Research Article

Differential effects of Urban Particulate Matter on BV2 microglial-like and C17.2 neural stem/precursor cells

Rebecca H. Morris[a,b], Gwladys Chabrier[a], Serena J. Counsell[b], Imelda M. McGonnell[a] and Claire Thornton[a,b]

[a] Department of Comparative Biomedical Sciences, Royal Veterinary College, London, UK

[b] Centre for the Developing Brain, School of Biomedical Engineering & Imaging Sciences, King's College London, London, UK

Short Title: Cell-specific responses to particulate matter exposure

Corresponding Author:

Claire Thornton
Department of Comparative Biomedical Sciences
Royal Veterinary College,
4 Royal College Street,
London, NW1 0TU, UK.
Tel: + 44 (0)20 7121 1919
E-mail: cthornton@rvc.ac.uk

Number of Tables: 0

Number of Figs: 4

Word count: 3365

Keywords: Air pollution, neurodevelopment, oxidative stress, mitochondria

Abstract

Air pollution affects the majority of the world's population and has been linked to over 7 million premature deaths per year. Exposure to particulate matter (PM) contained within air pollution is associated with cardiovascular, respiratory and neurological ill health. There is increasing evidence that exposure to air pollution *in utero* and in early childhood is associated with altered brain development. However, the underlying mechanisms for impaired brain development are not clear. While oxidative stress and neuroinflammation are documented consequences of PM exposure, cell-specific mechanisms that may be triggered in response to air pollution exposure are less well defined. Here we assess the effect of urban (U)PM exposure on two different cell types, microglial-like BV2 cells and neural stem / precursor-like C17.2 cells. We found that, contrary to expectations, immature C17.2 cells were more resistant to PM-mediated oxidative stress and cell death than BV2 cells. PM exposure resulted in decreased mitochondrial health and increased mitochondrial ROS in BV2 cells which could be prevented by mitoTEMPO antioxidant treatment. Our data suggest that not only is mitochondrial dysfunction a key trigger in PM-mediated cytotoxicity, but that such deleterious effects may also depend on cell type and maturity.

Introduction

Air pollution is a serious common public health concern, increasingly associated with morbidity and mortality and resulting in an estimated 7 million premature deaths per year, 4.2 million of which are directly related to outdoor air [1]. Air pollution comprises gases such as nitrogen dioxide (NO_2), ozone (O_3), sulphur dioxide (SO_2) and carbon monoxide (CO) as well as particulate matter (PM), composed of metals, soil/dust and organic chemicals derived from Traffic Related Air Pollution (TRAP), domestic fuel burning, industry and manufacturing [2]. Over 90% of the population routinely experience levels of $PM_{2.5}$ (PM comprising particles less than 2.5µm in size) that are significantly higher than World Health Organisation recommendations (5µg/m^3, annually[1]) with low-to-middle income countries (LMIC) such as India and China reporting levels up to ten-fold higher than Europe and the USA [3].

Exposure to air pollution increases the risk of developing cardiovascular (CV) and respiratory disease and the central nervous system is also a proposed target organ [4-7]. Numerous large-scale studies indicate an association between chronic exposure to high levels of PM and neurodegenerative disease such as Alzheimer's disease [7-10]. Within the last few years, concerns have increased that poor air quality and exposure to air pollution, both during pregnancy and in early life, can impact child health. Epidemiological studies link PM exposure during pregnancy and early childhood with reduced cognition, impaired learning and memory, attentiveness and communication [11-13]. Early life exposure to PM is also associated with an increased risk of neurodevelopmental disorders such as Attention Deficit Hyperactivity Disorder and Autism Spectrum Disorder (ASD; [14-16]).

