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Evaluating the effect of acute diesel exhaust particle exposure on P-glycoprotein efflux transporter in the blood–brain barrier co-cultured with microglia

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ABSTRACT

A growing public health concern, chronic Diesel Exhaust Particle (DEP) exposure is a heavy risk factor for the development of neurodegenerative diseases like Alzheimer's (AD). Considered the brain's first line of defense, the Blood–Brain Barrier (BBB) and perivascular microglia work in tandem to protect the brain from circulating neurotoxic molecules like DEP. Importantly, there is a strong association between AD and BBB dysfunction, particularly in the Aβ transporter and multidrug resistant pump, P-glycoprotein (P-gp). However, the response of this efflux transporter is not well understood in the context of environmental exposures, such as to DEP. Moreover, microglia are seldom included in *in vitro* BBB models, despite their significance in neurovascular health and disease. Therefore, the goal of this study was to evaluate the effect of acute (24 hr.) DEP exposure (2000 μg/ml) on P-gp expression and function, paracellular permeability, and inflammation profiles of the human *in vitro* BBB model (hCMEC/D3) with and without microglia (hMC3). Our results suggested that DEP exposure can decrease both the expression and function of P-gp in the BBB, and corroborated that DEP exposure impairs BBB integrity (i.e. increased permeability), a response that was significantly worsened by the influence of microglia in co-culture. Interestingly, DEP exposure seemed to produce atypical inflammation profiles and an unexpected general downregulation in inflammatory markers in both the monoculture and co-culture, which differentially expressed IL-1β and GM-CSF. Interestingly, the microglia in co-culture did not appear to influence the response of the BBB, save in the permeability assay, where it worsened the BBB's response. Overall, our study is important because it is the first (to our knowledge) to investigate the effect of acute DEP exposure on P-gp in the *in vitro* human BBB, while also investigating the influence of microglia on the BBB's responses to this environmental chemical.

Introduction

Traffic-related particulate matter (PM), such as Diesel-Exhaust PM (DEP), is a major contributor to air pollution world-wide. A growing public health concern, chronic DEP exposure is a heavy risk factor for the development of neurodegenerative diseases (ND) like Alzheimer's (AD). Once inhaled, the ultrafine (i.e. nanometer-sized) DEP fraction can reach the central nervous system (CNS) indirectly (via systemic circulation), and/or directly (via the olfactory bulb), circumventing the blood–brain barrier (BBB) altogether. Considered the brain's first line of defense, the BBB is made up of highly-selective, tightly-connected cerebrovascular endothelial cells (ECs) that form a physical, transport, and enzymatic barrier that protects the CNS from circulating pathogens and neurotoxic molecules like DEP Fig. 1.

Importantly, BBB function and permeability are regulated by several cell types within the neurovascular unit (NVU); namely, neurons, pericytes, astrocytes, and perivascular microglia. Of these cell types, microglia are the immune cell of the brain, and work in tandem with the

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BBB to maintain ionic homeostasis and debride the CNS of waste, like proteinaceous aggregates and foreign substances. However, microglia are seldom included in *in vitro* models of the BBB, despite their dual role in the protection against or promotion of cerebrovascular damage and neurodegeneration (Dudvarski Stankovic et al., 2016; Santiago et al., 2017). In recent years, exposure to DEP has been shown to cause BBB dysfunction and microglial activation resulting in neuroinflammation, oxidative stress, and other deleterious effects that can promote AD-like pathology and dementia (Calderón-Garcidueñas et al., 2008a; Calderón-Garcidueñas et al., 2004; Calderón-Garcidueñas et al., 2016a,b; Calderón-Garcidueñas et al., 2008b; Hullmann et al., 2017; Li et al., 2020; Naughton and Pasinetti, 2021; Steiner et al., 2016).

While decades of investigation have established the critical roles of the BBB and microglia in AD, the exact mechanism of disease is not fully understood. Of the various hypotheses of the etiology of AD, the Amyloid β (Aβ) Cascade Hypothesis dominates the field. This hypothesis states that Aβ accumulation/deposition in the brain is a key step in AD pathogenesis (He et al., 2020). Briefly, Aβ is composed of 36–46 amino acids and its insoluble extracellular aggregates have neurotoxic effects that can hamper neuronal signaling, damage the BBB, and activate microglia; all leading to neuroinflammation and neurodegeneration (Navarro et al., 2018; Tu et al., 2015; Wang et al., 2021). To prevent Aβ accumulation, the healthy brain is equipped with multiple Aβ clearance mechanisms; of which, Aβ transport across the BBB is the most significant. Specifically, the BBB's Aβ transport system is comprised of the low-density lipoprotein receptor-related protein 1 (LRP-1), the receptor for advanced glycation end-products (RAGE), and the critical membrane efflux transporter P-glycoprotein (P-gp) (Lam et al., 2001). Also known as the multidrug resistance protein 1 (MDR1), P-gp has a wide substrate specificity and restricts the distribution of lipophilic and amphipathic xenobiotics including CNS drugs into the brain; which contributes to the brain's therapeutic resistance and is a major hurdle in CNS pharmacotherapy. Like P-gp, LRP-1 removes extracellular Aβ from the brain, while RAGE uptakes peripheral Aβ into the brain (Deane et al., 2009). Thus, a decreased expression/activity of P-gp and LRP-1, and/or an increased expression/activity of RAGE could lead to the accumulation of Aβ in the CNS. Importantly, BBB dysfunction resulting from disease states and/or xenobiotic exposures can hamper this Aβ transport system, promoting cerebral amyloidosis, amyloid angiopathy, etc (See Fig 2.).

