**WORKING TITLE**

Differential cytotoxicity, oxidative stress, and pro-inflammatory markers in endothelial and microglial monocultures and mixed co-cultures exposed to low doses of ultrafine diesel exhaust particles

**AUTHORS**

Grace V. Aquino, Amjad Dabi, Gabriel J. Odom, Fan Zhang, Erica D. Bruce

**ABSTRACT**

**INTRODUCTION**

The 2013 Global Burden of Disease, Injuries, and Risk Factor Study found that atmospheric air pollution (AAP) from emissions of particulate matter (PM) is a leading cause of morbidity and mortality world-wide (Forouzanfar et al., 2015). Diesel exhaust particles (DEPs) generated by the combustion of diesel fuel by motor vehicles comprise a major fraction (>70%) of the PM in the atmosphere and are ubiquitous in the environment up to an altitude of 15 km (Peters et al., 2006). DEPs are composed of aerosols and particles measuring less than 10 μm in aerodynamic diameter (PM10), most of which are ultrafine particles (UF-DEPs) measuring less than 200 nm in aerodynamic diameter (Araujo and Nel, 2009; Donaldson et al., 2005). These combustion-derived nanoparticles are a particular public health concern because people in urban settings are widely exposed to them and once inhaled, UF-DEPs can translocate across the lung into systemic circulation, and can reach extra-pulmonary organs such as the brain (Elder et al., 2006; Nemmar et al., 2002; Nemmar et al., 2001; Oberdörster et al., 2009; Oberdörster et al., 2004; Terzano et al., 2010). Other routes of UF-DEP entry into the brain observed in animal studies include the nasal olfactory mucosa and nasal olfactory bulb (Genc et al., 2012; Lucchini et al., 2012; Oberdörster et al., 2004; Peters et al., 2006). Due to their large surface area-to-volume ratio and chemical composition -a carbonaceous core with adsorbed organic compounds like PAHs, endotoxin like lipopolysaccharide (LPS), sulfates, and trace elements-, UF-DEPs can be highly reactive and cause multiple cytotoxic effects (Alam et al., 2016; Heusinkveld et al., 2016; Levesque et al., 2013; Rohr and McDonald, 2016). In both peripheral and CNS UF-DEP exposures, oxidative stress and inflammation are the two chief processes by which air pollution exerts its toxicity (Block and Calderón-Garcidueñas, 2009; Brook et al., 2010; Genc et al., 2012; Lodovici and Bigagli, 2011). Upon intracellular uptake, DEPs generate reactive oxygen species (ROS), which is attributed to the chemical composition of the particles (Park et al., 2011). The resulting ROS can lead to oxidative stress, which in turn can trigger a multitude of cellular consequences, such as DNA damage, lipid peroxidation, proinflammatory signaling, antioxidant defense activation/depletion, and apoptosis (Cao et al., 2007; Li and Nel, 2006; Nemmar et al., 2010; Wan and Diaz-Sanchez, 2007; Wichmann, 2007). While recent studies have reported that exposure to DEP can increase the risk of stroke and cerebrovascular damage, it is well know that DEP exposures induce neuroinflammatory signaling and oxidative stress that can trigger neurodegenerative disorders like Multiple Sclerosis, Alzheimer’s, and Parkinson’s disease (Hartz et al., 2008; Levesque et al., 2011a; Levesque et al., 2013; Levesque et al., 2011b). Specifically, these studies reported that DEPs induce oxidative stress, proinflammatory signaling, up-regulation of P-glycoprotein (P-gp), and dysfunction in the blood-brain barrier.

The blood-brain barrier is a highly specialized barrier of brain microvascular endothelial cells (BMECs) that separates the blood from the central nervous system (CNS), thereby preventing the entry of blood-borne pathogens and xenobiotics, while selectively allowing nutrients and oxygen into the brain. The BBB’s endothelial cells do not function independently, but as a subunit within the greater context of the neurovascular unit (NVU), which includes perivascular pericytes, astrocytes, neurons, and microglia (Hawkins and Davis, 2005; Lok et al., 2007). While the non-endothelial cellular elements of the NVU contribute to the development and maintenance of the BBB phenotype and function, microglia are important because they act as the resident innate immune cell of the brain and are tasked with sensing their microenvironment, physiological housekeeping, and protecting against injurious agents (Hickman et al., 2018). While several groups have evaluated the effects of DEP exposure on the integrity and function of the BBB (Choi et al., 2014; Dekali et al., 2016; Heidari Nejad et al., 2015; Oppenheim et al., 2013; Pöss et al., 2013; Tobwala et al., 2013), the effect of microglia in co-culture with BMECs upon exposure to low concentrations of UF-DEP has not been evaluated.

Primary rat BMECs from cerebral cortices have been used extensively and provide a well-suited *in vitro* model for evaluating the basic molecular mechanisms of transport and dysfunction in the BBB (Audus and Borchardt, 1987; Franke et al., 1999; Weber et al., 1993; Wilhelm et al., 2011). Given their implication in the progression of several neurodegenerative diseases (Block et al., 2007; Cunningham, 2013; Kraft and Jean Harry, 2011; Schwab and McGeer, 2008; Tansey and Goldberg, 2010), primary microglia from rodent species have been used to evaluate DEP-induced dopaminergic (Block et al., 2004; Levesque et al., 2013) and cerebral granule (Roqué et al., 2016) neuron dysfunction *in vitro*. Furthermore, a multitude of *in vivo* and *in vitro* studies show that microglia, as well as endothelial cells (Terzano et al., 2010), are activated in response to DEP (Block and Calderón-Garcidueñas, 2009; Block et al., 2004; Levesque et al., 2011b; Morgan et al., 2011) and that microglia are important contributors to DEP-induced neurotoxicity (Roqué et al., 2016). Specifically, activated microglia (M2 phenotype) are the main producers of pro-inflammatory markers in the CNS that can lead to neuroinflammation, lymphocyte extravasation, and other adverse health effects (REFERENCE; does this result in O.S. too?). In isolated BMECs, oxidative stress from the activation of NADPH oxidase and pro-inflammatory signaling through TNFa specifically have been identified as key DEP-induced pathways that lead to alterations in the BBB, including up-regulation in P-gp (Hartz et al., 2008). P-gp is a broad-specificity efflux pump that is highly expressed in BBB endothelium and poses a significant neurotherapeutic impediment since it accounts for the high attrition rate of a many CNS-targeting drugs (REFERENCE). This DEP-induced up-regulation of P-gp further hinders the efficacy of CNS-acting drugs like brain chemotherapeutics and contributes to drug resistant epilepsy (Loscher and Potschka, 2005). Therefore, improving our understanding of DEP-induced effects on cells of the BBB is warranted in order to better understand the effects of DEP exposure on CNS drugs as well as on the onset or progression of CNS disease.