Routes of exposure of the brain to PM can be direct or indirect; in both human and animal studies, ultrafine PM and $PM_{2.5}$ have been found redistributed to the cortex and cerebellum via olfactory nerves and additional PM can arrive at the blood brain barrier (BBB) via the lungs and bloodstream [7]. An additional route of particular relevance to early life is *in utero* exposure via the placenta. Chronic nasal exposure to air pollution in pregnant rabbits resulted in impaired placenta function and growth retardation in the offspring [17]. PM was found in both the maternal and fetal blood stream indicating that nano-sized PM is capable of passing the placental barrier [17]. This initial evidence of fetal PM exposure has subsequently been supported in human studies through a label-free identification of black carbon particles in the placenta using femtosecond pulsed illumination. Every placenta screened showed evidence of black carbon particles accumulating on the fetal side, with the quantity of particles correlating with residential exposure [18]. *In vitro* and in adult rodent models of

fetal exposure, PM reduces the integrity of tight junctions at the BBB suggesting that PM circulating in the fetal blood stream may also penetrate the BBB, resulting in direct exposure [19,20]. Maternal exposure to air pollution is associated with epigenetic alterations in the placenta and cord blood [21-23]. In addition, maternal PM exposure is linked to offspring with low birth weight, increased incidence of preterm birth and increased risk of developmental disorders [24].

The cellular effects of exposure to PM include oxidative stress and neuroinflammation, which can negatively impact the highly regulated process of neurodevelopment [25,26]. Glial cell activation occurs in response to PM exposure of all sizes and types, increasing the production of proinflammatory cytokines and reactive oxygen species (ROS, [27-29]). *In vivo* and in mature rodent neurons *in vitro*, PM exposure results in increased ROS and reactive nitrogen species (RNS), as well as expression of markers of oxidative stress, leading to lipid peroxidation and membrane damage [30,31]. ROS and lipid peroxidation was also observed in offspring of rodents exposed to PM during gestation [32]. Cellular energy impairment and mitochondrial dysfunction has also been observed following PM treatment; ATP production was significantly decreased in primary neurons and dissipation of mitochondrial membrane potential, accompanied by reduced mitochondrial respiration has been reported *in vivo* [33,34]. Long-term PM exposure (< 10 months) significantly altered the rat brain proteome with significant changes in protein components of the electron transport chain [35].

In rodent models, gestational exposure to TRAP leads to anxiety-like behaviours and impaired spatial learning in offspring suggesting that PM-mediated cellular responses can alter neurodevelopment [36]. Here we have compared the effect of urban (U)PM exposure between microglial-like BV2 cells and neural stem / precursor-like C17.2 cells chosen to represent cell-types of differing origin and maturity. We hypothesized that neuroinflammation would be driven by BV2 and that neurotoxicity would be most apparent in C17.2, but that common mechanisms exist, such as oxidative stress, which would exacerbate the overall pathology.

Materials and Methods

BV2 and C17.2 Cell culture

BV2 cells, provided by Professor R. Donato (University of Perugia, Italy), were derived from *raf/myc*-immortalised female murine neonatal microglia [37]. C17.2 cells were derived from male murine cerebellar multipotent neural stem cells (ECACC 07062902). Both cell types were maintained in Dulbecco's Modified Eagle's Medium (Gibco, #2130737) supplemented with 10% Foetal Bovine Serum (FBS, Gibco, #15070-063) and 1% Penicillin/Streptomycin (Gibco, #15140122), incubated at 37˚C/5%CO_2 and passaged twice weekly. For treatments, BV2 cells (passages 2-17) were transferred to medium containing 5% FBS. C17.2 cells were used between passages 4 and 23.

UPM preparation and treatment

Stock solutions were prepared by suspending urban particulate matter (UPM, Sigma, #NIST1648A, certified reference material) in DMEM growth medium (100µg/ml and 100mg/ml) and stored in aliquots at -80°C until use. BV2 and C17.2 cells were seeded in 12-well plates with a density of 0.8 x 10^5 cells per well and allowed to settle overnight. Working solutions of UPM were prepared from the appropriate stock solution by sonication for an hour (22W, Fisherbrand Ultrasonic Bath) and subsequent dilution in DMEM growth medium containing 5% FBS, to final treatment concentrations (0-100µg/ml). Growth medium was removed from the cells and replaced with medium alone controls or UPM-containing medium. Plates were incubated for 24h at 37˚C/5% CO_2.

