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TITLE:

Neisseria meningitidis Infection of Induced Pluripotent Stem-Cell Derived Brain Endothelial Cells

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KEYWORDS:

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SUMMARY:

The protocol described here highlights the major steps in the differentiating induced pluripotent stem-cell derived brain-like endothelial cells, the preparation of *Neisseria meningitidis* for infection, and sample collection for other molecular analyses.

ABSTRACT:

Meningococcal meningitis is a life-threatening infection that occurs when *Neisseria meningitidis* (meningococcus, Nm) can gain access to the central nervous system (CNS) by penetrating highly specialized brain endothelial cells (BECs). As Nm is a human-specific pathogen, the lack of robust in vivo model systems makes study of the host-pathogen interactions between Nm and BECs challenging and establishes a need for a human based model that mimics native BECs. BECs possess tighter barrier properties when compared to peripheral endothelial cells characterized by complex tight junctions and elevated trans-endothelial electrical resistance (TEER). However, many in vitro models, such as primary BECs and immortalized BECs, either lack or rapidly lose their barrier properties after removal from the native neural microenvironment. Recent advances in human stem-cell technologies have developed methods for deriving brain-like endothelial cells from induced pluripotent stem-cells (iPSCs) that better phenocopy BECs when compared to other in vitro human models. The use of iPSC-derived BECs (iPSC-BECs) to model Nm-BEC interaction has the benefit of using human cells that possess BEC barrier properties, and can be used to examine barrier destruction, innate immune activation, and bacterial interaction. Here we demonstrate how to derive iPSC-BECs from iPSCs in addition to bacterial preparation, infection, and sample collection for analysis.

INTRODUCTION:

The blood-brain barrier (BBB), and the meningeal blood-CSF barrier (mBCSFB) are extremely tight cellular barriers that separate the circulation from the central nervous system (CNS) and are primarily comprised of highly specialized brain endothelial cells (BECs)[1, 2]. Together, BECs maintain proper brain homeostasis by regulating nutrients and waste products in and out of the brain, while excluding many toxins, drugs, and pathogens[1, 2]. Bacterial meningitis occurs when blood-borne bacteria are able to interact with, and penetrate the barrier formed by BECs and cause inflammation. *Neisseria meningitidis* (Nm, meningococcus) is a Gram-negative bacterium that colonizes the nasopharaynx of 10–40 % of healthy individuals, but in some cases can cause serious systemic disease[3]. In affected individuals, Nm can gain access to the blood stream where it can cause purpura fulminans as well as penetrate BECs gaining access to the CNS causing meningitis[3]. Nm is a leading cause of bacterial meningitis world-wide, and despite vaccination efforts, is still a primary cause of meningitis[4]. Modern medical intervention, such as antibiotic treatment, have made these conditions survivable, however those affected with meningitis often are left with permanent neurological damage[5, 6].

Previous studies have identified bacterial factors and host signaling that contribute to Nm-BEC interactions[7–11]. The identified adhesins and invasins such as the opacity protein Opc, and type-IV pili, as well as receptors such as CD147, have been conducted on various BEC models in vitro, however these models lack many defining BBB properties[7, 9, 11, 12]. Complete understanding of Nm-BEC interactions remain elusive due partially to the inability to utilize in vivo models, incomplete vaccination protection, and lack of robust human BEC models in vitro.

Modeling hBECs in vitro has been challenging due to the unique properties of BECs. Compared with peripheral endothelial cells, BECs have a number of phenotypes that enhance their barrier properties such as high trans-endothelial electrical resistance (TEER) due to complex tight junctions[12]. Once removed from the brain microenvironment, BECs rapidly lose their barrier properties limiting the usefulness of primary or immortalized in vitro models that only form a weak barrier[12, 13]. The combination of the human specificity of Nm infections, lack of robust in vivo models, and challenges modeling human BECs in vitro creates a need for better models to understand the complex host-pathogen interaction between Nm and BECs. Recently using model human induced pluripotent stem cell (iPSC) technologies, BECs have been derived from iPSCs that better mimic BECs in vivo[12–15]. iPSC-BECs are of human origin, easily scalable, and possess expected BEC phenotypes compared to their primary or immortalized counterparts[12–15]. Additionally we and others have demonstrated that iPSC-BECs are useful for modeling various diseases of the CNS such as host-pathogen interaction, Huntington's disease, and MCT8 deficiency that causes Allan-Hurndon-Dudley syndrome[16–21]. Here, we demonstrate how to derive iPSC-BECs from renewable iPSC sources and the infection of iPSC-BECs with Nm leading to activation of the innate immune response. We believe that this model is useful to interrogate host-pathogen interaction that is unable to be recapitulated in other in vitro models and is especially useful when examining interactions with human specific pathogens such as Nm.

PROTOCOL:

NOTE: All media / reagent preparation, stem-cell maintenance, and differentiation steps are adapted from Stebbins et al.[22].

1. Preparation of materials required for iPSC culture and BEC differentiation.

1.1. Matrix coating of tissue culture (TC) plastic for IMR90-4 iPSC culture

1.1.1. Aliquot basement membrane matrix gel (e.g., Matrigel) into 2.5 mg aliquots and store at -20 °C.

NOTE: Work quickly when handling the matrix gel and aliquot on ice, as it forms a gel above 4 °C and cannot be aliquoted once it has solidified.

1.1.2. For coating TC plastic, quickly add one aliquot of matrix gel to 30 mL of Dulbecco's Modified Eagle Medium (DMEM)/F12 medium in a 50 mL conical tube. Add 1 mL of the medium onto the frozen matrix gel, pipette up and down until it is thawed, and transfer immediately to the 50 mL conical tube with the remaining medium.

1.1.3. Use 1 mL of this matrix gel-coating solution per well of a 6-well plate and 12 mL per T75 flask.

NOTE: 30 mL coating solution can be used for 5x 6-well plates, 3x plates and 1x T75s, or 1x plate and 2x T75s. Substitute 1x T75 with 3x T25s if needed. Matrix gel-coated TC plastic can be prepared up to two weeks before it is used. It is, however, critical to avoid that the matrix gel solution dries out, which may require occasional addition of more DMEM/F12 on top of the wells.

1.2. Prepare stem-cell maintenance medium by adding 50 mL of 50x supplement to 450 mL of stem cell maintenance basal medium in the sterile environment of a biosafety cabinet.