In the context of AD, previous studies have demonstrated the association between disease progression and BBB dysfunction, specifically through altered P-gp function (Cai et al., 2018; Kuhnke et al., 2007; McCormick et al., 2021; van Assema et al., 2012). However, little is known about the effect of environmental chemical exposures, such as to DEP, on this Aβ and drug efflux transporter. Additionally, the influence

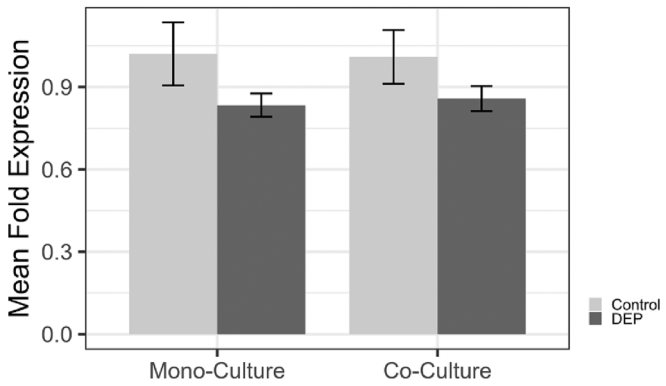

Fig. 2. Relative Gene Expression of P-gp after 24 hr. DEP exposure in monoculture and co-culture. RT-qPCR was used to assess the relative gene expression of P-gp (MDR1) in the endothelial monoculture and the endothelial-microglial co-culture after 24 hr. exposure to 2 mg/ml DEP. Bars represent mean fold change from control $2^{-\Delta\Delta CT}$ normalized with SDHA and expressed as a percentage of the unexposed control ± SEM. Statistical significance was considered $p \leq 0.05$ ($N \geq 3$, Mann-Whitney U Test). No statistical significance was detected.

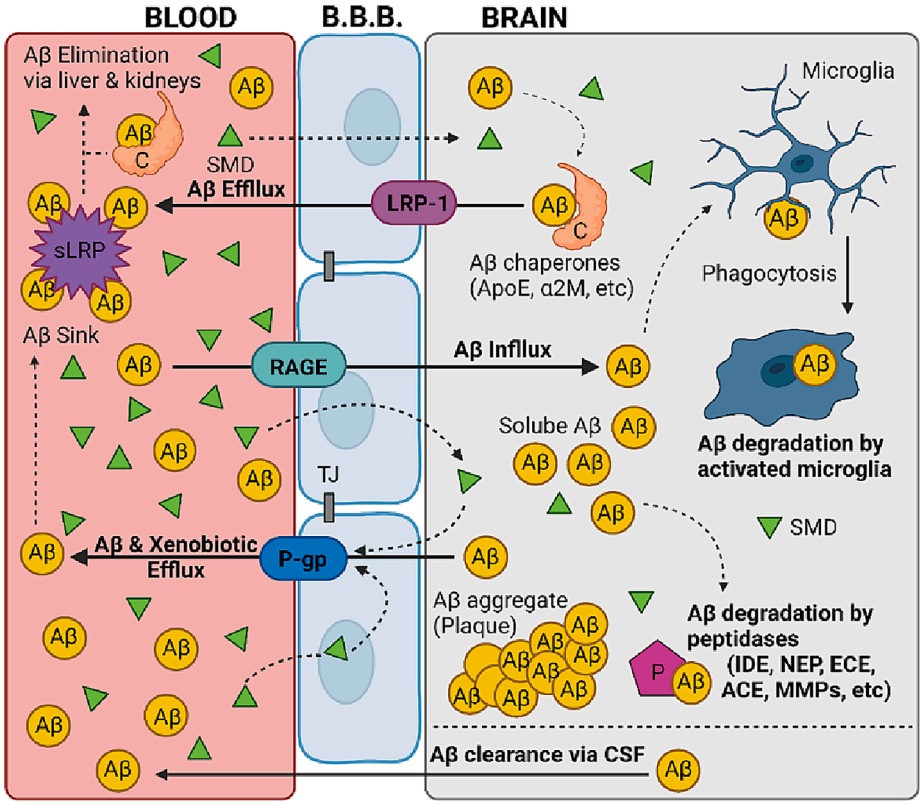

Fig. 1. The Role of P-glycoprotein and other Key Aβ Transporters in the Soluble Aβ Clearance Pathways of the BBB. Soluble Aβ in the brain parenchyma can undergo two basic fates: (1) aggregation into insoluble, neurotoxic Aβ aggregates (plaques) in the interneuron space, and (2) extrusion from the CNS across the BBB or via cerebrospinal fluid (CSF). Soluble Aβ can be eliminated from the brain via two main pathways: (1) enzymatic degradation and (2) receptor mediated clearance. In the first case, soluble Aβ is phagocytosed and then degraded by activated microglia (CNS macrophages) or peptidases (P) like Insulin-Degrading Enzyme (IDE), Neprilysin (NEP), Endothelin-Converting Enzyme (ECE), Angiotensin-Converting Enzyme (ACE), Matrix Metalloproteinases (MMPs), etc. In the second case, soluble Aβ is transported across the BBB and exported out of the CNS into systemic circulation through direct binding to P-gp and LRP-1 transporters, or through first binding Aβ chaperones (C) like apolipoprotein E (ApoE), apolipoprotein J (ApoJ), albumin, and α2-macrogobulin (α2M) that then deliver Aβ peptide to these efflux transporters. Importantly, P-gp is the most clinically-relevant multidrug resistant pump that besides extruding Aβ peptide, also extrudes xenobiotics and small molecule drugs (SMD), effectively limiting their accumulation in the CNS. Once in systemic circulation, Aβ can (a) re-enter the CNS via the RAGE receptor, (b) be sequestered by soluble LRP (sLRP), which acts as the main Aβ sink, and/or (c) be transported by chaperone molecules (ApoE and α2M) to the liver and kidneys for elimination from the body.

of naïve perivascular microglia on the BBB under such environmental exposures is not well understood. Therefore, the main goal of this study was to evaluate the effect of acute DEP exposure on the expression and function of P-gp in a human BBB *in vitro* model with and without microglia. A secondary goal was to evaluate the changes in the BBB model's paracellular permeability and in inflammation markers with and without microglia after DEP exposure. Overall, this study is significant because it offers deeper insight into how ambient air pollution like DEP can affect the BBB's AD-associated mechanisms and pharmacotherapy, which is a largely understudied area that warrants investigation (See Fig. 3).