Cytotoxicity detection

The presence of extracellular lactate dehydrogenase (LDH), released from UPM-treated BV2 and C17.2 cells, was used as a surrogate for cell damage/toxicity. Duplicate aliquots of medium (50µl) were mixed 1:1 with LDH reaction buffer (Cytotoxicity Detection LDH Kit, Roche, #11644793001), prepared following manufacturer's instructions and LDH release determined by colorimetric reduction of tetrazolium salt, measured at 490 nm (BMG), subtracting background 680nm measurements. A medium-alone blank was also included to compensate for residual LDH present in the FBS-supplemented growth medium.

Quantification of Cell Number

UPM-treated and control cells were incubated in DMEM growth medium containing Hoechst reagent (10µg/ml bisBenzimide H 33258, 30 min, 37˚C Sigma, B2883). Following incubation, the cells were rinsed with PBS, and then replaced with fresh, pre-warmed DMEM. BV2 and C17.2 cell nuclei were

visualised using the DAPI block on the EVOS M5000 microscope. Six fields of view were imaged per technical replicate (i.e. 18 images per treatment condition) for a minimum of three independent experiments. Images were analysed in Fiji (Image J) using the Cell Count plugin and mean data reported for each condition.

Gene Expression analysis

RNA from BV2 and C17.2 cells was prepared and analysed by qRT-PCR as described previously [38] scraped from 12-well plates into TRIzol Reagent (ThermoFisher) and RNA purified using the Direct-zol RNA miniprep kit (Zymo Research) as per manufacturer's instructions. Concentration and quality of column-eluted RNA were determined using a NanoDrop Spectrophometer, diluted to 100ng/µl in RNAse-free dH_2O and stored at -20C. One-step qRT-PCR (RNA-to-CT, Thermofisher) was carried out on 200ng RNA according to manufacturer's instructions and using the following Taqman primers (ThermoFisher): IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1), TNFα (Mm00443258_m1), IL10 (Mm01288386_m1), NRF2 (Mm00477784_m1) and GAPDH (Mm99999915_g1). Relative gene expression was determined by normalization to both GAPDH expression and experimental controls [39].

Detection of Reactive Oxygen Species (ROS) and mitochondrial membrane potential

Changes in intracellular ROS following UPM exposure were determined using dihydroethidium staining (DHE, 10µM, ThermoFisher), and mitochondrial membrane potential analysed by incubation with Tetramethylrhodamine (TMRM, 200nM, ThermoFisher). Following incubation (30 min, 37˚C/5%CO_2), cells were rinsed with PBS and imaged (RFP block, EVOS M5000). Levels of cellular fluorescence were determined using ImageJ analysis and calculating corrected total cell fluorescence (CTCF) where CTCF = integrated density – (area of selected cell x mean fluorescence of background reading).

MitoTEMPO antioxidant treatment

BV2 cells were pre-treated with mitochondria-targeted antioxidant, mitoTEMPO (10µM in DMEM, MedChemExpress USA) for 30 minutes prior to UPM exposure for 24 hours. Detection of ROS and quantification of cell number were carried out as above.

Statistics

Results are expressed as mean (± SEM) where a minimum of 3 biological replicates were performed (1-2 passages between each experiment), each experiment having 2-3 technical replicates. Data were

analysed using GraphPad Prism v9 Software (GraphPad Software, San Diego, USA) for statistical significance by one-way or two-way ANOVA dependent on the number of variables, and if significant, followed by appropriate *post hoc* tests (e.g. Sidak's, Dunnett's, shown in figure legend). A p-value of less than 0.05 was considered significant.