The co-culture system used in this present study is easily reproducible and represents cell-cell interactions of brain capillary endothelial cells and perivascular microglia that would be present at the BBB, better than monocultures. The primary goal of this study was to evaluate whether cell-cell interactions between endothelial cells and microglia have a mitigating or aggravating effect on UF-DEP toxicity. To evaluate this, we compared the responses of monocultures and mixed co-cultures of rat BMECs and microglia, exposed to low doses (2 ng/ml, 1 μg/ml, and 20 μg/ml) of UF-DEP. A secondary goal was to compare the response of primary rat microglia to that of highly aggressive proliferating immortalized (HAPI) microglia, a cell line that is frequently used as a proxy for primary microglia but has observable phenotypic and genotypic differences (Stansley et al., 2012). The three monoculture and two mixed co-culture groups evaluated in this study were: primary BMECs (E), primary microglia (M), HAPI microglia (H), primary BMECs and primary microglia (EM), and primary BMECs and HAPI microglia (EH). Specifically, this study evaluated cell viability from lactate dehydrogenase (LDH) leakage and cell metabolic activity (CMA), reactive oxygen species (ROS) production, and 27 pro-inflammatory chemokines and cytokines. Overall, this study provides a greater understanding of the sublethal, low concentration UF-DEP effects on cellular responses, and insight into whether UF-DEP elicits cell-type specific responses or generalized adverse effects independent of cell type.

**MATERIALS & METHODS**

**Cell culture and study design**

Primary rat brain microvascular endothelial cells (BMEC; R840-50a) were purchased from Cell Applications, Inc. and cultured on collagen IV and fibronectin-coated cultureware. Primary rat microglia (REMW) were purchased from BrainBits, LLC and highly aggressive proliferative immortalized (HAPI; SSC103) microglia, from MilliporeSigma (Burlington, MA). Both microglia cell types were cultured on poly-D-lysine-coated cultureware (Sigma-Aldrich). The primary endothelial and microglial cells were maintained and proliferated in their respective proprietary medium until the day the cells were dosed with UF-DEP: Rat Brain EC Growth Medium (R819H; Cell Applications, Inc., San Diego, CA) for BMEC, and NbMicroPRO Medium (NbPRO; BrainBits, LLC, Springfield, IL) for microglia. The HAPI microglia were maintained and proliferated in DMEM with low glucose (1 g/L), 1% Penicillin-Streptomycin, and 5% fetal bovine serum (Equitech-Bio., Inc, Kerrvile, TX) until the day the cells were dosed with UF-DEP. Cells were cultured in a humidified 5% CO2, 37°C incubator in their respective medium with serum, and the medium was replaced every other day.

For all experiments, endothelial cells passage 2-6, primary microglia passage 2-5, and HAPI microglia passage 5-10 were used. A total of five cell culture setups were evaluated: endothelial monoculture (E), primary microglial monoculture (M), HAPI microglial monoculture (H), endothelial-microglial mixed co-culture (EM), and endothelial-HAPI mixed co-culture (EH). Prior to experimentation, the monocultures (E, M, H) were seeded on collagen IV and fibronectin-coated 96-well plates or 12-well plates (Corning) at a density of 50,000 cells/cm2. The endothelial and microglial cells in the mixed co-cultures (EM, EH) were seeded 1:1 (25,000 cells/cm2 each cell type) for a total density of 50,000 cells/cm2. Both the monolayers and mixed co-cultures were used at 70-80% confluency in all assays.

**Preparation of ultrafine DEP working suspension and exposure protocol**

DEP from industrial forklift (SRM2975) were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). In our laboratory, we prepared the working suspensions of DEP according to Block *et al* [1]. Briefly, 2 mg of DEP was suspended in 10 ml of PBS, vortexed for 1 min, and sonicated for 45 min using an ultrasonicator (Ultrasonic Bath, Fisher Scientific, Waltham, MA). The suspension was then filtered through a 0.22-μm PES syringe filter (Thermo Fisher Scientific, Waltham, MA) prior to dosing the cells, and this was considered the UF-DEP 100% stock solution (v/v). The UF-DEP stock was then diluted to 2 ng/ml (0.001% v/v), 1 μg/ml (0.5% v/v), and 20 μg/ml (10% v/v) in Assay Medium (50% v/v MEM, 50% v/v Ham’s F-12 nutrient mix with non-essential amino acids, supplemented with 10 mM HEPES, 13 mM NaHCO3, 50 μg/ml gentamicin, 100 μg/ml heparin, without phenol-red, L-glutamine, or FBS). The monocultures and mixed co-cultures were dosed and incubated for 24 hours in standard conditions and then the four endpoints were evaluated.

**Cytotoxicity**

**LDH assay for cell viability.** After dosing the cells with UF-DEP and incubating the plate for 24 hours in standard conditions, cytotoxicity was determined using LDH assay. Briefly, 50 μL of the spent Assay Medium from each well were transferred into corresponding wells of a new, clear 96-well plate with 150 μL of LDH active reagent (total volume per well = 200 uL). The active LDH reagent was a 1:1:1 mixture of Lithium lactate (X moles; Sigma), Tris base (X moles; Sigma), and NAD-PMS-INT solution. To prepare 5 ml of the NAD-PMS-INT solution, 200 μL PMS (X moles;Sigma) and 200 uL INT (X moles; Sigma) were added to 4.6 ml of NAD. 5 ml NAD-PMS-INT, 5 ml Lithium lactate, and 5 ml of Tris base were mixed together to afford the complete active LDH reagent (15 ml is enough for one 96-well plate). Immediately after mixing the spent Assay Buffer and the active LDH reagent, the absorbance was measured at 490 nm using a spectrophotometric microplate reader (BioTek Synergy H1, Vermont, USA). As a positive control for cytotoxicity or cell damage, 0.2% Triton X 100X (Sigma) was used on the cells.

**MTT assay for cell metabolic activity.** After dosing the cells with UF-DEP and incubating the plate for 24 hours in standard conditions, cell metabolic activity was determined using MTT assay. Two hours before the completion of the 24-hour incubation time, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well of the clear 96-well plate with UF-DEP in Assay Medium for a final concentration of 0.5 mg/ml. The plate was incubated for an additional 2 hours in standard conditions. When the 24 hour incubation time ended, the spent Assay Media including UF-DEP and MTT was removed from each well and replaced with 100 μL of DMSO:ethanol (1:1) solution and the plate was incubated for an additional 10 minutes on a microplate shaker (Thermo Fisher) at 200 rpm, room temperature to solubilize the purple formazan product. Absorbance was measured at 595 nm using a spectrophotometric microplate reader (BioTek Synergy H1, Vermont, USA). As a positive control for cytotoxicity or cell damage, 0.2% Triton X 100X (Sigma) was used on the cells.

**Oxidative Stress**

**DCFH-DA assay for ROS production.** Oxidative stress was measured using the 6-Carboxy-2ʹ,7ʹ-Dichlorofluorescein Diacetate (DCFH-DA) assay, which measures activity of hydrogen peroxide (H2O2) produced inside the cell. Before dosing cells with UF-DEP, spent culture media was removed from all wells of the dark 96-well plate, and replaced with 5 mM DCFH-DA dye in Assay Medium (note: must be phenol-red free). Pre-incubation of cells with the dye in standard conditions (5% CO2, 37°C) lasted 45 minutes to ensure that the dye diffused into the cytosol. After the pre-incubation step, the cells were gently rinsed with PBS twice to remove residual dye in the extracellular space, and then cells dosed with UF-DEP. After 24 hours incubation in standard conditions, the plate fluorescence was read at ex/em 485/525 nm using a fluorometric microplate reader (BioTek Synergy H1, Vermont, USA). N-acetylcysteine (NAC; Sigma) and tert-butyl hydroperoxide (tBHP) were used as ROS-generation negative and positive controls, respectively. For the negative control, 10 mM NAC in Assay Medium was incubated with cells for one hour, two hours before pre-incubation with the dye; then removed, replaced and incubated with 50 μM tBHP for an additional hour, one hour before pre-incubation with the dye. For the positive control, 50 μM tBHP in Assay Medium was incubated with cells for one hour, one hour before pre-incubation with the dye. For both positive and negative controls, the cells were gently rinsed with PBS twice, prior to pre-incubation with dye, and then again before replacing dye with UF-DEP for 24-hour incubation.