NOTE: Other stem-cell maintenance media (mTeSR and E8) have been used in other studies[13–15, 22–25].

1.3. To prepare 500 mL of unconditioned medium (UM), combine 392.5 mL of DMEM/F12 with 100 mL of knock out serum replacement (KOSR), 5 mL of non-essential amino acids, L-glutamine at a final concentration of 1 mM, and 3.5 µL of β-mercaptoethanol. Filter-sterilize and store at 4 °C for up to 2 weeks.

1.4. To prepare 200 mL of endothelial cell (EC) medium plus retinoic acid (RA) plus bFGF, combine 198 mL of human endothelial serum free medium (hESFM), 2 mL of filter-sterilized platelet-poor derived serum (PDS), and 20 ng/mL bFGF. Filter sterilize and store for up to 2 weeks at 4 °C. Just before addition to cells, add 10 µM of RA to EC medium.

NOTE: As PDS has been discontinued and may therefore be limited, this protocol has been successfully conducted using B27 in place of PDS[15, 23, 26].

1.5. To prepare 200 mL of EC medium without RA or bFGF, combine 198 mL of hESFM and 2 mL of filter sterilized PDS and filter sterilize. Store for up to 4 weeks at 4 °C.

2. Maintenance IMR90-4 cell culture

NOTE: Here we use the IMR90-4 cell line as an example, however other induced pluripotent stem-cell lines such as CC3, CD10, CD12, DF19-9-11T, 83iCTR, 00iCTR, and CS03iCTRn2 have been successfully employed for differentiation into BECs[13–17, 23, 27, 28].

2.1. Culture iPSCs at 37 °C in 5% CO_2 and typically maintain on 6-well plates at various densities with 2 mL of stem-cell maintenance medium per well.

2.2. For maintenance of the iPSC culture, select a single well for passage that is not confluent and has open spaces between colonies.

2.2.1. Aspirate the culture medium, add 1 mL of non-enzymatic cell dissociation reagent, and incubate at 37 °C for 7 min. While incubation is ongoing, replace the matrix gel solution, on a new 6-well plate, with 2 mL of fresh stem-cell maintenance medium per well.

2.2.2. Carefully aspirate the non-enzymatic cell dissociation reagent taking care to not aspirate cells still attached to the plate.

2.2.3. Add 6 mL of stem-cell maintenance medium and rinse the well bottom a few times until all cells are completely detached. Then, seed the new 6-well plate with varying densities, typically 1:6 or 1:12 for normal maintenance.

NOTE: Using the aforementioned ratios, the cells are split approximately twice a week.

2.2.4. Move the plate to the incubator and distribute the seeded cells equally across the wells by shaking the plate back and forth and left to right, pausing in between alternating shaking motions until the medium has settled.

3. Differentiation of brain endothelial cells from human iPSCs

3.1. For the remaining wells to be split for differentiation (**day -3**) (from the same plate maintenance cells were split from), add 1 mL of enzymatic cell dissociation reagent per well and incubate at 37 °C for 7 min.

3.2. Inactivate the enzymatic cell dissociation reagent by transferring the 1 mL of dissociated cell suspension into a 15 mL conical tube with at least 2 mL of fresh stem-cell maintenance medium per 1 mL of cells. Spin down the cell suspension at 1,500 x *g* for 5 min.

3.3. Resuspend the cell pellet in 1 mL of stem-cell maintenance medium per well of IMR90-4 cells used, and count the cells using a hemocytometer.

NOTE: It may be helpful to dilute 1:1 with 0.4% trypan blue to distinguish between live and dead cells when counting. Depending on the density of iPSCs, one well of a 6-well plate usually yields 1–2 x 10^6 cells.

3.4. For differentiation in a T75 flask, add 7.5 x 10^5 cells to 12 mL of stem-cell maintenance medium and ROCK inhibitor (Y27632 dihydrochloride) at a final concentration of 10 µM. Aspirate matrix from the T75 flask and transfer the cell suspension to the flask. Distribute the cells equally by shaking the flask back and forth and left to right (see step 2.2.4) and incubate at 37 °C and 5% CO_2.

NOTE: It is critical to add ROCK inhibitor at this step to enhance survival of the dissociated single stem cells[25, 29]. Cells should be evenly distributed across the flask and in singlets exhibiting a spread, mesenchymal-like morphology due to the ROCK inhibitor treatment[22].

3.5. On **day -2** and **day -1,** change media to fresh stem-cell maintenance medium; 12 mL per T75.

3.6. On **day 0**, start differentiation by changing media to UM; 12 mL per T75.

3.7. On **day 1** to **day 5**, change UM daily.

NOTE: The cells typically reach confluence after 2 to 3 days in UM, which can be observed with the naked eye or through an inverted bright field microscope. As the differentiation progresses, nestin$^+$ "neural tracts" become visible with PECAM-1$^+$ cells in between as previously described[13, 14].

3.8. Selectively expand the endothelial cell population by switching to EC medium with 20 ng/mL bFGF and 10 µM retinoic acid (RA) (**day 6**) and incubating for two days. EC media with bFGF can be prepared up to two weeks prior. RA is added from frozen stock on the day of use (e.g., 1 µL of 10 mM RA stock per 1 mL of EC + bFGF).

NOTE: Successful differentiation can also be achieved without supplementation of RA at days 6 and 8. Omission of RA will, however, yield BECs with reduced TEER[12–14].

3.9. Coat cell culture plates and membrane inserts (e.g., Transwell) with collagen IV and fibronectin (**day 7**), for purification of the BECs and following experiments.

3.9.1. For coating of membrane inserts, combine 4 parts collagen IV (1 mg/mL in 0.5 mg/mL acetic acid), 1 part fibronectin (1 mg/mL) and 5 parts sterile tissue-grade water. ECM solution can be diluted 1:5 for coating cell culture plates (i.e., 4 parts collagen IV, 1 part fibronectin, 45 parts water). Incubate with coating solution at 37 °C overnight.

NOTE: Plates and membrane inserts can also be coated for at least 4 h prior to subculturing on the same day of the purification step.

3.10. Purify BECs by subculturing the differentiated cells on collagen IV and fibronectin-coated plates or membrane inserts (**day 8**).

3.10.1. Aspirate EC medium and add enzymatic cell dissociation reagent (12 mL per T75). Incubate at 37 °C until 90% of cells have detached from the flask.