Results

BV2 microglial cells are more sensitive to UPM exposure than immature neural stem/precursor-like C17.2 cells

The cytotoxic effect of UPM exposure was investigated using microglial (BV2) and neural stem/precursor (C17.2) cell lines. Cells were exposed to increasing concentrations of UPM for 24h and assessed for cell survival. BV2 toxicity, measured by LDH release, was significantly increased after exposure to both 30 μg/ml ($p<0.0001$) and 100μg/ml UPM ($p<0.0001$) compared with control untreated cells (Fig. 1A, $F=4.576$) and cell number also decreased, reaching significance for 10 ($p= 0.0448$), 30 ($p<0.0001$) and 100μg/ml ($p<0.0001$) UPM exposure (Fig. 1B, $F=34.32$). In contrast, although there was a slight increase in C17.2 cell toxicity at higher PM concentrations, (this did not reach significance, Fig. 1C) and there was little cell loss (Fig. 1D). UPM had little effect on doubling time of either BV2 or C17.2 cells. These data suggest that neural stem/precursor-like and microglial-like cell types have different tolerances to similar concentrations of UPM when evaluating toxicity.

UPM exposure evokes a concentration-dependent inflammatory response that is more profound in BV2 cells

Previous studies have highlighted that exposure to PM upregulates the inflammatory response particularly driven by microglia [27,40]. We therefore investigated whether UPM exposure induced a similar, concentration-dependent response in BV2 and C17.2 cells. We first confirmed that candidate gene targets (IL-6, IL-1β, TNFα, IL-10) were expressed to a similar extent in both C17.2 and BV2 cells by analyzing raw threshold values; comparable baseline gene expression was observed for both cell types (data not shown). Increases in proinflammatory gene expression in BV2 cells were apparent following exposure to 100 μg/ml UPM (IL-6, $p<0.0001$; IL-1β, $p=0.0208$; TNFα, $p<0.0001$; Fig. 2A-C) compared with control, with IL-1β expression also significantly increased at 30 μg/ml UPM ($p=0.005$; Fig. 2C). For C17.2 cells, no significant change was observed in IL-6, IL-1β or TNFα (Fig. 2A-C) and two-way ANOVA revealed global significant differences in the two cellular responses for IL-6 ($p=0.0340$, $F=4.950$) and IL-1β ($p=0.0263$, $F=5.460$). Anti-inflammatory IL-10 expression was not altered in either BV2 or C17.2 cells regardless of UPM exposure (Fig. 2D).

UPM exposure results in ROS production in BV2 cells but not C17.2 cells

Previous studies have also highlighted that oxidative stress may occur in the brain as a consequence of systemic exposure to PM [41,42]; production of Reactive Oxygen Species (ROS) can also contribute to the development of pollution-induced inflammation and *vice versa* [42]. To determine whether

UPM alters ROS production, UPM-exposed BV2 and C17.2 cells were incubated with DHE, which fluoresces in the presence of superoxide. BV2 cells exhibited increased fluorescence at 30 μg/ml (p=0.0045) and 100μg/ml UPM (p=0.0035, Fig. 3A, F=5.290) compared with control untreated cells. Unexpectedly, UPM exposure did not stimulate ROS production in C17.2 cells (Fig. 3B, F=2.572). In previous studies of rat brain, acute and chronic exposure to $PM_{2.5}$ triggers increased expression of anti-oxidant NRF2 [43,44]. We therefore measured NRF2 gene expression in our cell samples following a 24h UPM exposure. Although basal expression of NRF2 was similar between C17/2 and BV2, increased NRF2 expression was observed in C17.2 cells compared with BV2 cells (p=0.0003, F=15.74), most noticeably at the higher concentrations of UPM (30μg/ml, p=0.0085) whereas there was no change in BV2 cells (Fig 3C).