**Pro-inflammatory markers**

A panel of 27 chemokines and cytokines were quantified using commercially-available MILLIPLEX® MAP Rat Cytokine/Chemokine Magnetic Bead immunoassay kit following manufacturer’s instructions (Millipore Sigma, RECYTMAG-65K). Briefly, magnetic beads conjugated with chemokine/cytokine-specific antibodies were added to a dark 96-well plate, where they were mixed with 25 μl of sample (i.e. culture media) aliquots incubated at room temperature on a microplate shaker with agitation (500 rpm) for 2 hours, protected from light. The sample aliquots were taken directly from spent Assay Medium 24 hours after cells were dosed with UF-DEP. After the 2-hour incubation with magnetic beads, the plate was washed and incubated sequentially with chemokine/cytokine-specific Detection Antibodies for one hour, and then with streptavidin-phycoerythrin for 30 minutes. Fluorescence was measured using a xMAP Luminex 200TM system (Millipore Sigma, Burlington, MA) and cytokine/chemokine concentrations were calculated with the MILLIPLEX® Analyst 5.1 software. As a positive control for cytokine/chemokine production in cell culture, 10 μg/ml of lipopolysaccharide (LPS) from *E. coli* was used in Assay Medium without UF-DEP exposure. Potential immunoassay interference with UF-DEP was also evaluated and the detailed results are reported in Supplemental Information Table X. The panel of 27 pro-inflammatory markers was applied only to the primary E, M, and EM groups and not the H or EH groups because the HAPI microglia have been evidenced in previous *in vitro* studies to not produce relevant chemokines and cytokines, compared to primary microglia, under certain exposure conditions (REFERENCE).

**Statistical Analyses**

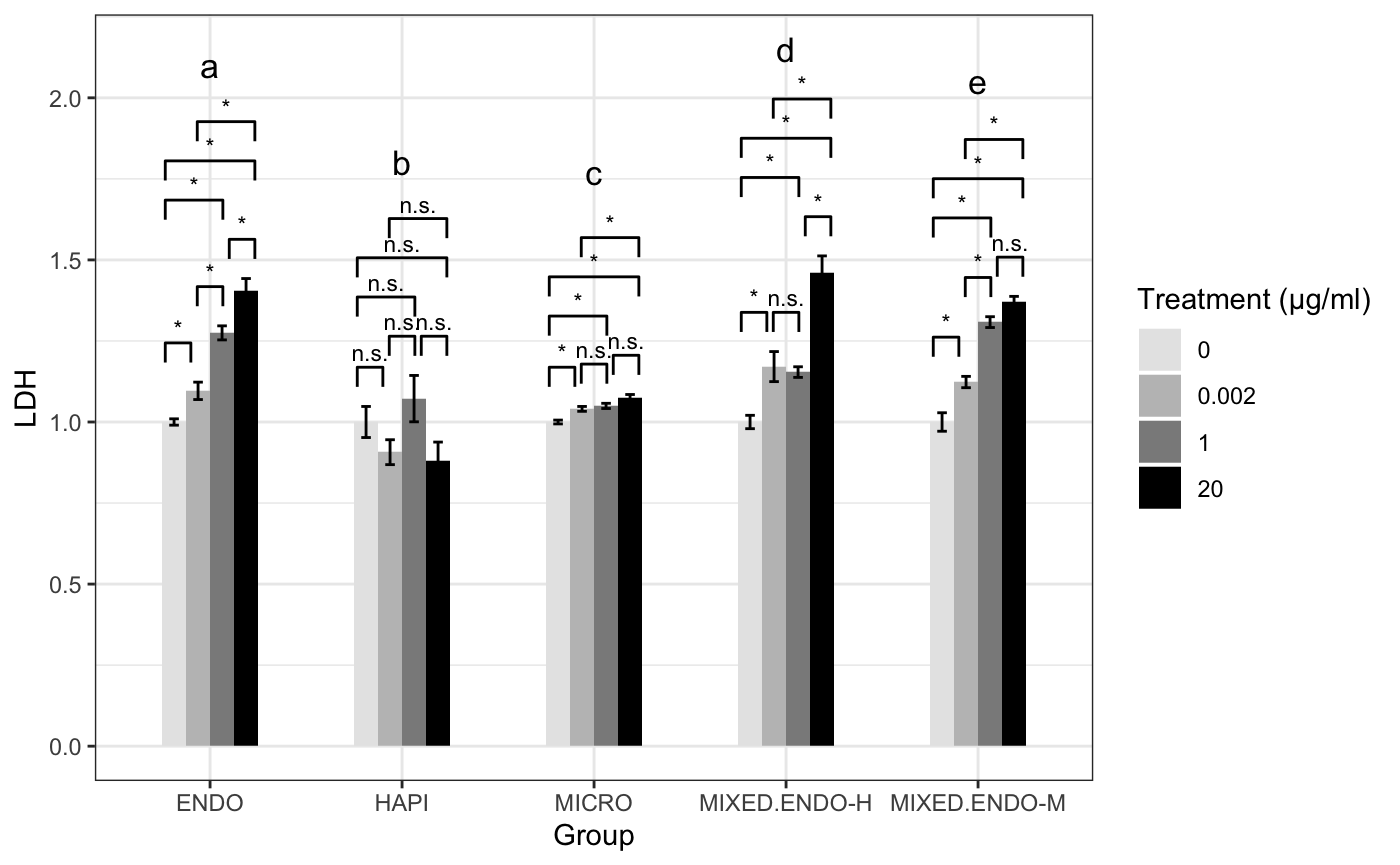
For LDH, MTT, and ROS data, we first used a Linear Mixed Model, which accounts for the random effect (UF-DEP treatment) and the fixed effect (cell type) in the data, using the endothelial group as the baseline. The Linear Mixed Model allowed the comparison among dose-response curves among the five cell culture setups. The model showed differences among slopes across the groups. To test the significance of the differences among slopes, a bootstrapping method was applied to the assay data set with 1000 iterations, fitting a linear slope for each iteration. Afterwards, an ANOVA test was performed which indicated a statistically significant difference in the means of the slopes, followed by the Games-Howell multiple comparison test. Two Šidák corrections were applied to the results to ensure that the Type I error of these *post hoc* tests are well controlled. This allowed us to compare the slopes of the dose-response curves (assuming local linearity) among all the cell groups and to determine specifically which dose-response curves (i.e. slopes) are significantly different.

To evaluate pro-inflammatory markers, and given that the number of data points for fluorescence was different for each analyte and across groups, another iterative sampling method was implemented. For each iteration, we randomly selected 800 data points for each analyte and in each group (the smallest number of data points available for any analyte was larger than 800). We then performed a MANCOVA test on the resulting sampled data set. This was repeated 100 times and the test statistics were averaged across all iterations. This allowed us to test significant differences between treated (20 μg/ml) and non-treated (0 μg/ml) groups among three cell culture setups (E, M, EM) across the 27 analytes, while accounting for the interdependence among analytes and the presence of treatment as a covariate. Significance was met at *p* < 0.05. All statistical analyses were computed using R programming language.