Materials & methods

Cell culture

hCMEC/D3

The human cerebral microvascular endothelial cell line (hCMEC/D3) was selected because it has been well characterized for brain endothelial phenotype and has been validated for drug transport studies, given that it expresses functional efflux transporters (i.e. ABC transporters), including P-gp (MDR-1/ABCB1), breast cancer resistance protein (BCRP/ABCG2), and multidrug resistance-associated proteins 4 and 5 (ABCC4 and ABCC5) (Ohtsuki et al., 2013; Poller et al., 2008; Tai et al., 2009; Weksler et al., 2013). This cell line also expresses typical endothelial markers (CD31, VE-cadherin, von-Willebrand factor, etc.) and BBB characteristics such as the formation of tight junction proteins (Weksler et al., 2013). In our lab, the cells were obtained from Millipore Sigma (SCC066) and maintained in EndoGRO^{TM}-MV Complete Media Kit (SCME004, EMD Millipore) with 100 IU/ml penicillin and 100 mg/ml streptomycin (1% Pen-Strep) on collagen-IV coated flasks in a humidified incubator at 37 °C with 5% CO_2. Passages 5–15 we were used for experiments (See Fig. 4).

hMC3

The human microglia clone 3 embryonic cell line (hMC3 or CHME-3) was selected because it has been extensively characterized for microglial phenotype, and have been shown to retain the phenotypic and morphologic properties of human microglia *in vivo* (Dello Russo et al., 2018). These cells positively express several myeloid-specific markers including cluster of differentiation (CD)11b, CD68, and CD14. The cells were obtained from ATCC (CRL-3304) and maintained in EMEM supplemented with 10% FBS and 1% Pen-Strep, in a humidified incubator at 37 °C with 5% CO_2. This medium was also used to prepare the DEP suspension (i.e. dosing media) for the experiments. Passages 10–20 were used for experiments.

Co-culture

To develop the BBB EC-microglia mixed co-culture, hCMEC/D3 cells and hMC3 cells were seeded at a 9:1 ratio (given that microglia are estimated to comprise 10% of the human brain) (Salter and Stevens, 2017). Briefly, 1.0×10^6 hCMEC/D3 cells and 1.0×10^5 hMC3 cells were seeded on collagen IV- and poly-D-lysine-coated culture flasks or clear-bottom black 96-well plates for genetic or functional assays, respectively. The co-culture was maintained in EndoGRO^{TM}-MV Complete Media for 24 h after plating, and then replaced with Dosing Media (EMEM low glucose, FBS-free, 1% P-S) with or without DEP for acute exposure studies.

Preparation of DEP working suspension and exposure protocol

DEP from industrial forklift (SRM297; CAS 1333-86-4) was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). For detailed physicochemical characteristics of this reference material, see the SRM2975 Certificate of Analysis provided by NIST in Supplemental Information 1. We prepared the working suspension following a modified method by Block et al. Briefly, 20 mg of DEP suspended in 10 mL of PBS with 1% DMSO v/v, vortexed for 1 min, and sonicated for 45 min using an ultrasonicator (Ultrasonic Bath, Fisher Scientific, Waltham, MA). A solvent tolerance test (cell viability) was performed previously to ensure that both cell types were insensitive to 1% DMSO in the DEP suspension (data not shown). The DEP suspension was then sequentially-filtered through 5-μm, 1.2-μm, 0.8-μm, and 0.45-μm PES syringe filters (Thermo Fisher Scientific, Waltham, MA) prior to dosing the cells. This stock suspension was then diluted to obtain a working concentration of 2 mg/ml (2000 μg/ml, or 2000 ppm) in Dosing Media. We chose this concentration as our exposure concentration because it is sublethal and less than the inhibitory concentration 10% (IC_{10}), which was experimentally derived through concentration–response analyses in our lab previously (data not shown, EC_{50} = 29.5 mg/ml). Finally, cells were dosed for 24 h with the DEP suspension and incubated in a humidified incubator at 37 °C with 5% CO_2, prior to assaying.

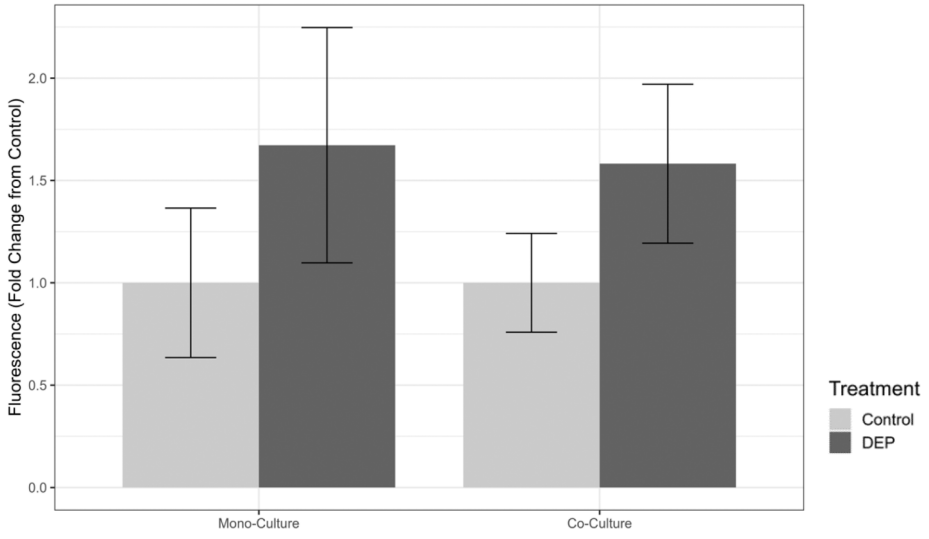

Fig. 3. P-gp function after 24 hr DEP exposure in monoculture and co-culture. The Rhodamine-123 Accumulation Assay was used to assess the transport activity or function of P-gp in the endothelial monoculture and the endothelial-microglial co-culture after 24 hr exposure to 2 mg/ml DEP. Bars represent mean fold change relative to the control ± SEM. Statistical significance was considered $p \leq 0.05$ ($N \geq 3$, Mann-Whitney U Test). No statistical significance was detected.