NOTE: Cell dissociation can take up to 1 h.

3.10.2. During the incubation time, remove the collagen IV/fibronectin coating solution from previously prepared plates/inserts and let them dry in a sterile hood. It takes approximately 20 min for the inserts to dry.

3.10.3. Once the cells have detached, wash them off the flask using a 10 mL pipette. Pipette up and down to achieve a single cell suspension.

NOTE: Single cell suspension is important for reliable cell counting and to achieve solid monolayers.

3.10.4. Dilute with at least equal volume of fresh hESFM in a 50 mL conical tube and count cells using a hemocytometer.

3.10.5. Pellet the cells at 1,500 x g for 10 min.

3.10.6. Resuspend cells in appropriate volume of freshly prepared EC + bFGF + RA to achieve a suspension of 2 x 10^6 cells/mL for seeding on membrane inserts. Add 500 µL (1 x 10^6 cells) on top of a 12-well insert and 1.5 mL of EC + bFGF + RA medium on the bottom. For seeding on 24 and 48-well plates, dilute cell suspension 1:2 and add 500 µL (5 x 10^5 cells) and 250 µL (2.5 x 10^5 cells) per well, respectively. Distribute cells evenly across the well/insert (see step 2.2.4) and incubate at 37 °C under 5% CO_2.

3.11. Change media on plates/transwells to EC **without** bFGF or RA (**day 9**).

3.12. Conduct infection experiments, TEER measurement, and immunofluorescence staining as described in the following sections (**day 10**).

NOTE: Successfully differentiated and purified BECs typically reach peak TEER on day 10 and express characteristic markers of brain endothelial cells such as PECAM-1 (CD31) and VE-cadherin, the glucose transporter GLUT-1, efflux transporters such as p-glycoprotein, and tight junction components ZO-1, Occludin, and Claudin-5[13, 14, 16, 17, 19, 22]. Refer to Lippmann et al., Stebbins et al. and others for further details and images of the cell types, morphologies, and expression of cell type specific markers during the differentiation process[13–17, 19, 22].

4. Transendothelial electrical resistance (TEER) as a measure of barrier tightness

NOTE: TEER is usually read on membrane inserts on **days 9** and **10** of differentiation to confirm successful generation of barrier forming iPSC-BECs (**Figure 1A**).

4.1. Place the epithelial volt-ohm meter (EVOM) in the sterile environment of a biosafety hood and connect the electrode to the EVOM.

4.2. Disinfect the electrode by submerging it in 70% EtOH for at least 5 min and let it completely dry.

NOTE: Longer incubation in 70% EtOH or decontamination of the electrode using 5% hypochlorite solution is possible if needed.

4.3. Retrieve the iPSC-BECs on membrane inserts from the incubator and measure TEER.

NOTE: It is important to read TEER rapidly after removal from the incubator as temperature change may impact TEER measurement.

4.4. Read TEER by dipping the electrode into the medium so that the shorter electrode is placed on top of the insert and the longer electrode reaches into the medium surrounding the insert.

NOTE: Make sure the electrodes at the tips of the "chopsticks" are completely covered by liquid. If needed, tilt the well to achieve this before setting the plate down again for measuring.

5. Immunofluorescence (IF) staining to validate BEC phenotype

NOTE: To validate the quality of the fully differentiated and purified cells, iPSC-BEC monolayers are stained for the characteristic markers of brain endothelial cells on **day 10** of the differentiation process as previously described (**Figure 1B–G**) [13–17, 19, 22].

5.1. Aspirate the medium and wash 1x with PBS.

5.2. Fix cells with ice cold methanol at room temperature (RT) for 15 min.

5.3. Wash 3x with phosphate buffered saline (PBS) and block with 10% fetal bovine serum (FBS) in PBS at RT for 1 h.

5.4. Aspirate and add primary antibodies diluted in blocking solution. Incubate at 4 °C overnight.

NOTE: Refer to the **Table of Materials** and Stebbins et al. for information related to source and dilution of the antibodies[22].

5.5. Wash 3x with PBS before adding secondary antibody diluted in blocking solution. Incubate at RT for 1 h. Protect samples from light from this point on.

5.6. Wash 2x with PBS. Then, add DAPI at a 1:5,000 dilution in PBS and stain at RT for 15 min.

5.7. Wash 1x leaving the PBS on and take images using a fluorescence microscope.

6. Preparation of bacteria and infection of iPSC-BECs

6.1. On the day before the infection experiment (**day 9** of differentiation), start an overnight culture of the bacteria from frozen stock. For infection with Nm, streak bacteria onto Columbia agar with 5% sheep blood (blood-agar). Incubate bacterial cultures at 37 °C and 5% CO_2 overnight.

6.2. The next day (**day 10**), prepare fresh PPM+ by supplementing Protease peptone media (PPM) with 50 µL of 2 M $MgCl_2$, 50 µL of 2 M $NaHCO_3$, and 100 µL Kellogg's supplement per 10 mL. Inoculate 10 mL of PPM+ medium in a 50 mL conical tube with Nm from the overnight culture plate using a sterile cotton swab. Incubate shaking at 200 rpm at 37 °C for 1.5 h (i.e., until bacteria are in logarithmic growth phase).

6.3. During bacterial incubation, prepare iPSC-BECs in a 24-well plate or on membrane inserts for infection by replacing the old medium with 400 µL of fresh EC medium (without bFGF or RA) per well/ top of membrane insert.

6.4. Centrifuge bacterial culture at 4000 x *g* for 10 min and aspirate the media. Resuspend the bacterial pellet in 250 µL of PBS.

6.5. In 4 mL of fresh PBS, use a portion of the bacterial cell suspension and adjust to an OD_{600} of 0.4 (approximately 1 x 10^8 CFU/mL).

6.6. Then, dilute the bacteria in cell culture medium (EC medium) according to the desired multiplicity of infection (MOI).

NOTE: For example, for an MOI of 10, dilute 1:10 or 1:5 when infecting iPSC-BECs in a 24-well plate or on membrane inserts, respectively (1 x 10^5 cells per monolayer in a 24-well plate). Addition of human serum is not included in the preparation of Nm for infection as was described in other manuscripts as it was observed to have a deleterious impact on the iPSC-BEC barrier phenotype as measured by TEER (data not shown)[30, 31]. However, the interaction of Nm and iPSC-BECs is unaffected with or without human serum (data not shown).