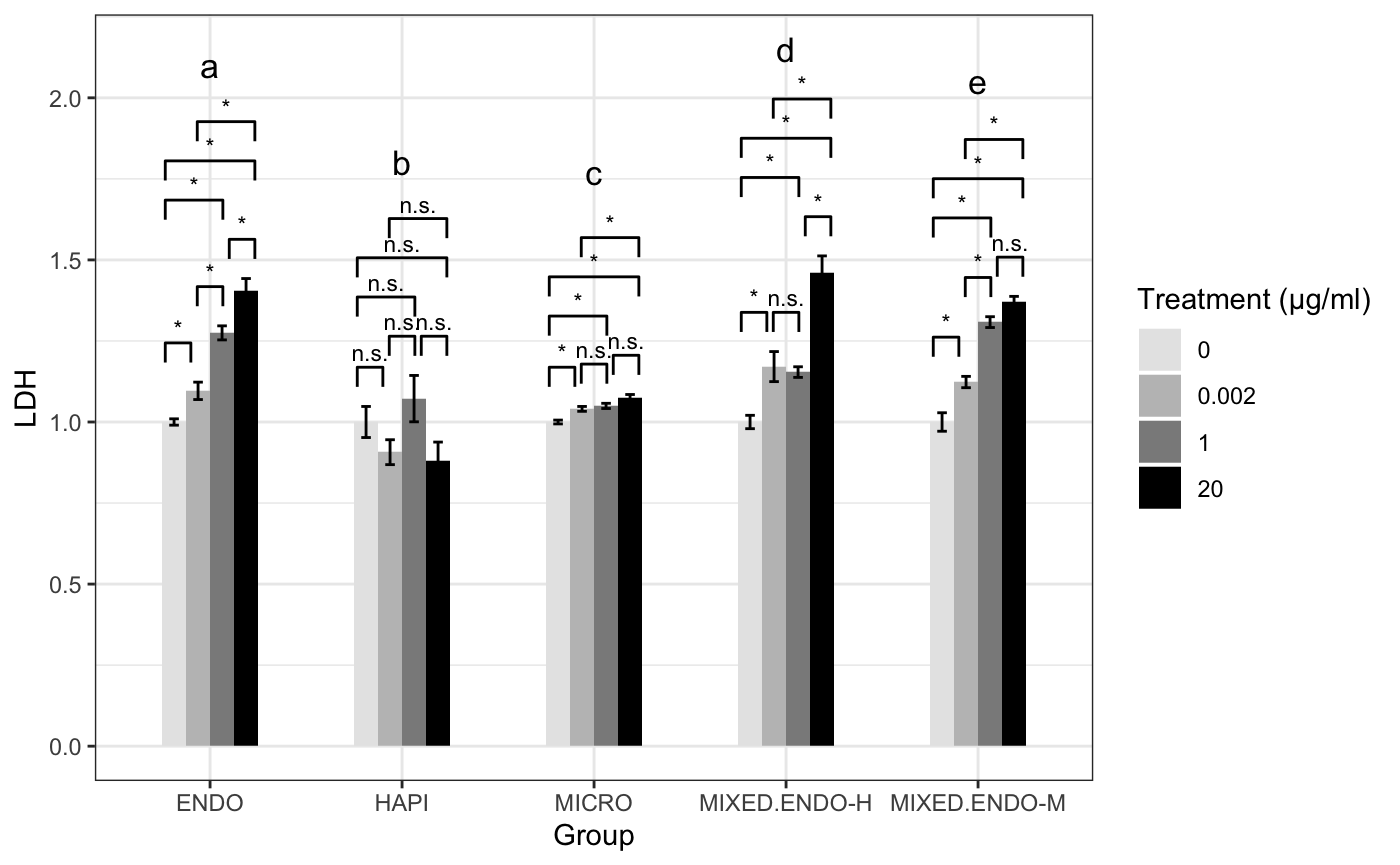
**RESULTS**

**Cytotoxicity**

Cytotoxicity was evaluated by measuring LDH leakage (Fig.1) and cell metabolic activity (CMA) (Fig.2). After 24 hr exposure to UF-DEP, LDH leakage increased with concentration in groups E, EM, EH; but no significant dose-response was observed in the microglial monocultures (M and H) (**Fig. 1**) Importantly, in this cytotoxic end-point, both EM and EH appear to respond similarly to E at the highest concentration. However, while EM was no different than E at 1 μg/ml, EH showed a significantly lower amount of LDH leakage at that concentration compared to E. Finally, the microglial monocultures (M and H) responded similarly except at the highest concentration, where M was unchanged but H was significantly lower, compared to their respective blank controls. In fact, at the highest dose (20 μg/ml), H released the least amount of LDH of all groups, and this amount was almost half of that released by the endothelial monoculture (E) at the same concertation level. The LOAEL according to LDH leakage for all cell culture setups was 0.002 ng/ml.



