillin-streptomycin (1%), and IL-2 (20 U/ml) (designated herein as “PBMC media”).

Co-culture studies were performed in “co-culture media.” Co-culture media consists of RPMI supplemented with heat-inactivated FBS (5%), penicillin-streptomycin (1%), ascorbic acid (5 µg/mL), chemically defined lipid concentrate (1%), HEPES (10 mM), basic fibroblast growth factor (1 ng/mL), and IL-2 (20 U/ml).

### 2.3 In vitro blood-brain barrier model

hCMEC/D3 cells were seeded on collagen-coated porous polytetrafluoroethylene (PTFE) transwell inserts (Corning, NY) at a density of  $4.5 \times 10^4$  cells/cm<sup>2</sup> and grown for 10 days to achieve confluence for use in FITC-D and PBMC transmigration assays. Of note, transwell inserts were purchased pre-coated with collagen, limiting the variability to which coating with collagen prior to each experiment may influence cell growth and membrane permeability. Addition of treatments to the upper chamber of the transwell inserts was used to mimic a peripheral exposure.

### 2.4 FITC-Dextran permeability assay

Any indicated treatments of the hCMEC/D3 monolayers were performed at confluence on porous PTFE transwell inserts (0.4 µm). The monolayers were subsequently washed with 10 mM HEPES in 1X HBSS and the FITC-dextran (FITC-D) permeability assay was performed as previously described (Weksler et al., 2005) with minor modification. The use of the FITC-D assay to monitor confluence and small-molecule permeability is the preferred method for hCMEC/D3 cells due to the well-published fact that many of the immortalized human BMEC cell lines have consistently low TEER values, therefore, necessitating the use of other tools to assess barrier tightness or integrity throughout the duration of culture while maintaining protein expression markers of the BBB (Deli et al., 2005; Eigenmann et al., 2013; Helms et al., 2016; Reichel et al., 2003; Weksler et al., 2005). Briefly, 70 kDa FITC-dextran (FITC-D; 2 mg/mL) in CMEC media was added to the upper chamber. Passage through the monolayer was monitored through sampling from the lower chamber at 5 minute intervals throughout a 30- minute time course (6 time points); these samples were read in optical bottom plates for fluorescence intensity. The increase in frequency of sampling from 10 to 5 minutes allows for increased accuracy in plotting the curves of cleared volume over time that are critical to the calculation of the permeability coefficient (Pe), or rate of passage. Some transwell inserts were maintained in the absence of cells, and these wells were used to determine the rate of passage across the filter alone, another necessary value in the calculation of Pe. Fluorescence intensity was then used to calculate the Pe of the monolayer utilizing the following equation:  $Pe = PS/s$ , where PS (clearance) is the permeability surface area of the endothelial monolayer and s is the surface area of the filter (1.12 cm<sup>2</sup>).

PS is given by  $1/PS = 1/m_e - 1/m_f$ , where  $m_e$  and  $m_f$  are the slopes of the curves corresponding to endothelial cells on filters and to filters only, respectively, with  $m_e$  and  $m_f$  calculated by plotting the cleared volume against time.

The cleared volume was calculated by  $(AU_a - AU_b)/F_i$ , where  $AU_a$  is the total fluorescence (arbitrary units) in the basal compartment,  $AU_b$  is the background fluorescence and  $F_i$  is the fluorescence of the initial solution (AU/ml).

## 2.5 PBMC viability assay

Following overnight non-adherent culture, as described above,  $1 \times 10^6$  PBMCs were incubated for 24 hours in the indicated media conditions in 12-well plates. An MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay System, Promega, Madison, WI) was then performed, as described by the manufacturer (Promega), to determine the viability of the PBMCs under selected culture conditions. Cell viability in normal PBMC media was set to 100% viability.