6.7. Infect iPSC-BECs in each well with 100 µL of the prepared bacteria suspension and incubate for the desired time of infection.

NOTE: Expression of a number of proinflammatory cytokines and chemokines is elevated in iPSC-BECs when infected with Nm, most prominently after 8 h of infection as previously described by Martins-Gomes et al (**Figure 2**)[19].

7. Innate immune activation by quantitative PCR

NOTE: Using a preferred RNA isolation, cDNA synthesis, and qPCR protocol, collect samples and run qPCR on selected cytokines.

7.1. Collect the RNA from BEC samples after infection on **day 10** of the differentiation protocol, using reagents form a commercially available RNA isolation kit (see the **Table of Materials**).

NOTE: Avoid nuclease contamination of samples by working carefully in a cleaned and sterilized environment.

7.1.1. Prepare lysis buffer and add 350 µL to each well/monolayer of iPSC-BECs.

7.1.2. Collect the samples by pipetting up and down numerous times (e.g., 20x) and transferring the suspension to a sterile microcentrifuge tube.

NOTE: Samples can be stored at -80 °C until ready for RNA isolation.

7.1.3. Follow the protocol provided with the RNA isolation kit for RNA purification from cultured cells and tissue.

7.1.4. After elution in nuclease-free water, keep RNA samples on ice to minimize any potential RNase activity.

7.1.5. Estimate RNA concentrations on a spectrophotometer (e.g., nanodrop).

7.2. Generate a cDNA library from the collected RNA using a cDNA synthesis kit (see **Table of Materials**).

7.2.1. Set up reactions consisting of cDNA synthesis master mix, at least 200 ng (ideally 500 ng) of sample RNA, and nuclease-free water in a defined total reaction volume as described in the protocol of the cDNA synthesis kit.

7.2.2. On a standard thermo cycler, run a program that is appropriate for the reagents of the cDNA synthesis kit used. For instance: 25 °C for 2 min, 55 °C for 10 min, 95 °C for 1 min.

7.2.3. After synthesis, dilute the cDNA up to 1:10 in nuclease-free water and move samples to 4 °C for short term or -20 °C for long term storage.

NOTE: Lower dilution may be necessary if RNA concentration was used was low (e.g., below 50 ng). The diluted cDNA can be stored at -20 °C for up to a year.

7.3. Perform qPCR on the cDNA samples targeting transcripts of innate immune response genes such as cytokines and chemokines with carefully designed and validated primers.

NOTE: As primer design is very important for the quality of qPCR results, primer efficiency should be tested conducting a dilution series and the absence of multiple products should be confirmed on a DNA gel.

7.3.1. Per 25 µL reaction, use 0.5 µL forward and 0.5 µL reverse primer (10 mM in nuclease-free water), 1 µL cDNA, 10.5 µL nuclease free H_2O, and 12.5 µL qPCR master mix.

7.3.2. Perform the reaction on a qPCR machine using the following cycler protocol: (a) 4 °C for 2 min; (b) 95 °C for 15 min; (c) 95 °C for 15 s; (d) 60 °C for 1 min; cycle through (c) and (d) 45 times; (e) optional melt curve: 30–99 °C in 1 °C increments; 25 °C for 5 min.

7.3.3. For data analysis, use the ΔΔCT calculation to compare cytokine and chemokine expression levels to a reference housekeeping gene such as 18S or GAPDH.

REPRESENTATIVE RESULTS:

The protocol described here is adapted from Stebbins et al. and highlights the process to differentiate iPSCs into brain-like endothelial cells that possess BBB properties, and how to utilize this model for infection studies using iPSC-BECs with Nm[19, 22]. The iPSC-BECs, when differentiated properly, exhibit tight barrier properties measured by TEER that are often greater than 2000 $\Omega \cdot cm^2$, and express endothelial markers such as VE-cadherin and CD31 (PECAM) (**Figure 1A–C**). Additionally, they express and localize the tight junction markers Claudin-5, Occludin, and ZO-1 (**Figure 1D–F**), and transporters such as Glut-1 (**Figure 1G**). Upon infection with Nm, iPSC-BECs respond to infection through the upregulation of neutrophilic proinflammatory cytokines as measured by qPCR such as IL-8 (*CXCL8*), *CXCL1*, *CXCL2*, *CCL20*, and *IL6* (**Figure 2A–E**). These representative results demonstrate how to ensure that iPSC-BECs are being differentiated reliably, and how to examine the response of iPSC-BECs to Nm infection.

FIGURE AND TABLE LEGENDS:

Figure 1: Characterization of iPSC-BECs. (**A**) TEER of two separate, individual differentiations, read on **days 9–12**. Data presented as mean of triplicates. Error bars represent ± SD. (**B–G**) Representative immunofluorescence data showing expression of endothelial cell markers VE-cadherin (**B**) and PECAM-1 (CD31; **C**), tight junction components Claudin-5 (**D**), Occludin (**E**), and ZO-1 (**F**), and glucose transporter GLUT-1 (**G**). Scale bar represents 50 µm. Panels B–G of this figure have been used with permission from Kim et al. originally published in Fluids and Barriers of the CNS, a BMC journal[17].

Figure 2: Upregulation of cytokines by iPSC-BECs upon infection with *Neisseria meningitidis*. Representative qPCR data showing relative expression of *CXCL8* (**A**), *CXCL1* (**B**), *CXCL2* (**C**), *CCL20* (**D**), and *IL6* (**E**) transcripts after 8 h of infection, comparing infected with uninfected iPSC-BEC monolayers. Data presented as mean of three independent experiments conducted in triplicate. Error bars represent ± S.D. Student's t-test used to determine significance. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. This figure has been modified and used with permission from Martins Gomes et al. originally published in Frontiers in Microbiology[19].

DISCUSSION:

Modeling BECs and the BBB has had challenges, as primary and immortalized human BECs, in vitro, tend to lack robust barrier phenotypes. The advent of human stem cell technologies has allowed for the generation of iPSC derived BECs that retain expected hallmark BBB phenotypes such as endothelial markers, tight junction expression, barrier properties, response to other CNS cell types, and functional efflux transporters[12–15, 22, 24, 25]. This has enabled researchers to utilize BECs in vitro that closely mimic in vivo BECs and model various diseases with reported BBB dysfunction[16, 17, 19–21, 32]. Nm is a leading cause of bacterial meningitis and is a human specific pathogen that lacks robust in vivo models[19]. This limitation has necessitated the use of better engineered models to drive the discovery of novel host-pathogen interaction between Nm and the BBB. Recently, we have demonstrated that iPSC-BECs are a viable model to interrogate Nm-BEC interaction[19].