Exposure to UPM decreases mitochondrial health

Although ROS can be produced in a variety of subcellular locations, the majority is generated within the mitochondrion through electron leakage during oxidative phosphorylation [45]. To determine whether mitochondrially-derived ROS was driving the increased DHE staining observed in the previous experiment, we treated BV2 cells with mitoTEMPO, a mitochondrially-targeted antioxidant, followed by exposure to UPM. There was a general decrease in DHE fluorescence observed in BV2 cells treated with UPM+mitoTEMPO compared with UPM alone (p<0.0001, F=6.009) and *post hoc* analysis determined a significant reduction for the highest UPM concentration (100μg/ml, p>0.0001; Fig. 4A). As mitochondrial ROS was clearly increased following UPM exposure, we measured mitochondrial membrane potential through the accumulation of TMRM in BV2 cells. There was a dose-dependent decrease in TMRM fluorescence, reaching significance at the highest UPM dose (p=0.155, F=3.015) indicating a dissipation of mitochondrial membrane potential and a decrease in active mitochondria within the cell (Fig. 4B). Finally, in cells receiving UPM + mitoTEMPO compared with UPM alone, cell numbers were sustained (p<0.0001, F=3.582). *Post hoc* tests revealed that this protection was most noticeable at the higher UPM concentrations (30 μg/ml UPM, p=0.0362; 100μg/ml UPM p=0.0003, Fig. 4C). Our data suggest that abrogation of mitochondrial ROS conferred cell survival benefits on BV2 microglial-like cells following UPM exposure.

Discussion/Conclusion

There is increasing evidence that the developing brain is sensitive to early life exposure to air pollution, in particular PM. As such, there is an urgent need to understand the cellular pathways triggered in response to this exposure. In this study, we examined the consequences of exposure to UPM on microglial-like BV2 cells and neural precursor stem cell-like C17.2 cells, initially predicting that C17.2 cells may be more vulnerable than BV2. However, we found that in measures of cytotoxicity, ROS generation and proinflammatory response was greater in BV2 cells than in C17.2 cells following exposure to UPM.

Activation of microglia following exposure to air pollution is a recognized outcome in both cell studies and animal models (reviewed in [26]). Indeed, microglia activation by PM can be direct (inhaled nasally, bypassing the blood brain barrier) or through systemic inflammation (via the respiratory system; [46]). Exposure in rodents to diesel exhaust particles or $PM_{2.5}$ alters microglial morphology and increases the expression of proinflammatory cytokines [47,48]. Our results also extend previous studies using BV2 exposed to black carbon and diesel exhaust particulate matter, which increased proinflammatory gene expression and which was abrogated with pretreatment by corticosteroids [49].

Although the majority of glial cell research has focused on alterations in the microglial response following exposure to air pollution, astrocytes and oligodendrocytes are also affected. Increased GFAP expression (a marker of astrocyte activation) was observed both in primary cells *in vitro* [50], as well as *in vivo* following $PM_{2.5}$ treatment [51,52]. A recent study found that chronic exposure to nanoscale-sized PM in mice results persistent microglial-mediated neuroinflammation accompanied by decreased oligodendrocyte cell number and damage to the white matter fibres in the corpus callosum [53]. However, increased cell death and neurotoxicity in response to PM exposure are more usually identified in neurons [27]. Our data suggest that neural stem/precursor cells may be more resistant to the pathological effects of UPM than terminally differentiated neurons or microglia. Previous studies have highlighted that resistance to environmental stress may be a property of stem cells contributing to their potential as therapeutics [54]. In the CNS, stem cells are partially resistant to metabolic stress and subsequent toxicity, supporting the survival of surrounding neurons [55,56]; this property may have contributed to our observations in C17.2 cells. Such cellular robustness in this immature cell type may be attributed to their role in development [54]. However, toxicity has been reported in microglia-neuron co-culture experiments in response to pollution [28,57], highlighting a critical role for microglia in pollution-driven neurodevelopmental impairment.