UF-DEP μg/ml



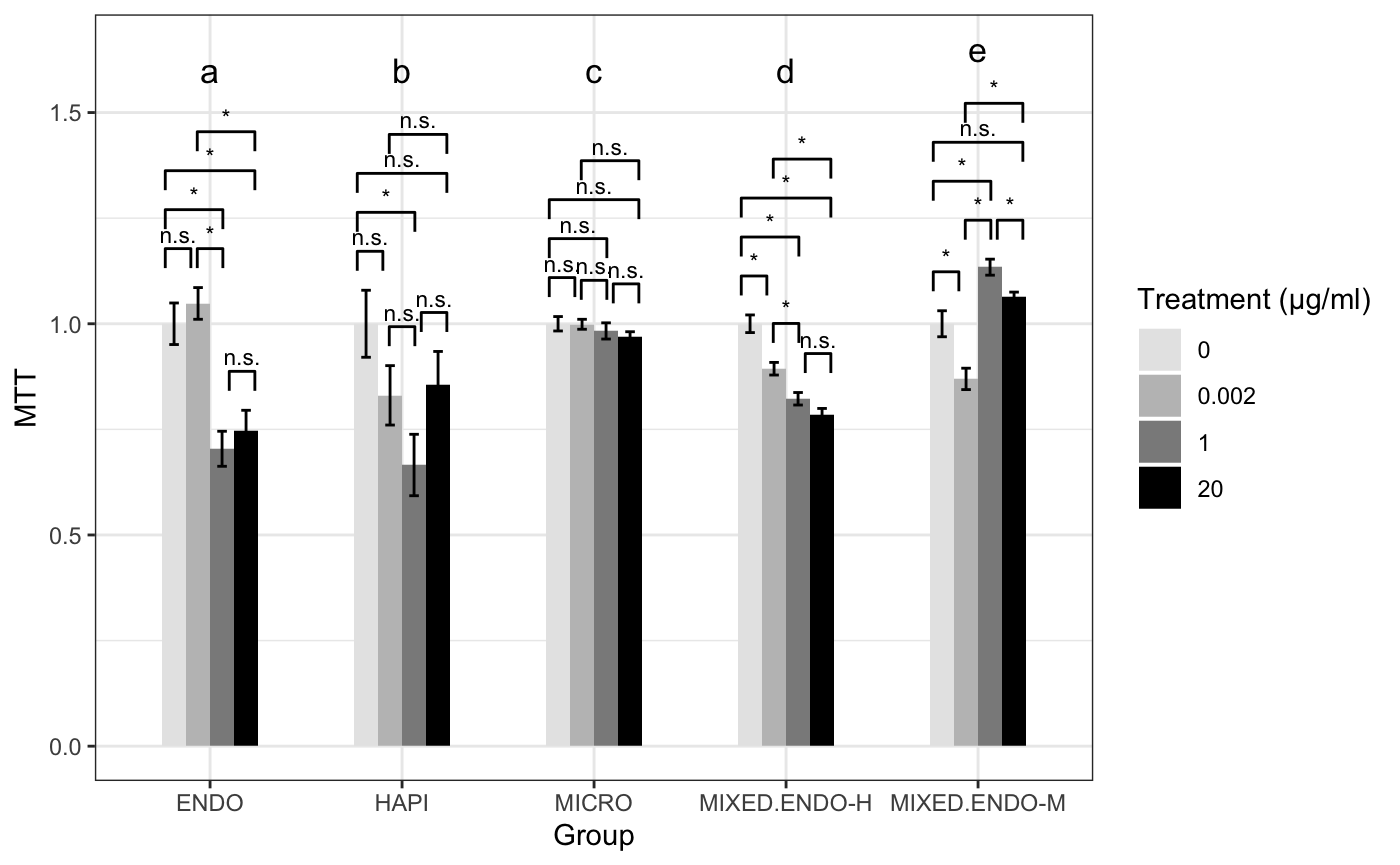
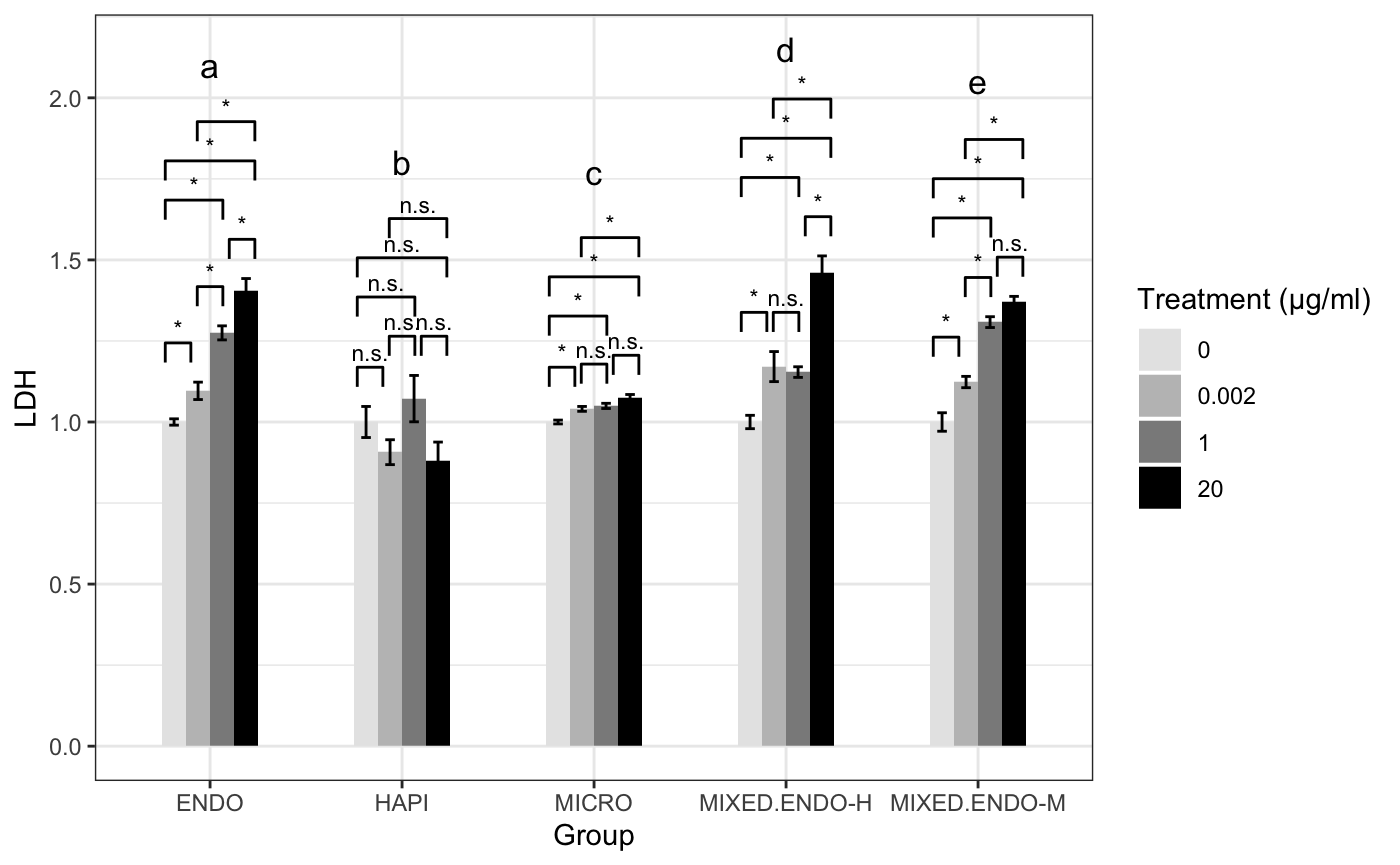
Relative LDH Leakage

**Fig 1. Relative LDH leakage.** RelativeLDH Leakage after 24 h exposure to low concentrations (2 ng/ml, 1 ug/ml and 20 ug/ml) of UF-DEP in five different cell culture setups (primary endothelial, primary microglial, HAPI microglial monocultures, and endothelial-microglial and endothelial-HAPI microglial mixed co-cultures). Bars represent the cells’ LDH leakage compared to their respective unexposed control group + SEM of the experimental averages. Asterisks indicate groups that are significantly different from blank control. # represents mixed co-culture groups that are significantly different from the endothelial monoculture group at the corresponding dose level. Different lettersdenote significantly different cell groups. Statistical significance was considered p-value < 0.05.

**DISC: How does the E monoculture compare to the mixed co-cultures?**

In terms of LDH leakage, both co-cultures (EM, EH) appear to behave similarly to E, but EM moreso than EH.