## 2.6 PBMC transmigration assay

Primary human PBMCs were obtained from the Comprehensive NeuroAIDS Center (Basic Science Core 1, Temple University School of Medicine) and cultured at  $1 \times 10^6$  cells/mL in the presence of IL-2 (20 U/ml) in polypropylene 50 mL conical tubes to minimize monocyte differentiation and adherence. Meanwhile, hCMEC/D3 cells were grown to confluence on porous PTFE transwell inserts (3  $\mu$ m). At confluence, a suspension of  $1 \times 10^6$  PBMCs in co-culture media were then added to the upper chamber. As a positive control of enhanced PBMC transmigration, SDF-1 $\alpha$  (100 ng/mL) was added to the lower chamber. In both cases, untreated or SDF-1 $\alpha$ -treated, the media in the lower chamber was co-culture media. Following a 24 hour incubation, cellular transmigration to the lower chamber was determined using flow cytometry. Briefly, media from the lower chamber was collected, and transmigrated cells were counted using the Beckton Dickson FACSCalibur and the results were analyzed using FlowJo software.

## 2.7 Statistical analysis

Statistics were calculated using the student's t-test. P values less than 0.05 were considered significant in all assays.

# 3. Results

## 3.1 hCMEC/D3 cells as an in vitro model of the BBB

To establish the growth pattern of hCMEC/D3 cells on collagen-coated PTFE transwell inserts, cells were seeded at  $4.5 \times 10^4$  cells/cm<sup>2</sup> and monitored microscopically for 10 days (Figure 1A). After 2 days in culture, hCMEC/D3 cells remained on the membrane with a rounded morphology. On day 4, the cells were partially confluent with areas of cells still rounded and proliferating. By 10 days in culture, hCMEC/D3 cells were flat against the membrane, and appeared to be fully confluent. To complement the visual analysis, confluence was monitored through a FITC-D permeability coefficient assay. FITC-D is available in multiple sizes ranging from 4 – 70 kDa, and for these studies the 70 kDa size

was used since it most closely mimics tracer molecules, such as Evan's blue labeled albumin, often used in in vivo models. Following 2, 4, 6, or 10 days in culture, 70 kDa FITC-D (2 mg/mL) in CMEC media was added to the upper chamber of the transwell inserts, and passage through the monolayer to the lower chamber was monitored at 5 minute intervals over a 30 minute period and permeability coefficients ( $P_e$ ) were calculated (Figure 1B). After 10 days in culture, a  $P_e$  comparable to published values for this cell line was observed, and, notably, this  $P_e$  was lower than those of other commonly used in vitro BBB models (Weksler et al., 2005). Additionally, treatment of the confluent monolayer with mannitol (1.4 M), a compound commonly used clinically to enhance transport of therapeutics across the BBB, induced a significant increase in  $P_e$  (Figure 1C), indicating that the low  $P_e$  observed at 10 days was not due to cell piling and a physical blockade of the insert pores. From these studies it can be concluded that hCMEC/D3 cells form a functionally confluent monolayer on collagen-coated PTFE transwell inserts after 10 days of growth. For all subsequent transwell studies, hCMEC/D3 cells were grown for 10 days prior to any treatments or experiments.

### 3.2 CMEC media decreases PBMC Viability

Assays of BBB function requires co-culture of both PBMCs and hCMEC/D3 cells; therefore, necessitating the merging of the cell culture requirements of the cell types. Due to the differences between PBMC and CMEC media components (Table 1), PBMC viability was assessed to establish optimal co-culture conditions.

PBMCs were grown under selected combinations of PBMC and CMEC media conditions for 24 hours prior to an MTS assay. Compared to PBMC media, PBMC viability in CMEC media was 40% (Figure 2A). Importantly, the addition of IL-2 did not rescue viability (35%), nor did any ratio combinations of PBMC and CMEC media. The low viability (59%) observed when PBMCs were grown in 3 parts PBMC media and 1 part CMEC media suggested that one or more components of CMEC media may be toxic to PBMCs (Figure 2A). To identify toxic components, PBMCs were grown under 24 media conditions, each with either an RPMI or EBM-2 media base and the indicated combinations of CMEC media supplements, for 24 hours prior to an MTS assay. All media conditions included IL-2 and Pen/Strep (Figure 2B). Any condition with less than 75% viability was considered toxic.