Here we describe a general method to derive iPSC-BECs and infect with Nm resulting in the upregulation of proinflammatory cytokines that are typically induced by bacterial infection[19]. For the derivation of iPSC-BECs we generally follow the protocol as described in Stebbins et al. for the generation of iPSC-BECs, with minor modifications[22]. In particular here we use StemFlex media instead of mTesR1, however either media can be used for the maintenance of the stem cell culture[17]. It has been established that this protocol works well with may iPSC lines, however it is important that the optimum seeding density is determined for each individual iPSC line[15, 24]. For this manuscript we used the IMR90-4 cell line and it was previously established that 1×10^5 cells/cm^2 was the optimum initial seeding density[24]. Finally as a demonstration of the identity of BECs generated, iPSC-BECs express expected endothelial cell markers and tight junctions while exhibiting high TEER (**Figure 1**)[13–15, 24]. These phenotypes, as well as being of human origin, make iPSC-BECs a powerful tool to interrogate Nm-BEC interaction.

The preparation of Nm for infection was adapted from previously published methods[19, 33]. To ensure that the bacterial growth media was not introduced into the cell culture experiments, a washing step and resuspension in PBS was conducted as described in the methods. Finally, an MOI of 10 had been previously observed to result in the activation of iPSC-BECs through an upregulation of pro-inflammatory cytokines[19]. Activation of BECs in response to various bacteria have been observed namely through the upregulation of neutrophilic chemokines and cytokines[6]. It has been previously observed that iPSC-BECs upregulate the potent neutrophil chemoattractants IL-8, CXCL1, and CXCL2 after infection with Group B *Streptococcus*, and Nm[16, 19]. This observed response of iPSC-BECs demonstrate that these cells are able to detect bacteria and activate an innate immune program resulting in the upregulation of cytokines. The methods

to detect the upregulation of these cytokines by qPCR are well established and are briefly described above. However interestingly, while these pro-inflammatory cytokines are detected by qPCR, the coordinate protein products are either undetected or very low[16, 19]. At present, it is unclear if this is an artifact of the iPSC-BEC models, or if the observed low abundance of cytokines is biologically relevant. Future research will be required to determine a mechanism behind the disconnect between expression and secretion.

A major strength of the iPSC-BEC model is the expression and localization of tight junctions that contribute to barrier function as read be TEER[12–15, 22]. Previous work with *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) has demonstrated that the upregulation of Snail1 contributes to the destruction of BBB tight junctions in vitro and in vivo[34]. More recently, this finding was confirmed in the iPSC-BEC model both with GBS and Nm suggesting a mechanism for how bacteria are able to disrupt BBB integrity during infection[16, 19]. Additionally, it was demonstrated that Nm interacts with CD147 on endothelial cells that promote bacterial attachment, and ultimately reorganization of tight junctions leading to barrier dysfunction[9]. We have demonstrated that Nm colocalizes with CD147 in the iPSC-BECs potentially making this model ideal for the future elucidation of Nm-CD147 interactions as they pertain to BBB dysfunction[19].

The method presented here demonstrates the differentiation of iPSC-BECs from a pluripotent stem cell source, and application with Nm infection. The iPSC-BECs are of human origin, express endothelial markers, and possess BBB specific phenotypes making them an ideal model for the examination of human specific pathogens such as Nm. Finally, we are able to demonstrate that the iPSC-BEC model respond to bacterial infection through the upregulation of a neutrophilic cytokine response. Taken together, the iPSC-BEC model has certain advantages over primary and immortalized model systems to examine the host-pathogen interactions at the BBB. Further work should be aimed at elucidating mechanisms of BBB destruction during bacterial meningitis.

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DISCLOSURES:

The authors have nothing to disclose.