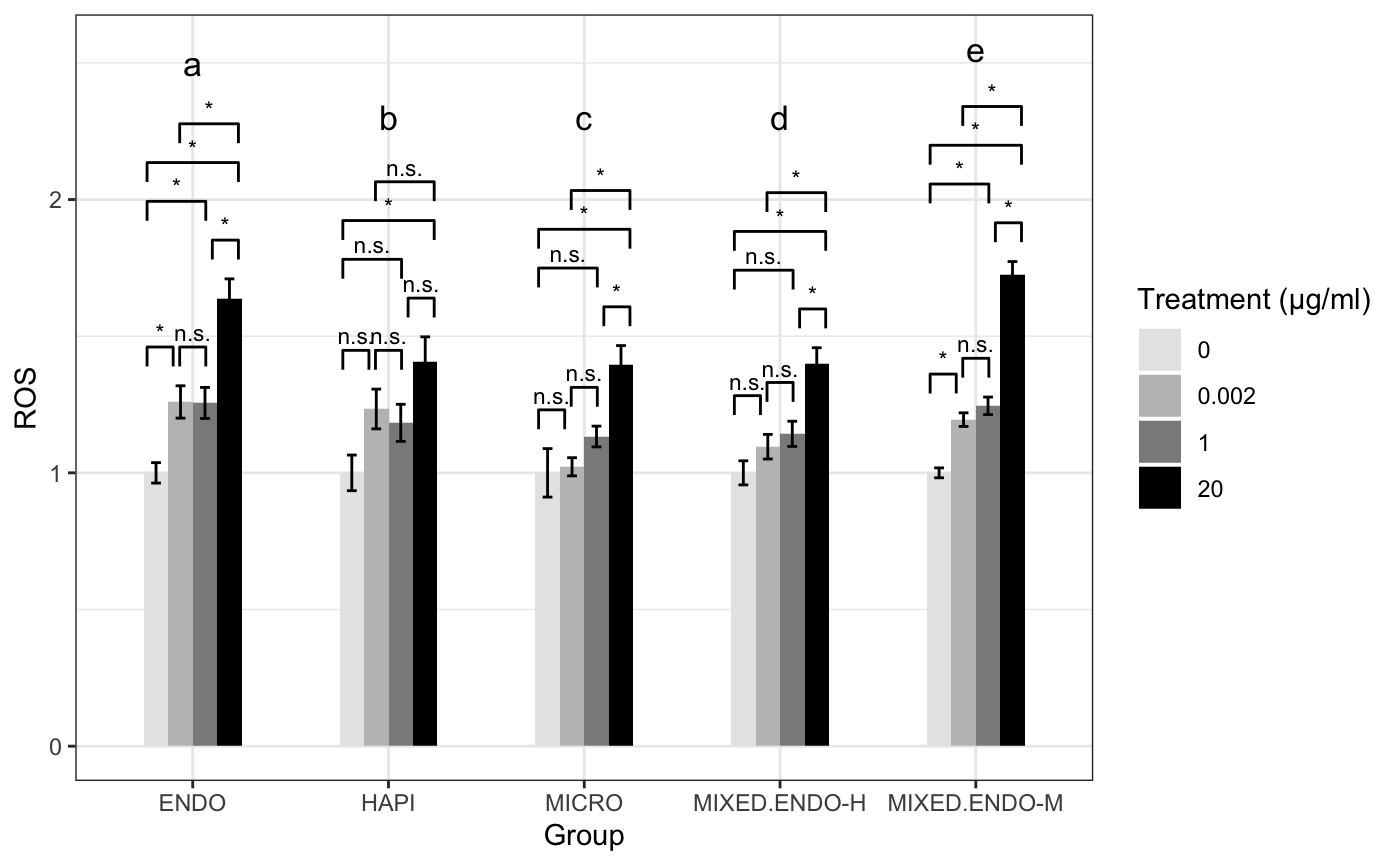
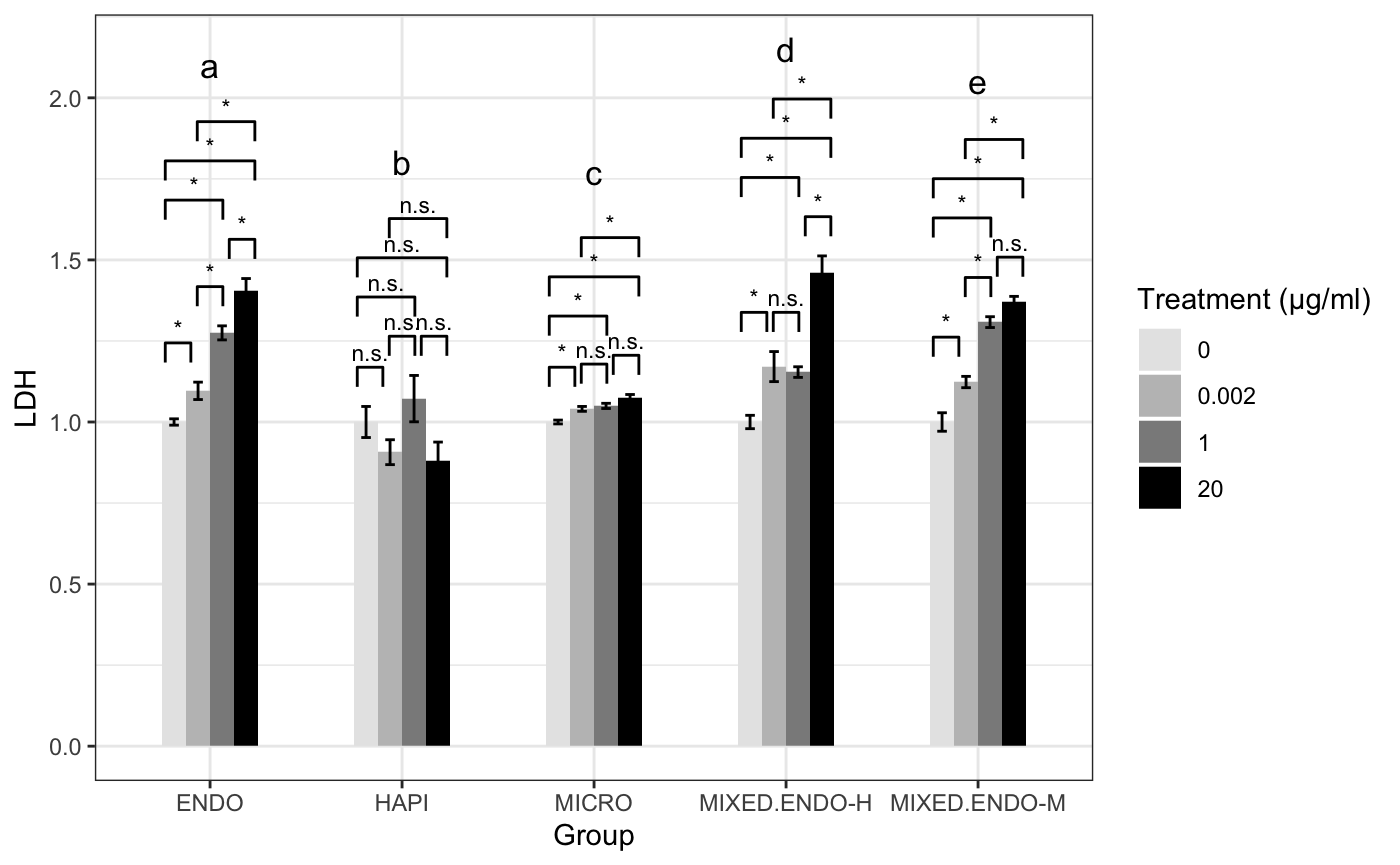
How do M and H compare? In terms of LDH, they beahve similarly except for in the highest dose (20 μg/ml, where H decreases LDH leakage significantly compared to both M at that concentration and to the H blank control. This decrease in LDH leakage technically indicates a decrease in cytotoxicity or an increase in viability in HAPI microglia, which is not expected because UF-DEP has shown *in vitro* toxicity in XYZ (REFERENCE).