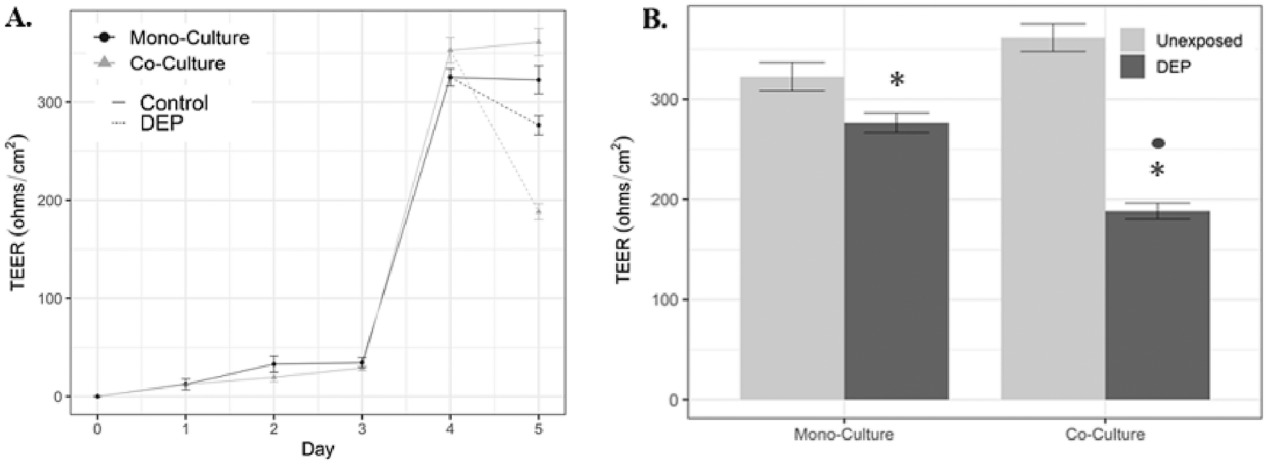

Fig. 4. A. TEER development in BBB models over time and after DEP exposure. Monoculture and co-culture BBB models were developed following a modified method from Förster *et al*, and their TEER were measured daily for 6 days. When the BBB models reached peek TEER on day 4, the models were exposed luminally to 2 mg/ml DEP or control media for 24 hr. Lines represent mean TEER ± SEM. B. Change in TEER after 24 hr. DEP exposure in monoculture and co-culture. Monoculture and co-culture BBB models were exposed to 2 mg/ml DEP for 24 hr. Bars represent mean fold change relative to the control ± SEM. Statistical significance was considered $p \leq 0.05$ ($N \geq 3$, Mann-Whitney U Test). Asterisk denotes significance from unexposed control. Black dot denotes significance from DEP-exposed monoculture.

Total RNA isolation and cDNA synthesis

After exposing the cells to DEP suspension or control medium for 24 h, total RNA was extracted using the PureLink RNA Mini Kit (12183018A, Thermofisher) with PureLink DNase On-Column treatment to remove genomic DNA (12185010, Thermofisher) following the manufacturer's instructions. RNA was eluted from the column with 50 μL RNase-free water and stored at −80 °C. RNA concentration (ng/μL) and quality ($A_{260/280}$ and $A_{260/230}$) were measured by UV spectrophotometry on NanoDrop One (13-400-519, Thermo Scientific, USA). RNA yield ranged from 800 to 2000 ng. The absorbance ratios ranged between 1.8 and 2.2. For cDNA synthesis, 1 μg of RNA template per cell group was retrotranscribed to cDNA using the SuperScript IV VILO Master Mix with ezDNase Enzyme kit (11766050, Thermo Fisher) following the manufacturer's instructions. cDNA samples were stored at −20 °C.

Reverse transcription quantitative real-time PCR (RT-qPCR)

To evaluate the relative genetic expression of P-gp in the DEP-exposed and control groups, we used Hs00184500_m1 (human Pgp/ABCB1) pre-designed Taqman Genetic Expression Assay (ThermoFisher). To verify that our reference gene and gene of interest (GOI) were independent of each other in order to satisfy the assumptions for multiple independent univariate testing, a correlation matrix was calculated using RNAseq data from healthy patients obtained from the Religious Orders Study and Memory and Aging Project (ROS/MAP) database (for correlation matrix results, see Supplementary Information 2). SDHA was selected as our reference gene because it was not correlated to our GOI. For SDHA, we used Hs99999905_m1 Taqman Genetic Expression Assay (ThermoFisher). The probes (i.e. context sequences) for P-gp and SDHA were the following:

AGACATGACCAGGTATGCCTATTAT (P-gp).

TGTTGTTGCCACAGGAGGCTACGGG (SDHA).

For each cell group, the number of biological replicates was at least 3, and the number of technical replicates for all reactions was 3. No Template Controls (NTCs) were included in all experiments. RT-qPCR was performed with Taqman Fast Advance Master Mix (4444557, Thermo Fisher) following the manufacturer's instructions, using 25 ng cDNA and 1X Taqman Genetic Assay in 20 μL reaction volume. The thermal protocol consisted of initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 10 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min. The RT-qPCR experiments were performed in one 384-well plate using the QuantStudio™ 6 Flex RT-qPCR System (ThermoFisher, USA).

P-gp function (Rhodamine-123 accumulation assay)

To measure P-gp function after exposure to DEP suspension or control medium, we used the Rhoamine-123 (RHO-123) Accumulation Assay described previously by Tai et al. (2009). RHO-123 is a fluorescent molecule that passively diffuses into the cell and is a known substrate of P-gp, whose efflux activity can be arrested at 4 °C or inhibited with chemical inhibitors (e.g. Tariquidar). Briefly, after 24-hour exposure to DEP or control cell culture media, the cells in black, clear-bottom 96-well plates were pre-incubated with 20 μM RHO-123 (Cat. No. 83702, Sigma) in Ringer-Hepes Buffer at 4 °C for 60 min, protected from light. Subsequently, warmed Ringer-Hepes Buffer was added and cells were incubated at 37 °C for 60 min. After this incubation, the RHO-123 solution was removed and cells were rinsed twice with DPBS to remove excess extracellular RHO-123. Finally, cells were lysed by adding ethanol and DMSO (1:1) solution, and fluorescence was measured on a spectrophotometer at 505/534 nm excitation/emission wavelengths. Tariquidar was used as a positive control for RHO-123 accumulation (i.e. P-gp inhibition) to ensure adequate assay performance (data not shown).