Interestingly, all toxic conditions included either EBM-2 or hydrocortisone (HC) (Figure 2C). These results suggested that EBM-2 and HC are specifically cytotoxic for PBMCs. The toxicity of HC may be due to an induced decrease in IL-2 receptors on T cells within the PBMCs. Based on these observations, a co-culture media formulation was developed and included an RPMI base with IL-2, Pen/Strep, 10% FBS, ascorbic acid, chemically defined lipid concentrate (CDLC), HEPES, and bFGF.

### 3.3 hCMEC/D3 monolayers maintain low permeability in co-culture media

A critical next step was to confirm that a media lacking HC and made with RPMI would not alter proper hCMEC/D3 monolayer function. To establish the impact of the new co-culture media formulation on the hCMEC/D3 monolayer a FITC-D permeability coefficient assay was performed. The hCMEC/D3 monolayer was grown to confluence in full CMEC media

on 0.4  $\mu\text{m}$  pore size transwell inserts. Following 10 days in culture in CMEC media, when confluence was reached, media in the upper and lower chambers was changed to the new co-culture media and cells were incubated for 24 hours. The absence of HC and substitution of an RPMI base did not significantly increase permeability of the hCMEC/D3 monolayer after 24 hours of culture (Figure 3). Therefore, these results suggest that co-culture media can be used for assays of BBB function.

### 3.4 Co-culture media supports PBMC and hCMEC/D3 transmigration assays

To confirm that the co-culture media supports functional assays utilizing both cell populations, transmigration of PBMCs across the hCMEC/D3 monolayer was examined. hCMEC/D3 cells were grown to confluence in CMEC media on collagen-coated PTFE transwell inserts (3  $\mu\text{m}$  pores), as described above. At confluence, media in lower chambers was replaced with either co-culture media or co-culture media supplemented with SDF-1 $\alpha$  (100 ng/mL), added as a chemoattractant to promote PBMC transmigration. Additionally, primary human PBMCs suspended in co-culture media were added to the upper chamber and incubated for 24 hours at 37°C in 5% CO<sub>2</sub> to allow transmigration to occur. After 24 hours, the media in the lower chambers was collected, and the number of transmigrated cells was quantified by flow cytometry. Events were gated based on forward and side scatter to distinguish PBMCs, and the number of events within this gate was quantified for each sample. Of note, this method can be expanded to include staining the transmigrated PBMCs for surface proteins of interest in order to assess subpopulations of cells that migrate preferentially in response to stimuli. As expected, SDF-1 $\alpha$  significantly increased PBMC transmigration (Figure 4). This result demonstrated that the untreated hCMEC/D3 monolayer maintained functional integrity and that both the hCMEC/D3 cells and the PBMCs maintained the ability to become activated and participate in the transmigration process in co-culture media for 24 hours. Overall, these studies demonstrate that a co-culture media formulation for the hCMEC/D3 cell line and primary human PBMCs allows for functional studies of the two populations and this co-culture model will provide an additional tool to examine the structure and function of the BBB and its relationship with other cellular compartments and pathogenic agents.

## 4. Discussion

The development of the hCMEC/D3 cell line has allowed for great advances in the field of BBB research, due in part to the ease of acquisition, amplification of cell number, and extended culture time to facilitate experimentation (Chen et al., 2015; Daniels et al., 2013; Helms et al., 2016; Urich et al., 2012; Weksler et al., 2013b; Weksler et al., 2005). However, despite these benefits, this cell line has been shown to require numerous media supplements for optimal growth and function. This requirement has led to a unique challenge for co-culturing hCMEC/D3 cells with primary human PBMCs, and perhaps other cell types, due to the contrasting simplicity of typical PBMC culture media. We have been able to identify a unique media formulation for co-culturing both hCMEC/D3 and PBMC cells to permit *in vitro* investigations focused on mechanisms that may facilitate cellular transmigration. In this regard, we have first identified specific components of the CMEC media that are cytotoxic to PBMCs and a co-culture media was then formulated that was shown to maintain

both cell populations for use in co-culture studies. Importantly, both the hCMEC/D3 cells and PBMCs were grown initially in complete CMEC and PBMC medias, respectively, and were then transferred into co-culture media at the onset of the functional assay. This process ensures normal growth and proliferation of the two cell types individually before their behavior is examined under selected experimental conditions. Additionally, the studies performed in this investigation demonstrated that co-culture media alone did not induce altered BBB permeability or PBMC viability.