Epidemiological and systematic reviews of data suggest that there is a marked association between gestational exposure to PM and risk of ASD, and that this linkage is driven by exposure to smaller particles e.g. <$PM_{2.5}$ [58,59]. However, data on the risks associated with other air pollutants such as NO_2 and O_3 are more variable as is the identification of a critical "exposure window" during gestation. Given our data from the immature C17.2 cells, the deleterious effects of gestational exposure to ultrafine PM may depend not only on pathological activation of glial cells but also on neuronal maturity. Future work will extend our study to primary neurons to establish the effect of repeated low dose PM exposure on neuronal maturation.

In this study we identified that cellular exposure to PM increased ROS production in microglial cells, in line with previous findings investigating a variety of sources of PM including UPM, DEP, $PM_{2.5}$ and PM_{10} [41,43]. We also found that, in contrast with C17.2, BV2 cells were not able to mount an antioxidant NRF2-mediated response. This may mimic the situation in the neonatal brain where there is reduced antioxidant capacity in the compared with adult brain, due to limited scavenging and increased free iron [60]. However, the NRF2 antioxidant response alone may be insufficient to counteract the impact of PM as protection by NRF2 can be overcome by fine and ultrafine PM exposure [61]. Left unchecked, the consequences of oxidative stress include lipid peroxidation, energy impairment and cell death and therefore these may play a more significant role in the developing brain compared with adult.

We found that PM-mediated ROS production in BV2 cells emanated primarily from mitochondria, and that this was accompanied by decreased mitochondrial membrane potential. Mitochondria may be emerging as new targets in mediating the effects of PM exposure [26]. In line with our mitoTEMPO findings, administration of a mitochondrially-targeted antioxidant (MitoQ) to rats prior to exposure to simulated vehicle exhaust prevented mitochondrial ROS production, restored ATP production and rebalanced mitochondrial dynamics [62]. Similar to our observation of impaired mitochondrial membrane potential, recent studies suggest that mitochondrial morphology and dynamics are also vulnerable to the effects of PM exposure [61,63]. A longitudinal brain proteomics study of rats exposed to coarse, fine or ultrafine PM identified evidence of electron transport chain impairment at all timepoints (1/3/10 months), limited to the fine / ultrafine PM groups [35]. Evidence of air pollution-mediated mitochondrial dysfunction in humans is also emerging. Increased levels of the mitochondrial oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) were found in maternal blood sampled throughout pregnancy and in cord blood of newborns, correlating with exposure to PM [64]. Metabolomics analysis of serum taken from women during pregnancy correlated oxidative stress and inflammation with exposure to traffic-related air pollution [65].

Further interrogation of these biobanked samples correlated impairments in the TCA cycle and mitochondrial function with increased exposure to traffic-related air pollution and an increased risk of ASD in their offspring [66]. Such evidence, combined with *in vitro* and preclinical PM exposure data suggest that maintaining mitochondrial function may provide protection following chronic exposure to air pollution. More work is required to determine whether intervention post exposure can be effective.

In conclusion, we provide *in vitro* evidence of cell-specific effects of UPM exposure that impact pathways including inflammation, oxidative stress and mitochondrial impairment, dependent on cell maturity. In microglial-like BV2 cells, mitochondrial dysfunction is apparent through increased ROS production and reduced mitochondrial membrane potential. Mitochondrially-targeted antioxidants may improve microglial cell health and have the potential to reduce chronic inflammation. However, high-level strategies are clearly required to limit maternal exposure to air pollution during pregnancy to reduce the risk of lifelong neurological impairment.

Statements

Statement of Ethics

There is no involvement of human or animal samples in this work

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by the Biotechnology and Biological Sciences Research Council as a London Interdisciplinary Doctoral (LIDo) programme studentship to R.H.M. (BB/M009513/1) held jointly between the RVC and KCL.