Transwell BBB model development and Transendothelial Electrical resistance (TEER)

To develop the *in vitro* Transwell BBB models, we followed a method previously developed by Förster *et al* (Förster et al., 2008). Briefly, on Day 0, cells were plated at 2.5×10^4 cells per cm^2 on collagen IV-coated Transwell inserts (polyethylene terephthalate (PET), 12-well format, 1 μm pore; 353103, Falcon, Germany) in EBM-2 basal medium (from EGM-2 BulletKit, Lonza, UK), supplemented with 2.5% FBS and the growth factors included in the kit except hydrocortisone (HC) (VEGF, IGF-1, EGF, bFGF, heparin, ascorbate, gentamycin), following the manufacturer's recommended concentrations. To develop the BBB Transwell model, this HC-free medium was replenished on Day 2. Then on Day 3 (when the cells formed a confluent monolayer), the medium was switched to EBM-2 basal medium with reduced (0.25%) FBS and

100 nM hydrocortisone, with no other growth factors (Serum-Reduced HC Medium). To develop the BBB-microglial Transwell model, the microglia were seeded in poly-D-lysine-coated 12-well plates at 2.5 × 10^3 cells per cm^2 on Day 2. On Day 3, the corresponding, confluent BBB monolayer Transwells were transferred to the microglial plates and the medium was exchanged for Serum-Reduced HC Medium. 24 h later (Day 4), both the BBB and the BBB-microglia Transwell models reached maximum TEER (~300 Ohm/cm^2), at which point the cells were dosed apically with DEP suspension for 24 h. TEER was measured daily using the EVOM2 Meter with the EndOhm Chamber (ENDOHM-12G, World Precision Instruments, USA). The cell's TEER was baseline-corrected by subtracting the TEER of a blank Transwell insert coated with collagen-IV.

Inflammatory marker production

The production (fluorescence) of six pro-inflammatory (IL-1α, IL-1β, IL-6, TNFα, IFNg, GM-CSF) and four anti-inflammatory (IL-4, IL-10, IL-13, Fractalkine) cytokines/chemokines (C/Cs) was measured with a customized MILLIPLEX Magnetic Microsphere immunoassay (HCYTA-60 K-PX48, Millipore, USA). The assay was performed following manufacturer's instructions with a MAGPIX multiplexing system (Luminex Corp., Austin, TX), using cell culture media samples collected 24 h post-exposure to DEP suspension or to control media. The samples were stored in −80 °C until day of assay. A DEP interference test was used to confirm that the DEP suspension alone had no effect on fluorescence (data not shown).

Statistical analyses

All results are expressed as mean ± SEM, and all assays had a sample size N ≥ 3 (biological replicates). For the RT-qPCR assay, relative gene expression was calculated using the delta-delta Ct method. The difference in means between the unexposed control and DEP-exposed groups within each cell culture type (i.e. monoculture and co-culture) and between the two cell culture types for DEP exposure were tested using the Mann-Whitney U Test. Second, for the P-gp (RHO-123 Accumulation) Assay, the data were standardized as fold-increases from the mean for each group. Then, the difference in means between the unexposed control and the DEP-exposed groups for each cell culture type was tested using the Mann-Whitney U Test. Next, for the Luminex (Inflammatory C/Cs) Immunoassay, the median fluorescence intensity of all events for each sample was first standardized as fold-increase from the mean of the control of each group. Then, the difference in means between the unexposed control and the DEP-exposed groups for each cell culture type was tested using the Mann-Whitney U Test. Finally, for the Paracellular Permeability Assay, the difference in means between the unexposed control and DEP-exposed groups, and between the two cell culture types for DEP exposure were tested with a Mann-Whitney U Test. The *p*-values were corrected using the Benjamini-Hochberg FDR method.

Results

RT-qPCR assay

After 24 h of DEP exposure, RT-qPCR was performed to measure P-gp's (MDR1) relative genetic expression. In the DEP-exposed monoculture (83 ± 4%), P-gp was downregulated ~ 17%, compared to its unexposed control (100 ± 10%). Similarly, in the DEP-exposed co-culture (86 ± 5%), P-gp was downregulated ~ 14% compared to its unexposed control (100 ± 10%). Although the P-gp expression level in the DEP-exposed co-culture was slightly attenuated compared to that of the DEP-exposed monoculture, this difference was not significant.

P-gp activity (rhodamine-123 accumulation assay)

After 24 h of DEP exposure, P-gp function (i.e. transport activity) was assessed using the Rhodamine-123 Accumulation Assay. Results indicated that DEP caused a moderate increase in intracellular RHO-123 accumulation in both the monoculture (~67%; DEP-exposed mono. = 1.67 ± 0.57%) and co-culture (~58%; DEP-exposed co-c. = 1.58 ± 39%)(Control mono. = 100 ± 37%; Control co-c. = 100 ± 24%). This DEP-induced increase in RHO-123 accumulation indicates a decrease in the activity or function of P-gp, since RHO-123 accumulation and P-gp activity are inversely proportional; however, this response was not considered significant. Additionally, there was no difference in RHO-123 accumulation between the DEP-exposed monoculture and DEP-exposed co-culture, which suggests that the microglia's influence did not affect the response of the BBB monoculture in this assay.

Paracellular permeability (TEER) assay

After 24 h of DEP exposure, the paracellular permeability or Trans-endothelial Electrical Resistance (TEER) of the barriers were measured. Overall, DEP exposure caused a significant decrease in both the monoculture and the co-culture's TEER. Specifically, DEP exposure caused a moderate reduction (-14%; −46.1 Ohm/cm^2) in the monoculture's TEER (DEP-exposed mono. = 276.5 ± 9.7 Ohm/cm^2); and a large reduction (-48%; −173 Ohm/cm^2) in the co-culture's TEER (DEP-exposed co-c. = 188.3 ± 7.9 Ohm/cm^2), relative to their respective unexposed controls (Control mono. = 322.6 ± 14.3 Ohm/cm^2; Control co-c = 361.3 ± 13.7 Ohm/cm^2). This also suggests that the microglia's influence in co-culture exacerbated the loss of barrier integrity (i.e. increased permeability) in the BBB after DEP exposure, indicating that the microglia had a detrimental effect on barrier tightness.