Numerous in vitro BBB models have been established and studied. Primary BMECs of animal origin, including bovine (Salmeri et al., 2013), murine (Ludewig et al., 2013), and porcine (Pieper et al., 2013), have often been used to mimic the human BBB. While in vitro cell culture models share common characteristics with the human BBB, they are certainly not optimal or ideal. Culture of primary human BMECs in combination with primary human astrocytes has widely been thought to be a relevant model of barrier function, and has been utilized in many different types of studies (Bethel-Brown et al., 2012; Gandhi et al., 2010). A difficulty of primary cell models is the limited window beyond confluence during which the cells maintain viability and function. Consequently, primary cell models of the BBB are incompatible with extended duration exposure studies. The hCMEC/D3 cell line, which has been characterized for several days beyond establishing confluence, has been previously used for co-culture studies with PBMCs, and has been shown to yield results comparable to primary human BMECs both in the absence or presence of astrocytes (Daniels et al., 2013). Previous studies combining hCMEC/D3 cells with PBMCs introduced PBMCs to the upper chamber of the transwell system in PBMC media while CMEC media, minus HC, was maintained in the lower chamber (Daniels et al., 2013). While these culture conditions were sufficient for showing the capacity of the model to recapitulate a primary cell culture model, the mixed conditions are not ideal for studies concerning the impact of selected treatments on barrier function due to the added variable of media differences. The comparable results obtained from hCMEC/D3 cells and primary human BMECs in the previous investigations open the possibility that the co-culture media described here may additionally optimize PBMC co-culture with primary human BMECs and astrocytes.

## 5. Conclusions

These studies have established the optimal conditions for the co-culture of the hCMEC/D3 cell line with primary human PBMCs for use in BBB functional assays, in vitro. It was demonstrated that EBM-2 media and HC are cytotoxic to PBMCs, and the removal of these two components from a new media formulation yields an improved co-culture media. The results demonstrate that PBMCs cultured in this co-culture media remain viable for at least 24 hours. Additionally, incubation of hCMEC/D3 cells in co-culture media for 24 hours did not induce leakiness of the monolayer.

The new media formulation can thus be used for the simultaneous exposure of the two cell populations to additional treatment conditions or potential BBB insult of interest. Another key benefit of this co-culture media formulation has been the ability to study the interaction of the two cell populations following such treatments, allowing for the assessment of BBB function at both the endothelial and migrating cell level. Proper BBB function involves not



only small molecule and ion exclusion, but, importantly, the regulation of cellular passage and immune surveillance between the periphery and the CNS. In the future, this co-culture media formulation may be used for the study of therapeutics, infectious diseases, autoimmunity, exogenous proteins, neuroinflammation, and other disease processes involving deregulation of cellular passage between the periphery and the CNS.

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## List of abbreviations

<b>BBB</b>	Blood-brain barrier
<b>BMEC</b>	Brain microvascular endothelial cells
<b>CNS</b>	Central nervous system
<b>FITC-D</b>	Fluorescein isothiocyanate dextran
<b>PBMC</b>	Peripheral blood mononuclear cells
<b>P<sub>e</sub></b>	Permeability coefficient
<b>PTFE</b>	Polytetrafluoroethylene
<b>TEER</b>	Transendothelial electrical resistance