UF-DEP μg/ml

Relative Cell Metabolic Activity

**Figure 2. Relative cell metabolic activity.** Relative cell metabolic activity (CMA) after 24 h exposure to low concentrations (2 ng/ml, 1 ug/ml and 20 ug/ml) of UF-DEP in five different cell culture setups (primary endothelial, primary microglial, HAPI microglial monocultures, and endothelial-microglial and endothelial-HAPI microglial mixed co-cultures). Bars represent mean CMA compared to respective unexposed control group + SEM of the experimental averages. Asterisks indicate groups that are significantly different from blank control. # represents mixed co-culture groups that are significantly different from the endothelial monoculture group at the corresponding dose level. Different lettersdenote significantly different cell groups. Statistical significance was considered p-value < 0.05.



UF-DEP μg/ml

Relative ROS Production

**Figure 3. Relative ROS production.**

**Figure 4. Pro-inflammatory cytokines and chemokines. 6 group comparison.**

**Cytotoxicity**

**LDH assay.** 24 hours after exposure to UF-DEP, LDH leakage was measured in the spent media of cells. All cell groups except H, showed a statistically significant but small dose response (insert p value for linear model here). EH showed the greatest dose-response, followed by EM,

Lactate dehydrogenase (LDH) is an intracellular enzyme thatis released into the extracellular space when the cell membrane becomes damaged and is a measure of membrane integrity and cell viability.

**MTT assay.**

**Oxidative Stress**

**DCHF-DA assay.**

**Pro-inflammatory markers**

**Cytokine and chemokine production.**

**DISCUSSION**

***Did monocultures and co-cultures respond differently to exposure?***

***Did microglia exacerbate or mitigate cytotoxicity?***

The main objective of this study was to evaluate whether cell-cell interactions between primary ECs and microglia ameliorate or exacerbate the toxicity of acute (24h) low concentration UF-DEP exposure *in vitro*.

***Did HAPI and MICRO cells respond differently across end-points?***

While the association between DEP exposures and cardiorespiratory morbidity and mortality has been well established (Brook et al., 2004; Pope et al., 2002), recent

It has been established that DEPs are known to generate ROS on intracellular uptake, and ROS is attributed to the chemical composition of the particles such as transition metals and organic chemicals (Park et al., 2011)

* ROS follows dose response, TAC not so much. Which groups show a “normal” response? Which groups show an abnormal response?
* LDH follows dose response, MTT not so much. Which groups show a “normal” response? Which groups show an abnormal response?
* Pro-inflammatory markers were NOT produced in any group or any sublethal concentration. WHY? Talk about LPS’s effect differences in co-cultur (EM) vs monocultures (E, M) still show validity in using the co-culture instead of mono.

Talk about Hartz, Kasurinen Nejad, Choi et al, and Tseng, and Terzano

Kasurinen et al evaluated differences between co-cultures and monocultures of human alveolar A549 cells and THP-1 human monocyte cells exposed to different types of PM derived from log wood and pellet combustion. Several pro-inflammatory markers including IL-8, TGF-B, GM-CSF, IL-1 and TNF-a were evaluated in the co-culture exposed to 25 – 200 ug/ml of various types of PM1. Interestingly, while ROS generation and pro-inflammatory responses are typically observed concomitantly, in certain cases the exposure did not result in significant ROS production at any dose, but did result in IL-8 production in the co-culture and in the monocultures. Unlike ours, the PM used in Kasurinen et al’s study was unfiltered given that the lungs are exposed to PM1 - PM2.5.

Non-toxic doses (1–50 μg/mL) of suspended DEP showed a significant stimulatory effect on IL-8 and GM-CSF production by airway epithelial cells (Takizawa et al, 2001)

Exposure of primary human bronchial epithelial cells (*GSTM1*+) to 25-100 μg/ml DEP for 24 h significantly increased IL-8 and IL-1β protein expression. Knockdown of *GSTM1* in these cells further elevated DEP-induced IL-8 and IL-1β expression, implying that *GSTM1* deficiency aggravated DEP-induced pro-inflammatory response. *GSTM1* regulates DEP-induced IL-8 and IL-1β expression in primary human bronchial epithelial cells by modulation of ROS, ERK and Akt signaling.

The pathogenesis of many respiratory diseases is characterized by airway inflammation, which is driven by a plethora of pro-inflammatory mediators released from airway resident and infiltrating inflammatory cells [[5](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR5)]. The airway epithelium represents the interface between the external environment and the tissue of the airway wall [[6](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR6)]. The production of pro-inflammatory mediators from airway epithelium plays a critical role in the pathogenesis of pulmonary diseases [[5](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR5), [7](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR7)]. Exposure to air pollution particles has been shown to evoke pro-inflammatory mediator production in airway epithelial cells [[8](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR8), [9](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR9), [10](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR10)]. It has been demonstrated that the pro-inflammatory effect of air particles is affected by many factors, such as particle size, concentration, composition, duration of exposure, and co-pollutants [[11](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR11)]. Increasing evidence indicates that the host susceptibility factors may also play an important role in air pollutant-induced lung inflammation [[12](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR12), [13](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR13)]. Susceptibility to the adverse effects of air pollutants is an intrinsic trait most probably related to genotypes [[14](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR14)]. Animal studies have shown that prolonged low-dose DEP exposure induces airway inflammatory responses that differ remarkably among mouse strains with different genetic backgrounds of oxidative stress response [[15](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR15)]. It has been proposed that host responses to DEP are regulated by a balance between antioxidant defenses and pro-inflammatory responses [[16](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR16)]. The lung has multiple anti-oxidative defense systems including the glutathione *S*-transferases (GSTs) [[17](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR17)]. The GSTs are a supergene family of phase II conjugating enzymes that consist of a number of sub-classes such as GSTM1 and GSTP1, and catalyze the conjugation of reduced glutathione with hydrophobic electrophiles and reactive oxygen species (ROS) [[18](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR18)]. *GSTM1* is mapped to the *GST mu* 1 gene cluster on chromosome 1p13.3. Genetic variants that regulate the availability and functionality of the GST enzymes determine the levels of oxidative effects in the airway and associated injury [[19](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR19)]. *GST* gene polymorphisms, particularly the *GSTM1* null genotype, are frequent in the population with reported frequencies from 18 to 66% in different ethnic groups [[20](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR20)]. The deletion variants or null alleles that exist for the *GSTM1* gene present biochemically as a failure to express protein [[21](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR21), [22](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR22)]. Individuals with the *GSTM1* null genotype completely lack the GSTM1 enzyme activity and their susceptibility to asthma and lower lung function is increased [[23](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR23), [24](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR24), [25](https://particleandfibretoxicology.biomedcentral.com/articles/10.1186/1743-8977-9-31#CR25)]. (Wu et al, 2012)

Look for Ohtoshi et al, 2003

REFER TO Roque et al, 2016 printed version for your notes. Compare their neuro+micro+DEP high dose results to your endo+micro+DEP low dos. They did NOT filter out irrelevant size fraction

(Wang et al., 2014) Look up this paper and read: The blood-brain barrier (BBB) is composed of capillary endothelial cells, pericytes, and perivascular astrocytes, which regulate central nervous system homeostasis. Sonic hedgehog (SHH) released from astrocytes plays an important role in the maintenance of BBB integrity. BBB disruption and microglial activation are common pathological features of various neurologic diseases such as multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease. Interleukin-1β (IL-1β), a major pro-inflammatory cytokine released from activated microglia, increases BBB permeability. Here we show that IL-1β abolishes the protective effect of astrocytes on BBB integrity by suppressing astrocytic SHH production. Astrocyte conditioned media, SHH, or SHH signal agonist strengthened BBB integrity by upregulating tight junction proteins, whereas SHH signal inhibitor abrogated these effects. Moreover, IL-1β increased astrocytic production of pro-inflammatory chemokines such as CCL2, CCL20, and CXCL2, which induce immune cell migration and exacerbate BBB disruption and neuroinflammation. Our findings suggest that astrocytic SHH is a potential therapeutic target that could be used to restore disrupted BBB in patients with neurologic diseases.