Inflammatory marker production

After 24 h of DEP exposure, 10C/Cs (pro- and anti-inflammatory) were measured in the monoculture and co-culture. In the monoculture, DEP exposure caused an overall downregulation in C/Cs except in IL-1α, which showed the greatest induction strength (16 ± 22%); and in GM-CSF, which showed no change. On the other hand, pro-inflammatory C/Cs IL-6 (11 ± 2%) and TNFα (76 ± 2%) showed the greatest downregulation after DEP exposure (~89%, and ~ 24% reduction, respectively). Additionally, pro-inflammatory IL-1β (93 ± 3%) and IFNg (93 ± 3%) were equally downregulated in the monoculture after DEP exposure (~7% both). Similar to most pro-inflammatory C/Cs here, the anti-inflammatory C/Cs IL-4 (68 ± 2%), Fractalkine (83 ± 2%), IL-10 (86 ± 1%), and IL-13 (88 ± 1%) showed a large to moderate downregulation after DEP exposure (~32%, ~17%, ~14%, and ~ 12% reduction, respectively).

In the co-culture, DEP exposure caused the greatest induction strength in pro-inflammatory C/Cs GM-CSF (50 ± 20%), and minimal induction or no change in IL-1β and IFNg (5 ± 5% and 0 ± 3%, respectively). On the other hand, DEP exposure caused a large to moderate downregulation in pro-inflammatory C/Cs IL-6 (53% ± 9%), TNFα (76% ± 4%), and IL-1α (90 ± 5%) (~47%, ~24%, ~10% reduction, respectively). The anti-inflammatory C/Cs IL-4 (69% ± 4%), IL-13 (92 ± 3%), and Fractalkine (96 ± 4%) showed moderate to minimal downregulation after DEP exposure (~31%, ~8%, ~4% reduction, respectively); while IL-10 showed no change (0 ± 4%). Table 1 (below) summarizes the C/Cs mean percent change for the DEP-exposed groups, relative to their corresponding unexposed controls.

Last, compared to the monoculture, the co-culture showed greater C/C production levels, though still downregulated, in all anti-inflammatory (Fractalkine, IL-4, IL-10, IL-13) and several pro-inflammatory (IFNg, IL-1β, IL-6) C/Cs. This suggests that the microglia's influence in the co-culture increased C/C production relative to the monoculture, as we expected; but the overall effect

Table 1
Mean percent change (i.e. induction strength) represents the C/C fluorescent ratio of the DEP-exposed group to the unexposed control group. Values represent mean ± SEM.

Type	Cyto./Chemo.	Monoculture	Co-culture
Anti-Inflammatory	Fractalkine	0.83±0.02	0.96 ± 0.04
	IL-4	0.67 ± 0.02	0.69 ± 0.04
	IL-10	0.86 ± 0.01	1.00 ± 0.04
	IL-13	0.88 ± 0.01	0.92 ± 0.03
Pro-Inflammatory	GM-CSF	1.02 ± 0.07	1.50 ± 0.20
	IFNg	0.93 ± 0.03	1.00 ± 0.03
	IL-1α	1.16 ± 0.22	0.90 ± 0.05
	IL-1β	0.93 ± 0.03	1.05 ± 0.05
	IL-6	0.11 ± 0.02	0.53 ± 0.09
	TNFα	0.76 ± 0.02	0.76 ± 0.04

(downregulation) of DEP exposure on most C/Cs in both cell culture groups, we did not expect. Interestingly, DEP exposure caused an elevated production of IL-1α in the monoculture that was not observed in the co-culture, and an even greater production of GM-CSF in the co-culture that was not present in the monoculture. This suggests that the microglia's influence in the co-culture limited the BBB model's production of IL-1α, oddly, but increased the production of GM-CSF.

Discussion

There is robust evidence that chronic exposure to PM like DEP is associated with BBB dysfunction and microglial activation, both of which can contribute to the onset and development of neurodegenerative diseases like AD over time (Babadjouni et al., 2017). Despite the BBB and microglia's physiological relevance as the brain's first line of defense (Alexander, 2018), most *in vitro* toxicology and pathophysiology studies fail to include microglia in their BBB models. To investigate the effect of DEP in the context of AD, the goal of this study was to evaluate the effect of acute DEP exposure on P-gp, a key Aβ- and drug-efflux transporter, and on barrier permeability and inflammation using an *in vitro* human BBB model with and without microglia.

Taken together, the results of the P-gp assays suggest that acute DEP exposure may cause changes in P-gp expression and function that can alter the BBB's Aβ transport equilibrium, potentially leading to the decreased excretion of soluble Aβ and drugs/xenobiotics from the brain to some degree. Whether this degree of P-gp inhibition leads to toxic levels of cerebral Aβ accumulation likely depends on the length of exposure, the efficiency of other Aβ clearance mechanisms (LRP-1 and RAGE, microglia, proteases, vascular smooth-muscle cells, etc.), and the inflammation status of the brain, etc. Specifically, the results of the RT-qPCR assay indicated that DEP exposure decreased the mRNA expression of P-gp in the monoculture and the co-culture to a similar extent (though not significantly), suggesting that the microglia's presence in co-culture did not influence this parameter. At the phenotypic level, this finding was corroborated by the results of the P-gp Function Assay, which measured the intracellular accumulation of RHO-123, a known P-gp substrate. Notably, Rho-123 accumulation after DEP exposure increased over 50% in both the monoculture and co-culture. Importantly, an increase in intracellular RHO-123 accumulation is indicative of a downregulation in P-gp activity and/or expression; which means that DEP exposure appeared to inhibit P-gp's function to an extent in this study (though not statistically-significant). An essential, ATP-dependent, polyspecific efflux pump in the BBB, P-gp is responsible for the extrusion of soluble Aβ from the brain and the high attrition rate of CNS drugs. Therefore, a DEP-induced decrease in P-gp's efflux function could potentially result in the increased penetration of not just CNS-targeting drugs but of unwanted, harmful molecules also. This unwanted P-gp inhibition is not a beneficial effect and can potentially result in heightened Aβ peptide accumulation in the brain and CNS susceptibility to toxic insult, adverse drug interaction, and numerous pharmacokinetic issues.