Author Contributions

Author contributions are based on ICMJE recommendations. Rebecca Morris, Gwladys Chabrier, Serena Counsell, Imelda McGonnell and Claire Thornton made substantial contributions to the conception or design of the work and/or the acquisition, analysis, or interpretation of data. Claire Thornton and Rebecca Morris drafted the work with intellectual contributions from Serena Counsell

and Imelda McGonnell; additionally they were responsible for its revision along with Gwladys Chabrier. Rebecca Morris, Gwladys Chabrier, Serena Counsell, Imelda McGonnell and Claire Thornton proof read and gave final approval of the version to be published. Rebecca Morris, Gwladys Chabrier, Serena Counsell, Imelda McGonnell and Claire Thornton agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

References

1 WHO: [Internet] Air pollution [Accessed 2nd October, 2021]. Available: https://www.who.int/health-topics/air-pollution#tab=tab_1,

2 Karagulian F, Belis CA, Dora CFC, Prüss-Ustün AM, Bonjour S, Adair-Rohani H, Amann M: Contributions to cities' ambient particulate matter (PM): A systematic review of local source contributions at global level. Atmospheric Environment 2015;120:475-483.

3 Burnett R, Cohen A: Relative Risk Functions for Estimating Excess Mortality Attributable to Outdoor PM2.5 Air Pollution: Evolution and State-of-the-Art. Atmosphere 2020;11:589.

4 Huangfu P, Atkinson R: Long-term exposure to NO_2 and O_3 and all-cause and respiratory mortality: A systematic review and meta-analysis. Environ Int 2020;144:105998.

5 Cohen AJ, Brauer M, Burnett R, Anderson HR, Frostad J, Estep K, Balakrishnan K, Brunekreef B, Dandona L, Dandona R, Feigin V, Freedman G, Hubbell B, Jobling A, Kan H, Knibbs L, Liu Y, Martin R, Morawska L, Pope CA, 3rd, Shin H, Straif K, Shaddick G, Thomas M, van Dingenen R, van Donkelaar A, Vos T, Murray CJL, Forouzanfar MH: Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015. Lancet 2017;389:1907-1918.

6 Costa LG, Cole TB, Coburn J, Chang YC, Dao K, Roque PJ: Neurotoxicity of traffic-related air pollution. Neurotoxicology 2017;59:133-139.

7 Costa LG, Cole TB, Dao K, Chang YC, Coburn J, Garrick JM: Effects of air pollution on the nervous system and its possible role in neurodevelopmental and neurodegenerative disorders. Pharmacol Ther 2020;210:107523.

8 Calderon-Garciduenas L, Herrera-Soto A, Jury N, Maher BA, Gonzalez-Maciel A, Reynoso-Robles R, Ruiz-Rudolph P, van Zundert B, Varela-Nallar L: Reduced repressive epigenetic marks, increased DNA damage and Alzheimer's disease hallmarks in the brain of humans and mice exposed to particulate urban air pollution. Environ Res 2020;183:109226.

9 Lee JH, Byun MS, Yi D, Ko K, Jeon SY, Sohn BK, Lee JY, Lee Y, Joung H, Lee DY, Group KR: Long-Term Exposure to PM10 and in vivo Alzheimer's Disease Pathologies. J Alzheimers Dis 2020

10 Seelen M, Toro Campos RA, Veldink JH, Visser AE, Hoek G, Brunekreef B, van der Kooi AJ, de Visser M, Raaphorst J, van den Berg LH, Vermeulen RCH: Long-Term Air Pollution Exposure and Amyotrophic Lateral Sclerosis in Netherlands: A Population-based Case-control Study. Environmental health perspectives 2017;125:097023.

11 Allen JL, Klocke C, Morris-Schaffer K, Conrad K, Sobolewski M, Cory-Slechta DA: Cognitive Effects of Air Pollution Exposures and Potential Mechanistic Underpinnings. Curr Environ Health Rep 2017;4:180-191.