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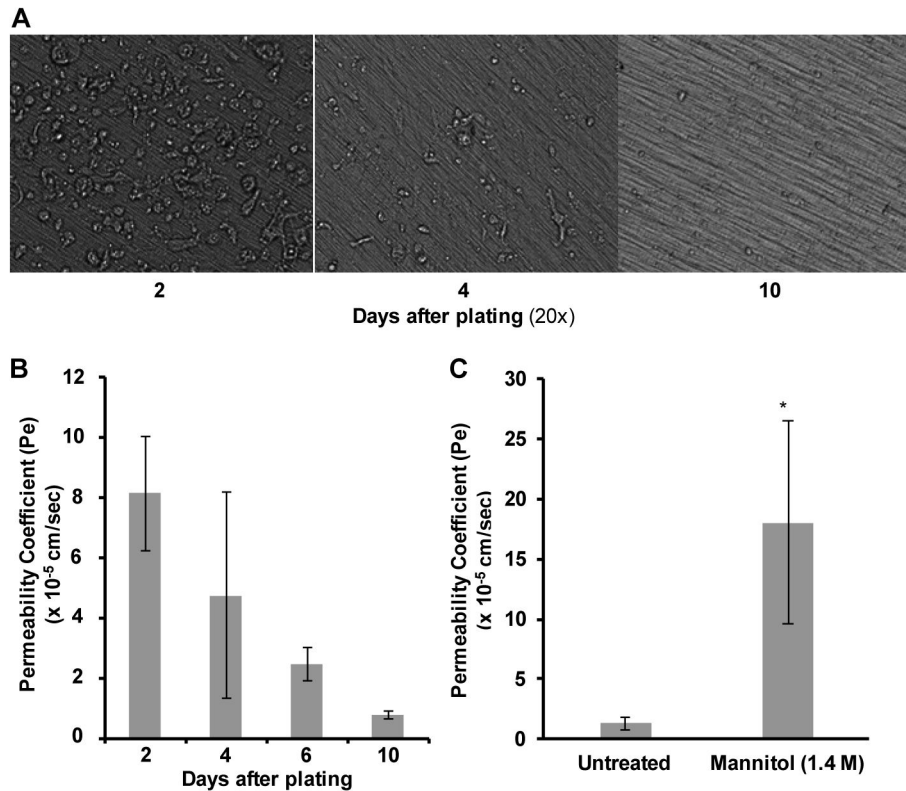
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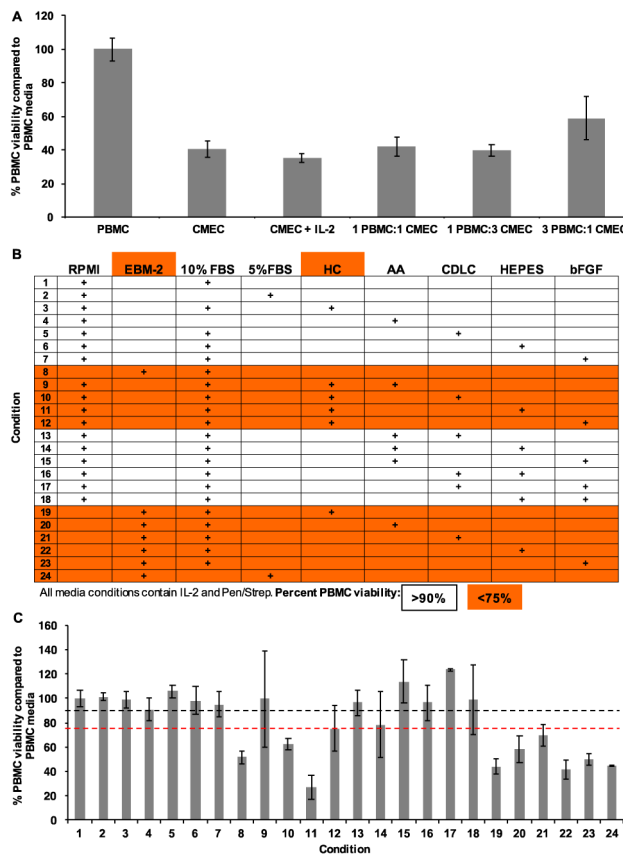
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### Highlights

- The immortalized human brain microvascular endothelial cells (BMEC) cell line, hCMEC/D3, in vitro blood brain barrier model is useful in long-term functional studies
- hCMEC/D3 media components EBM-2 and hydrocortisone are cytotoxic to peripheral blood mononuclear cells (PBMCs)
- Co-culture media excluding the toxic components, EBM-2 and hydrocortisone, support hCMEC/D3 barrier function as well as continued PBMC viability
- Co-culture media can be used in assays of PBMC transmigration