Notably, P-gp activity has been shown to be modulated by a variety of non-ligand agents, including physiological compounds, like progesterone, and curcumin (Sreenivasan et al., 2013; Zhou, 2008) as well as environmental toxins like sterigmatocystin (mycotoxin)(Wang et al., 2016). Transcriptionally, P-gp is regulated by several pathways such as Notch signaling, Wnt/β-catenin signaling, peroxisome proliferator-activated receptors (PPAR) signaling, and the pregnane X receptor (PXR) (Apostoli and Nicol, 2012; Hartz and Bauer, 2010). Importantly, PXR is the ligand-activated master transcription factor that controls xenobiotic- and drug-inducible expression of key genes (e.g. MDR1, BCRP1, etc.) that encode numerous phase I and phase II metabolic enzymes and drug transporters (Bauer et al., 2005; Bauer et al., 2006; Pavek, 2016). Therefore, one plausible signaling pathway for DEP-induced P-gp modulation is through PXR activation, but this hypothesis has yet to be confirmed.

Interestingly, the P-gp findings in our human *in vitro* model differ from what a seminal study by Hartz *et al* found in a rat *in vitro* model. In said study, P-gp showed an upregulation in expression and activity, and in TNF-α production, in freshly isolated rat brain capillaries exposed to DEP (5–200 μg/ml) (Hartz et al., 2008). Notably, they demonstrated that P-gp upregulation was associated specifically with DEP exposure and not PM (i.e. carbon black) exposure in general. This P-gp upregulation was abolished by inhibition of TNF-receptor 1, c-Jun N-terminal kinase (JNK), and NADPH oxidase; which highlighted the role of inflammation and oxidative stress pathways in DEP-induced P-gp changes in rat ECs. However, other studies have demonstrated that DEP-induced cytotoxicity and oxidative stress can be inflammatory or noninflammatory (Aquino et al., 2021; Block et al., 2004; Levesque et al., 2013; Levesque et al., 2011; Tseng et al., 2017). In the human BBB-microglia *in vitro* model of our study, DEP exposure appears to be noninflammatory. Interestingly, a different study by Le Vee *et al* that exposed human breast cancer cells (MCF-7/R) to organic DEP extract (1–20 μg/ml) showed no altered P-gp activity (Le Vee et al., 2015). Not surprisingly, the discrepancy in P-gp activity results among these *in vitro* DEP exposure studies may be due to differences in cell type sensitivities and/or interspecies variability, a phenomenon that has been documented in other xenobiotic/drug toxicity and disease mechanism studies (Navarro et al., 2018; Shanks et al., 2009; Uhl and Warner, 2015).

In addition to evaluating the changes in P-gp function under DEP exposure, a second objective in our study was to evaluate the effect of DEP exposure on BBB paracellular permeability, an important measure of BBB integrity and health. Overall, the results of the TEER Assay suggested that acute DEP exposure significantly impaired the *in vitro* BBB, as evidenced by a moderate reduction in the monoculture's TEER (−14%) and a large reduction in the co-culture's TEER (−48%). These findings corroborate the results of numerous other studies that have demonstrated reduced BBB tightness and function following exposure to PM (Choi et al., 2014; Heidari Nejad et al., 2015; Lucero et al., 2017; Oppenheim et al., 2013; Suwannasual et al., 2018; Tobwala et al., 2013). Additionally, these results further suggest that the more severe BBB impairment in the co-culture is a result of microglial influences, which have been shown both *in vitro* and *in vivo* to worsen BBB dysfunction, oxidative stress, neuroinflammation, and other mechanisms involved in the progression of CNS diseases (Blasko et al., 2004; Block et al., 2004; Block et al., 2007; da Fonseca et al., 2014; Sumi et al., 2010; Yenari et al., 2006).

The last objective of our study was to evaluate the inflammatory response of the BBB model to DEP exposure by measuring both pro-inflammatory and anti-inflammatory C/Cs. As observed in our previous study (Aquino et al., 2021), DEP exposure caused mixed, atypical responses in both the monoculture and the co-culture (Fig. 5). In our current study, the majority of C/Cs in both cell culture groups were downregulated after DEP exposure, save for IL-1α (16% upregulation) in the monoculture, and GM-CSF (50% upregulation) in the co-culture. In

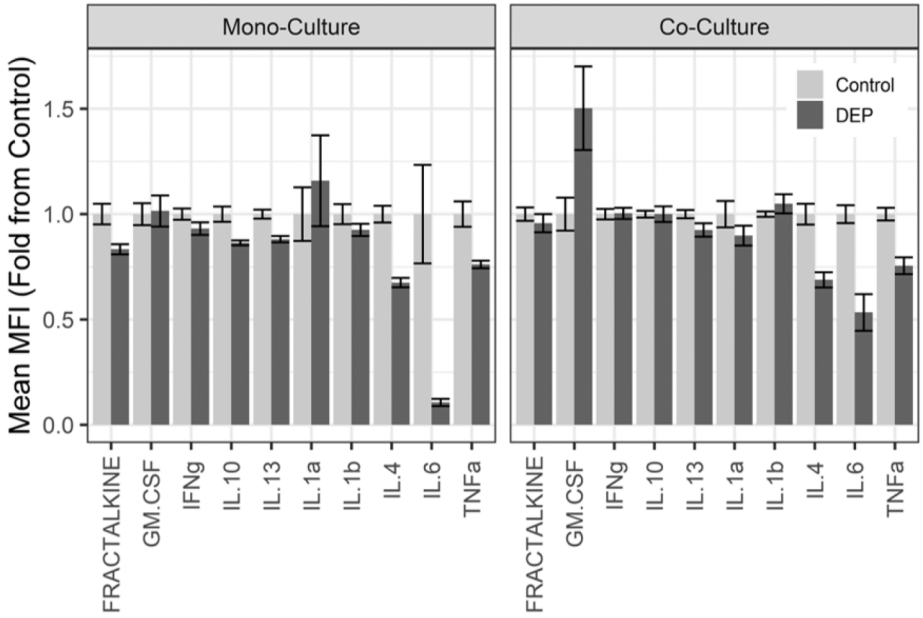

Fig. 5. Inflammatory marker profiles of monoculture and co-culture BBB models after 24 hr DEP exposure. Luminex bead immunoassays were used to measure 10 cytokines/chemokines in the endothelial monoculture and the endothelial-microglial co-culture after 24 hr exposure to 2 mg/ml DEP. Bars represent mean fold change from unexposed controls ± SEM. Statistical significance was considered $p \leq 0.05$ (N = 4, Mann-Whitney U Test). No statistical significance was detected.

other words, while both cell culture groups experienced an overall C/C downregulation after DEP exposure, they differentially produced GM-CSF and IL-1α (although these changes were not statistically significant). It is important to note that a downregulation in C/Cs is not necessarily a beneficial response and may point to the role of DEP as a possible suppressor of cytokine signaling (SOCS) or as an inhibitor of NF-κB, MAPK, and/or JAK/STAT3 signaling, which are essential signal transduction pathways that control the production of many inflammatory mediators (Hu et al., 2016; Malik and Kanneganti, 2018; Planas et al., 2006).