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615

Figure 1

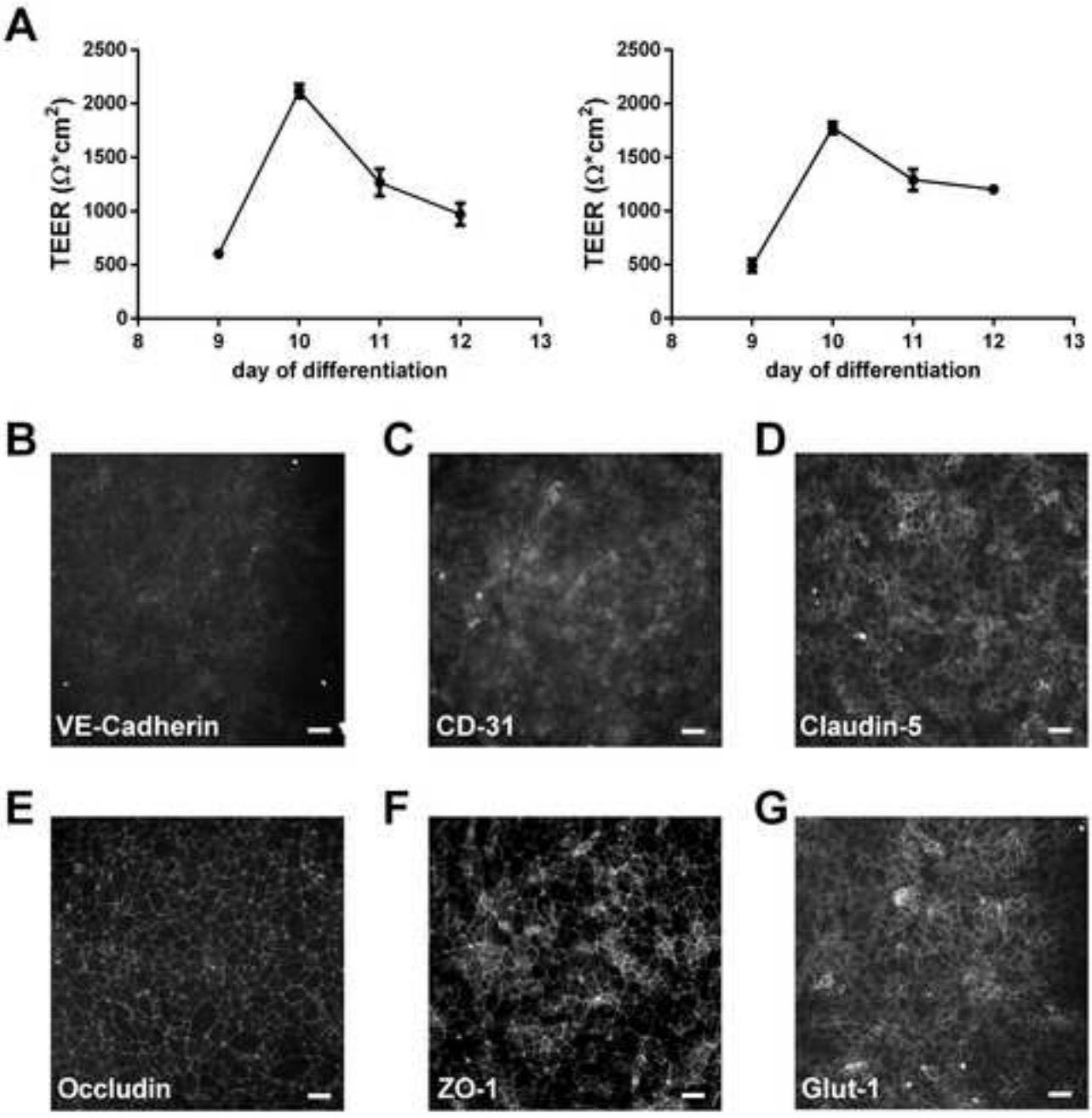

Figure 2

Figure 2

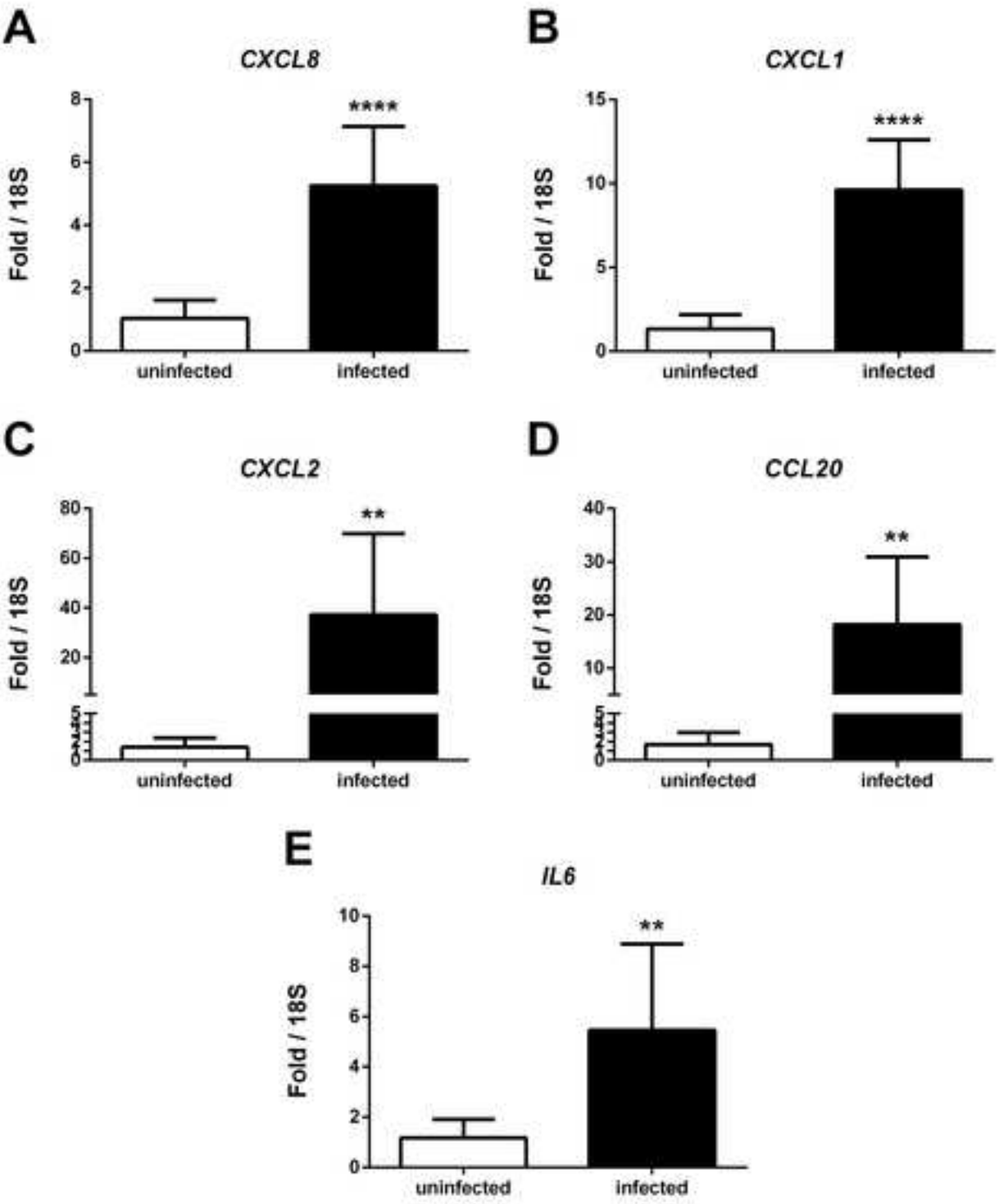

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials revision v3.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Accutase (1x)	Sigma	A6964	Enzymatic cell dissociation reagent
Acetic acid	Sigma	A6283	
All-trans retinoic acid (RA)	Sigma	R2625	
Anti-CD31 (PECAM-1)	Thermo Scientific (Labvision)	RB-10333	
Anti-Claudin-5	Invitrogen	4C3C2	
Anti-Glut-1	Thermo Scientific (Labvision)	SPM498 (MA5-11315)	
Anti-Occludin	Invitrogen	33-1500	
Anti-VE-cadherin	Santa Cruz	sc-52751	
Anti-ZO-1	Invitrogen	33-9100	
Bacto Proteose Peptone	BD	211684	
b-Mercaptoethanol	Merck (Sigma-Aldrich)	805740	
Cell culture plates and flasks	Sarstedt		
Centrifuge (Heraeus Megafuge 1.0R)	Thermo Scientific		
Class II biosafety cabinet	Nuaire	NU-437-400E	
CO2 Incubator (DHD Autoflow CO2 Air-Jacketed Incubator)	Nuaire		
Collagen IV	Sigma	C5533	
Columbia aaer + 5 % sheep blood	Biomerieux	43049	
Costar Transwell polyester filters (12- or 24-well)	Corning	3460, 3470	
D(+)-Glucose	Merck (Sigma-Aldrich)	G8270	
DAPI	Invitrogen	D1306	
DMEM/F12	Gibco	31330-038	
DMSO	ROTH	A994.1	
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	21600-069	
Epithelial Volt-Ohm Meter (Millicell ERS-2) with STX electrode	Merck (Millipore)	MERS00002	
$Fe(NO_3)_3$	ROTH	5632.1	
Fibronectin	Sigma	F1141	
Fluoresence microscope (Eclipse Ti)	Nikon		
Hemacytometer (Neubauer)	A. Hartenstein	ZK06	
Human basic fibroblast growth factor (bFGF)	PeproTech	100-18B	
Human Endothelial Serum Free Medium (hESFM)	Gibco	11111-044	
Inverted microscope (Wilovert)	Hund (Will Wetzlar)		
iPS(IMR90)-4 cells	WiCell		
Kellogg's supplement			To prepare 110 ml of Kellogg's supplement, prepare 100 ml of 4 g/ml glucose, 0.1 g/ml glutamine, and 0.2 mg/ml thiamine pyrophosphate and 10 ml of 5 mg/ml $Fe(NO_3)_3$ and combine the solutions. Filter sterilize and store aliquoted at -20 °C.
Knockout serum replacement (KOSR)	Gibco	10828-028	
L-glutamine (GlutaMAX)	Invitrogen	35050-038	
LunaScript RT SuperMix Kit	NEB	E3010L	cDNA synthesis kit
Matrigel Matrix	Corning	354230	
Methanol	ROTH	4627.5	
$MgCl_2$	ROTH	KK36.1	
Microplatten (Research Plus)	Eppendorf		
$NaHCO_3$	ROTH	6329	
Nonessential amino acids (NEAA)	Gibco	11140-035	
NucleoSpin RNA isolation kit	Machery-Nagel	740955	RNA isolation kit
Pipette boy (Accu-Jet Pro)	Brand		
Platelet poor plasma-derived serum, bovine (PDS)	Fisher	50-443-029	
PowerUp SYBR Green Master Mix	Applied Biosystems	A25742	qPCR master mix
qPCR film (MicroAmp Optical Adhesive Film)	Applied Biosystems	4311971	
qPCR plates (MicroAmp Fast 96-well)	Applied Biosystems	4346907	
ROCK inhibitor, Y27632 dihydrochloride	Tocris	1254	
RT-PCR thermo cycler (StepOnePlus)	Applied Biosystems	4376600	
Serological pipettes	Sarstedt		
StemFlex basal medium + 50x StemFlex supplement	Gibco	A3349401	Stem-cell maintenance medium
Swinging Bucket Rotor (Heraeus #2704)	Thermo Scientific		
Thiamine pyrophosphate	Sigma	C8754-5G	
Trypan Blue Solution, 0.4%	Gibco	15250061	
Versene	Gibco	15040-033	Non-enzymatic cell dissociation reagent (EDTA)