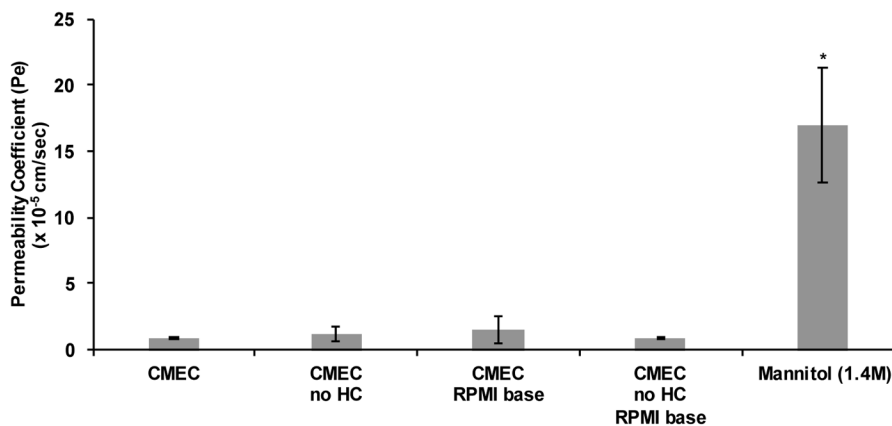


**Figure 1. hCMEC/D3 cells form a confluent monolayer following 10 days in culture**  
 (A) hCMEC/D3 cells were seeded on porous PTFE transwell inserts ( $0.4 \mu\text{M}$ ) at time zero and cultured for 10 days. Throughout this time course, monolayer growth was visualized under 20X Relief Contrast Microscopy using an Olympus IX81 de-convolution microscope. (B) hCMEC/D3 cells remained in culture 2, 4, 6, or 10 days (as in A) prior to addition of 2 mg/mL 70 kDa FITC-D to the upper chamber. Samples were taken from the lower chamber at 5 minute intervals over 30 minutes in order to calculate Pe. (C) hCMEC/D3 cells were cultured (as in A) for 10 days prior to treatment of the monolayer with mannitol (1.4 M) for 30 minutes at  $37^\circ\text{C}$  in  $\text{CO}_2$  (5%). Following treatment, a FITC-D assay was performed (as in B) to calculate Pe. Bars represent the mean of three independent experiments performed in triplicate with standard deviation. Statistical analysis was performed with a two-tailed Student's t-test. \* $p < 0.0001$ .



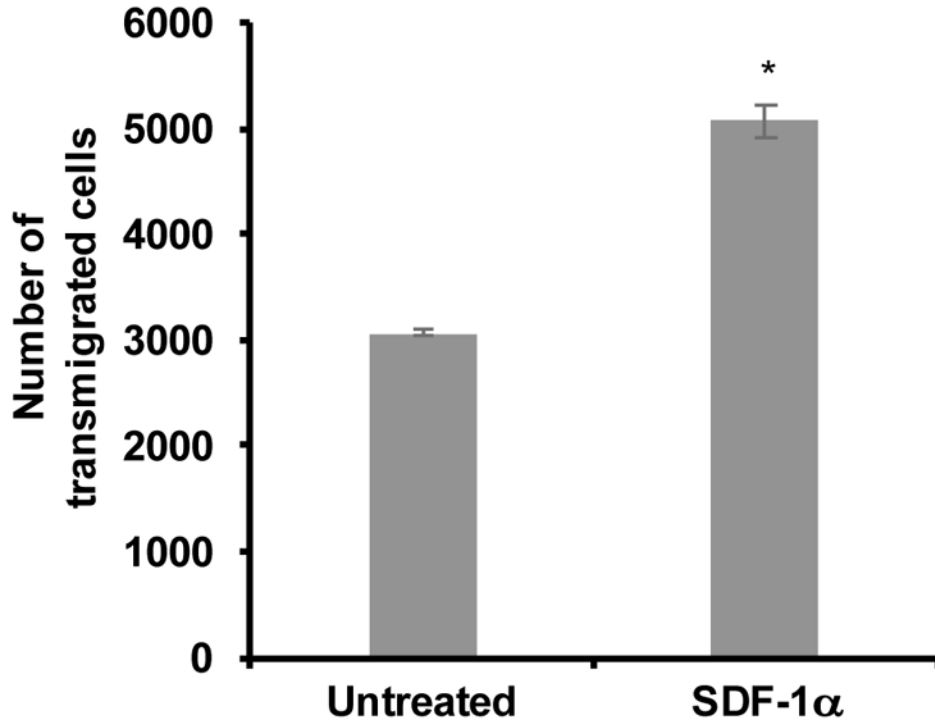
**Figure 2. CMEC media decreases PBMC viability**