In the context of PM exposures *in vivo*, IL-1α and TNF-α have been shown to be elevated in different regions of rodent brains after prolonged exposure to DEP (Campbell et al., 2005; Gerlofs-Nijland et al., 2010). Similarly, GM-CSF is considered a pro-inflammatory cytokine, but it can also be anti-inflammatory depending on its concentration, the presence of other relevant C/Cs, and the context of the immune response (Bhattacharya et al., 2015). In the brain, it is a major mediator of the inflammatory process but has been shown to trigger microglial proliferation (microgliosis) without the release of pro-inflammatory IL-6 or TNF-α (Dikmen et al., 2020), two cytokines that were absent in our DEP-exposed cells' profiles. Notably, other studies have also reported a decreased or no production of pro-inflammatory C/Cs from DEP-exposed macrophages (Aquino et al., 2021; Saito et al., 2002).

Given the complexity of inflammatory signaling and the general decrease in inflammatory marker levels, it is difficult to determine whether the ECs and microglia assumed a pro-inflammatory or anti-inflammatory state in our study. However, the results seem to indicate that the microglia were partially but atypically activated, given the increased production of GM-CSF but the low abundance of classic (IL-1, IL-6, TNF-α) pro-inflammatory C/Cs in the co-culture. Importantly, we previously confirmed that HMC3 microglia can be activated towards the pro-inflammatory phenotype *in vitro*, as evidenced by IL-6 production from exposure to LPS and IFNg, two potent pro-inflammatory activators (data not shown).

Finally, the last question we sought to answer was, how does the microglia's influence in the co-culture alter the response of the BBB, relative to the monoculture? Interestingly, in both the genotypic and functional P-gp assays, there was no observable difference between the monoculture's and co-culture's responses; suggesting that the microglia did not play a role in the transcriptional or functional modulation of the P-gp efflux transporter. In the TEER assay, however, the DEP-exposed co-culture's TEER was reduced more than 2-fold that of the DEP-exposed monoculture; suggesting that the microglia's influence was detrimental by exacerbating the negative effect of DEP exposure on barrier permeability. Last, in terms of inflammation, the microglia in the DEP-exposed co-culture did not significantly increase the production of inflammatory markers compared to the DEP-exposed monoculture, though it should be noted that the levels of GM-CSF, IL-1β, and IL-6 were relatively higher in the co-culture. Given the general downregulation of most inflammatory markers in the DEP-exposed groups, however, we cannot conclude that the microglia's influence in this parameter was detrimental (pro-inflammatory). Taken together, these results suggest that the microglial influence on the BBB EC's under DEP exposure was assay-dependent and that these naïve (un-primed) microglia *in vitro* do not readily respond to PM.

Finally, while the results of the P-gp genotypic and functional assays were not statistically-significant, they trend towards significance, and they are important because they are the first documented attempt at elucidating the effect of DEP exposure on the human P-gp pump, a crucial AD-related Aβ- and drug-efflux transporter that is severely understudied in the context of environmental exposures. Moreover, given that the human *in vitro* BBB-microglia model in this study applied immortalized cells, which may or may not exhibit similar responses and sensitivities to exposures as primary cells, future studies should incorporate improved human *in vitro* BBB/NVU models, such as iPSC-derived ECs and microglia, to investigate P-gp dysfunction under DEP exposure (and environmental chemicals in general). Last, to better study the inflammation profile of microglia exposed to DEP and other toxicants, phenotypic analyses such as through flow cytometry can provide deeper insight of the inflammation status of the cell population according to the M1-M2 continuum.

Conclusion

In summary, this study evaluated the effect of acute DEP exposure on P-gp expression and function, paracellular permeability, and inflammation profiles of the *in vitro* human BBB with and without microglia. Our results suggested that DEP exposure may modify the genotypic

expression and function of P-gp *in vitro* to some extent, which may promote increased xenobiotic and Aβ entry/accumulation in the CNS; though these findings should be validated *in vivo*. Our results also corroborated that DEP exposure impairs BBB integrity, evidenced by increased paracellular permeability (i.e. loss of barrier tightness); a response that was worsened by the influence of microglia in co-culture. Interestingly, DEP exposure seemed to produce atypical inflammation profiles and a general downregulation in inflammatory markers in both the monoculture and co-culture, which differentially expressed pro-inflammatory IL-1β and GM-CSF. Additionally, the microglia in co-culture did not appear to influence the response of the BBB to DEP exposure, save in the barrier permeability assay; indicating that the BBB-microglia interaction is important in some mechanisms of BBB dysfunction but not all. Overall, this pilot study is important because it is the first (to our knowledge) to investigate the effect of acute DEP exposure on the P-gp efflux transporter in the *in vitro* human BBB, while also investigating the influence of microglia on the BBB's responses to this environmental exposure.

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CRediT authorship contribution statement

Grace V. Aquino: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Amjad Dabi:** Formal analysis, Data curation, Visualization. **Gabriel J. Odom:** Formal analysis, Data curation. **Ramon Lavado:** . **Kaitlin Nunn:** Methodology, Validation, Investigation. **Kathryn Thomas:** Methodology, Validation, Investigation. **Bennett Schackmuth:** Methodology, Validation, Investigation. **Nazeel Shariff:** Methodology, Validation, Investigation. **Manogna Jarajapu:** Investigation. **Morgan Pluto:** Investigation. **Sara R. Miller:** Methodology, Validation, Investigation. **Leah Eller:** Investigation. **Justin Pressley:** Investigation. **Rishi R. Patel:** Investigation. **Jeffrey Black:** Investigation. **Erica D. Bruce:** Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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