Other applications have sought to use multicellular BBB

models to understand the response of the NVU to inflammatory

stimuli. Brown et al. constructed a NVU

model from primary human BMECs, pericytes, and astrocytes,

and human iPSC-derived neurons in a twochamber

microfluidic device [40]. After exposure of

the vascular (apical) chamber to lipopolysaccharide

(LPS) or a cocktail of inflammatory cytokines, the authors

harvested media from the vascular and brain

chambers and used LCeMS-based metabolomics to

identify metabolic pathways influenced by inflammatory

stimuli, including several that were differentiallyaffected

between the two chambers, indicating the

impact of the multicellular configuration. Similarly, a

microfluidic NVU model comprising a tubular monolayer

of primary human BMECs surrounded by pericytes

or astrocytes was employed to evaluate cytokine release

upon TNF-a stimulation [41]. The authors demonstrated

that astrocyte and pericyte co-cultures showed

increased basal levels of the pro-survival cytokine granulocyte

colony stimulating factor (G-CSF) compared to

BMEC monoculture, and co-cultures also displayed

increased induction of G-CSFupon TNF-a stimulation.

They further showed that these effects were not

detectable in an analogous Transwell model. Taken

together, these examples demonstrate the unique ability

of multicellular in vitro models of the NVU to provide

novel biological insights that would be difficult or

impossible to discern with BMEC-only models. (Gastfriend et al., 2018)

Use Lipopolysaccharide-activated microglia lower P-glycoprotein function in brain micorvascular endothelial cells (Matsumoto et al., 2012; Mo et al., 2009)

In this study, DEP exposure was found to cause an obvious increase in the TNF-a levels in the BAL fluid to 4.1-fold that in the control, with a marked increase in CD8+ T-cell distribution on day 1 after treatment, but induced B-cell dominance with rapid recovery of the ratio of CD4+/CD8+ T cells on day 7. In addition, on day 7, the concentrations of IL-1, IL-6, and IL-5 in the blood were 4.1-, 12.8-, and 5.2-fold that in the control, respectively, whereas the IL-12 concentration in the BAL fluid increased to 10.4-fold that in the control. (RAT IN VIVO STUDY INHALATION, Park et al, 2011)

For a detailed list of pro-inflammatory markers that occur together in microglia from LPS exposure see (Song and Colonna, 2018)

Mice exposed to nanoparticle-enriched DE show elevated neuroinflammation and

performance deficits in hippocampal-dependent spatial learning and memory tasks (Win-Shwe et al. 2011). Short term studies (up to 1-month exposure) show pro-inflammatory

factors, such as TNFα, in the adult brain with DE exposure, using month-long inhalation

models (Gerlofs-Nijland et al. 2010, Levesque et al. 2011b, Cassee et al. 2012), intratracheal

administration directly into the lung (Levesque et al. 2011b), and a 2 hr-long exposure by

nose-only inhalation (van Berlo et al. 2010). DE exposure also causes elevated

neuroinflammation with subchronic (6 month) exposure in certain vulnerable brain regions

(Levesque et al. 2011b). In fact, we have previously shown that DE elevates α synuclein

levels in the midbrain, indicating that DE may impinge on PD pathology. Thus, while there

are clear CNS effects with DE exposure, the underlying mechanisms are poorly understood.

From Levesque et al, 2013

RESULTS: Rats exposed to DE by inhalation demonstrated elevated levels of whole-brain IL-6 (interleukin-6) protein, nitrated proteins, and IBA-1 (ionized calcium-binding adaptor molecule 1) protein (microglial marker), indicating generalized neuroinflammation. Analysis by brain region revealed that DE increased TNF[alpha] (tumor necrosis factor-[alpha]), IL-l[beta]\ IL-6, MlP-1[alpha] (macrophage inflammatory protein-1[alpha]) RAGE (receptor for advanced glycation end products), fractalkine, and the IBA-1 microglial marker in most regions tested, with the midbrain showing the greatest DE response. Intratracheal administration of DEP increased microglial IBA-1 staining in the substantia nigra and elevated both serum and whole-brain TNF[alpha] at 6 hr posttreatment. Although DEP alone failed to cause the production of cytokines and chemokines, DEP (5 [micro]g/mL) pretreatment followed by lipopolysaccharide (2.5 ng/mL) in vitro synergistically amplified nitric oxide production, TNF[alpha] release, and DA neurotoxicity. Pretreatment with fractalkine (50 pg/mL) in vitro ameliorated DEP (50 [micro]g/mL)-induced microglial hydrogen peroxide production and DA neurotoxicity.

From Levesque et al, 2011b

INCLUDE THESE CHEMO/CYTO IN ANALYSIS FOR GABRIEL

**F\_u\_n\_c\_t\_i\_o\_n\_a\_l\_ \_v\_a\_l\_i\_d\_a\_t\_i\_o\_n\_ \_o\_f\_ \_i\_M\_G\_L\_s\_—**Next, iMGLs were validated as surrogates of microglia using both functional and physiological assays. To this end, cytokine/chemokine secretion by iMGLs was measured following stimulation by Lipopolysaccharide (LPS), IL-1β \_or IFNγ. IL-1β \_and IFNγ \_are two cytokines that are elevated in AD patients and mouse models (Abbas et al., 2002; Blum-Degen et al., 1995; Patel et al., 2005; Wang et al., 2015) (Figure 3C). Basally, iMGLs secrete 10 of the examined cytokines at low but detectable levels (Table S2). However, in response to IFNγ \_or IL-1β, iMGLs secrete 8 different chemokines including TNFα, CCL2, CCL4, and CXCL10. As expected, iMGLs robustly responded to LPS with induction of all measured cytokines except for CCL3 (Table S2 for values). Collectively, iMGLs differentially release cytokines/chemokines based on their cell-surface receptor stimuli, a finding that closely aligns with the responses observed in acutely isolated primary microglia (Rustenhoven et al., 2016). (Abud et al., 2017)

iMGLs express the microglial-enriched purinergic receptor P2ry12, which sense extracellular nucleotides from degenerating neurons, and is critical for microglial homeostatic function (De Simone et al., 2010; Moore et al., 2015) (Figure S2A,B). In response to ADP, iMGLs chemotax toward ADP and produce detectable calcium transients (Figure 3D,E), that were both negated by a P2ry12-specific inhibitor, PSB0739. These physiological findings further underscore that iMGLs respond appropriately to stimuli and express functional surface receptors, such as P2ry12, enabling quantitative analyses of microglial physiology.

(Abud et al., 2017)

In fact, we have previously shown that DE elevates α synuclein

levels in the midbrain, indicating that DE may impinge on PD pathology. Thus, while there

are clear CNS effects with DE exposure, the underlying mechanisms are poorly understood. (Levesque et al, 2013)

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