We thank the editor for their critical reading of the manuscript and the constructive comments concerning content and format. Please find a point-by-point response to the comments below (bold text).

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Keywords: Please provide at least 6 keywords or phrases.

The manuscript now contains the 6 keywords that were included in the online submission form.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercialnames. Examples of commercial sounding language in your manuscript are: Matrigel, StemFlex, Glutamax, Versene, Accutase, Millicell, LunaScript, etc.

We have replaced terms of commercial sounding language to the best of our ability.

For instance: "Stem-cell maintenance medium" to replace StemFlex (lines 114, 143, 150, 155, 172, 175, 182, 192); "L-glutamine" instead of GlutaMAX (line 121); "non-enzymatic cell dissociation reagent" for Versene (lines 148, 152); "enzymatic cell dissociation reagent" for Accutase (lines 168, 171, 224); "Epithelial volt-ohm meter (EVOM)" replacing Millicell (line 269).

We have also removed commercial sounding language such as NuceoSpin, LunaScript and SYBR green from the qPCR section (Section 7; lines 356 and following).

Matrigel, however, is a commonly used extracellular matrix (ECM) mix that we do not consider commercial language and find written in other JOVE reports (see below).

- **Ghogha, A., Bruun, D. A., & Lein, P. J. (2012). Inducing dendritic growth in cultured sympathetic neurons. Journal of visualized experiments : JoVE, (61), 3546. https://doi.org/10.3791/3546**
- **Winkler, M., Trieu, N., Feng, T., Jin, L., Walker, S., Singh, L., & Ku, H. T. (2011). A quantitative assay for insulin-expressing colony-forming progenitors. Journal of visualized experiments : JoVE, (57), e3148. https://doi.org/10.3791/3148**

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

Personal pronouns have been removed from the Protocol text.

5. 1.2: Please specify the volumes/concentrations of the supplement used.

The volumes of basal medium and supplement used in the preparation of the stem-cell maintenance medium are now mentioned in the text, as follows:

Lines 114-115: "Stem-cell maintenance medium is prepared by adding 50 ml of 50 x supplement to 450 ml of stem cell maintenance basal medium in the sterile environment of a biosafety cabinet."

6. 3.12: What experiments? Please specify.

We have specified experiments that are done on day 10 of the differentiation protocol.

The text in lines 253-254 now reads: "Conduct infection experiments, TEER measurement, and immunofluorescence staining as described in the following sections (day 10)."

7. Figures: Please define the error bars in the figure legend.

Error bars representing ± S.D. are now individually defined in the figure legends.

8. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

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Citations in the figure legends have been change to the appropriate format.

Lines 436-438: "Panels B-G of this figure have been modified and used with permission from Kim et al. (2019)18."

Lines 445-446: "This figure has been modified and used with permission from Martins Gomes et al. (2019)20."