(A) PBMCs were grown under selected media conditions at  $1 \times 10^6$  cells/mL at 37°C in 5% CO<sub>2</sub> for 24 hours prior to an MTS assay to assess cellular viability. Media conditions: complete PBMC media; complete CMEC media; CMEC media supplemented with IL-2; combination of one part PBMC and one part CMEC; combination of one part PBMC and three parts CMEC; three parts PBMC and one part CMEC. PBMC viability within complete PBMC media was normalized to 100%, and all further media conditions were compared to complete PBMC media. (B) PBMCs were grown as in (A). Media conditions included either an RPMI or EBM-2 media base with the addition of selected components of complete CMEC media. HC – hydrocortisone, AA – ascorbic acid, CDLC – chemically defined lipid concentrate, and bFGF – basic fibroblast growth factor. All media conditions included IL-2 and Pen/Strep. An MTS assay was performed to assess PBMC viability. PBMC viability within complete PBMC media (condition 1) was normalized to 100%, and all further media conditions were compared to complete PBMC media (condition 1). Media formulations and components highlighted in orange indicate conditions which yielded less than 75% viability. All conditions with less than 75% viability contain either EBM-2 or HC. (C) Percent PBMC viability as determined by MTS assay. Conditions refer to those defined in (B). Black line: 90%. Red line: 75%.



**Figure 3. Co-culture media does not induce leakiness of the hCMEC/D3 barrier**

The effect of co-culture media on hCMEC/D3 structure and function was assessed through a FITC-D permeability coefficient assay as previously described (Figure 1). Media conditions: complete CMEC media; CMEC media without HC; CMEC media made with an RPMI base (no EBM-2); CMEC media without HC made with an RPMI base. A 30 minute treatment with mannitol (1.4 M) is included as a positive control of barrier compromise for the assay. Statistical analysis was performed with a two-tailed Student's t-test. \* $p = 0.04$



**Figure 4. Co-culture media supports the PBMC transmigration assay**

hCMEC/D3 cells were cultured for 10 days on porous PTFE transwell inserts (3  $\mu$ M). Separately, PBMCs were maintained in non-adherent culture in the presence of IL-2 for 24 hours. At confluence of the monolayer, the CMEC media was removed from both chambers, new co-culture media was added to the lower chambers, in the absence or presence SDF-1 $\alpha$  (100 ng/mL), and a suspension of  $1 \times 10^6$  PBMCs in co-culture media was added to the upper chambers. Transmigration was allowed to occur over a period of 24 hours during incubation at 37°C in 5% CO<sub>2</sub>. Following transmigration, cells were collected from the lower chambers and analyzed by flow cytometry. Cells were characterized based on forward and side scatter, to identify the PBMC population, and quantified. All bars represent the average two independent experiments performed in triplicate treatments  $\pm$  standard deviation. All treatments were significantly increased when compared to untreated as determined by student's t-test. \* $p < 0.0013$



**Table 1**

Components of PBMC and hCMEC/D3 complete media

<b>PBMC media</b>	<b>hCMEC/D3 media (CMEC)</b>
RPMI-1640	EBM-2
Fetal Bovine Serum (10%)	Fetal Bovine Serum (5%)
Penicillin/Streptomycin	Penicillin/Streptomycin
IL-2	Hydrocortisone
	Ascorbic Acid
	Chemically Defined Lipid Concentrate
	HEPES
	bFGF

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