We thank the reviewers for their critical reading, their generally positive feedback and constructive comments. Please find a point-by-point response (bold text) to the comments and concerns raised during the review. Changes to the manuscript have been exactly stated below to facilitate review.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript provides a detailed description for the differentiation of human induced pluripotent stem cells (iPSCs) to brain endothelial cells and their subsequent infection with the *Neisseria meningitidis* pathogen. The protocol is well-written and comprehensive. I have a few suggestions on technical details that would broaden its applicability to the research community.

We appreciate the reviewer's comments and agree that the suggested changes and additions better reflect the broad applicability of the protocol and increase its value to the research community.

Minor Concerns:

1) On lines 113-114, culture is described using StemFlex medium. In the discussion it is noted that other media can be used, but since this is a protocol paper, it would probably be appropriate to note here the other media that have been used in the literature (mTeSR and E8).

We have added a note to reflect the previous use of other stem-cell maintenance media for this protocol.

Lines 117-118: "NOTE: Other stem-cell maintenance media (mTeSR and E8) have been used in other studies[13–15, 22–25]."

2) On lines 120-123, the preparation of EC medium is described with platelet poor plasma-derived serum (PDS). Since PDS has been discontinued throughout the world, a protocol that utilizes it will not be feasible. It would be appropriate to note other available protocols that circumvent the need for PDS so readers are aware.

We agree and have now included a note mentioning use of other supplements such as B27, as described in literature, to make the reader aware of options for PDS substitution.

Find the added text in lines 130-131 stating: "NOTE: As PDS has been discontinued and may therefore be limited, this protocol has been successfully conducted using B27 in place of PDS[15, 23, 26]."

3) The protocol is centered around the use of the IMR90-4 line (which is incorrectly labeled as just IMR90 on line 128). In the discussion it is noted that other lines can be used, but it would probably be appropriate to note here some of the other lines that have been used extensively (can be limited to control lines, not disease-specific lines, since these are likely to be of the most interest for this protocol).

We have added a note stating that various other non-disease stem cell lines have been used for differentiation into BECs in the literature.

Lines 138-140 state: "NOTE: Here we use the IMR90-4 cell line as an example, however other induced pluripotent stem-cell lines such as CC3, CD10, CD12, DF19-9-11T, 83iCTR, 00iCTR, and CS03iCTRn2 have been successfully employed for differentiation into BECs[13–17, 23, 27, 28]."

4) On lines 184-187, it is noted that VE-cadherin+ cells become visible as differentiation progresses. VE-cadherin does not usually appear until after retinoic acid treatment. Thus, readers might be confused if they assume they will observe VE-cadherin during the UM differentiation phase. I would recommend replacing VE-cadherin with PECAM-1, which is commonly observed during the UM phase.

We thank the reviewer for catching this error. We have updated the note.

Now on lines 198-201, the note states: "NOTE: The cells typically reach confluence after 2 to 3 days in UM, which can be observed with the naked eye or through an inverted bright field microscope. As the differentiation progresses, nestin$^+$ "neural tracts" become visible with PECAM-1$^+$ cells in between as previously described[13, 14]."

5) In the references, the seminal paper from Lippmann and colleagues in Nature Biotechnology appears to be cited twice in different forms.

We thank the reviewer for spotting this error and have adjusted the references accordingly.

Reviewer #2:

Manuscript Summary:

Endres et. al. provided a protocol that clearly demonstrates how to derive human brain microvascular endothelial cells from iPSCs, prepare Neisseria meningitis for infection, and how to collect samples for future assays and analysis. The manuscript is well-written, clear, cited relevant works, and was concise.

Major Concerns:

None

Minor Concerns:

1) Section 3.9 was unclear in coating trans-wells as two different ratios were provided. Additionally, more information for the collagenIV/fibronectin/water ratio would be appreciated as some components arrive in liquid others in powder...or a final concentration would suffice.

This is a very good point made by the reviewer. We have amended section 3.9 to include more information on the preparation of the coating solution and clarify the difference between the two ratios used when coating transwells or cell culture plates. Please find the amended section:

Lines 211-216: "3.9. Coat cell culture plates and transwell inserts with collagen IV and fibronectin (day 7), for purification of the BECs and following experiments. For coating of transwells, combine 4 parts collagen IV (1 mg/ml in 0.5 mg/ml acetic acid), 1 part fibronectin (1 mg/ml) and 5 parts sterile tissue-grade water. ECM solution can be diluted 1:5 for coating cell culture plates (i.e. 4 parts collagen IV, 1 part fibronectin, 45 parts water). Incubate with coating solution at 37 °C overnight."

2) Figure 1 Immuno images were unclear and difficult to see in the provided .pdf. The downloaded .jpg figure added clarity but the VE-Cadherin image is still very dim. Additionally a magnified insert for each image would provide additional insight for the correct morphology/localization expected.

Generally we agree with the reviewer on this point. However the PDF provided is still not the full resolution file that is provided with the manuscript and is only meant for the review process. The actual file is a 600 dpi JPEG file with high resolution presentation of the images. Finally, as these results are meant to be "representative results" for JOVE, we have included the unaltered images from a previous publication (Kim et al 2019 Supplement Figure 1). Additionally, this protocol has been presented in many other papers also demonstrating the stainings. Therefore, we respectfully believe that our images are sufficient for the presentation of this protocol in general. To make this clear we have included in a note referring to the many other manuscripts also showing these markers.

Please find this note on lines 256-262 and the statement that addresses this comment is as follows: "NOTE: Successfully differentiated and purified BECs typically reach peak TEER on day 10 and express characteristic markers of brain endothelial cells such as PECAM-1 (CD31) and VE-cadherin, the glucose transporter GLUT-1, efflux transporters such as p-glycoprotein, and tight junction components ZO-1, Occludin, and Claudin-5[13, 14, 16, 17, 19, 22]. Refer to Lippmann et al. (2012), Stebbins et al. (2016) and others for further details and images of the cell types, morphologies, and expression of cell type specific markers during the differentiation process[13–17, 19, 22]."

Reviewer #3:

Manuscript Summary:

This manuscript provides a detailed differentiation protocol that is a variant of the original work by Lippmann and colleagues (Lippmann ES et al., Nature Biotech 2012) including the differentiation of the iPSC-derived BMECs into a T-75 flask model (instead of the 6-well plate), the validity of StemFlex iPSC maintenance medium instead of mTeSR1 or Essential-8 medium and its use for pathogen-host interaction.

Major Concerns:

No major concerns.

Minor Concerns:

No minor concerns.

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