12 Basagana X, Esnaola M, Rivas I, Amato F, Alvarez-Pedrerol M, Forns J, Lopez-Vicente M, Pujol J, Nieuwenhuijsen M, Querol X, Sunyer J: Neurodevelopmental Deceleration by Urban Fine Particles from Different Emission Sources: A Longitudinal Observational Study. Environmental health perspectives 2016;124:1630-1636.

13 Shehab MA, Pope FD: Effects of short-term exposure to particulate matter air pollution on cognitive performance. Scientific reports 2019;9:8237.

14 Frye RE, Cakir J, Rose S, Delhey L, Bennuri SC, Tippett M, Melnyk S, James SJ, Palmer RF, Austin C, Curtin P, Arora M: Prenatal air pollution influences neurodevelopment and behavior in autism spectrum disorder by modulating mitochondrial physiology. Mol Psychiatry 2020

15 Costa LG, Chang YC, Cole TB: Developmental Neurotoxicity of Traffic-Related Air Pollution: Focus on Autism. Curr Environ Health Rep 2017;4:156-165.

16 Allen JL, Oberdorster G, Morris-Schaffer K, Wong C, Klocke C, Sobolewski M, Conrad K, Mayer-Proschel M, Cory-Slechta DA: Developmental neurotoxicity of inhaled ambient ultrafine particle air pollution: Parallels with neuropathological and behavioral features of autism and other neurodevelopmental disorders. Neurotoxicology 2017;59:140-154.

17 Valentino SA, Tarrade A, Aioun J, Mourier E, Richard C, Dahirel M, Rousseau-Ralliard D, Fournier N, Aubriere MC, Lallemand MS, Camous S, Guinot M, Charlier M, Aujean E, Al Adhami H, Fokkens PH, Agier L, Boere JA, Cassee FR, Slama R, Chavatte-Palmer P: Maternal exposure to diluted diesel engine exhaust alters placental function and induces intergenerational effects in rabbits. Part Fibre Toxicol 2016;13:39.
18 Bove H, Bongaerts E, Slenders E, Bijnens EM, Saenen ND, Gyselaers W, Van Eyken P, Plusquin M, Roeffaers MBJ, Ameloot M, Nawrot TS: Ambient black carbon particles reach the fetal side of human placenta. Nature communications 2019;10:3866.
19 Woodward NC, Haghani A, Johnson RG, Hsu TM, Saffari A, Sioutas C, Kanoski SE, Finch CE, Morgan TE: Prenatal and early life exposure to air pollution induced hippocampal vascular leakage and impaired neurogenesis in association with behavioral deficits. Translational psychiatry 2018;8:261.
20 Kang YJ, Tan HY, Lee CY, Cho H: An Air Particulate Pollutant Induces Neuroinflammation and Neurodegeneration in Human Brain Models. Adv Sci (Weinh) 2021;8:e2101251.
21 Ladd-Acosta C, Feinberg JI, Brown SC, Lurmann FW, Croen LA, Hertz-Picciotto I, Newschaffer CJ, Feinberg AP, Fallin MD, Volk HE: Epigenetic marks of prenatal air pollution exposure found in multiple tissues relevant for child health. Environ Int 2019;126:363-376.
22 Saenen ND, Martens DS, Neven KY, Alfano R, Bove H, Janssen BG, Roels HA, Plusquin M, Vrijens K, Nawrot TS: Air pollution-induced placental alterations: an interplay of oxidative stress, epigenetics, and the aging phenotype? Clin Epigenetics 2019;11:124.
23 Maghbooli Z, Hossein-Nezhad A, Adabi E, Asadollah-Pour E, Sadeghi M, Mohammad-Nabi S, Zakeri Rad L, Malek Hosseini AA, Radmehr M, Faghihi F, Aghaei A, Omidifar A, Aghababei Y, Behzadi H: Air pollution during pregnancy and placental adaptation in the levels of global DNA methylation. PLoS One 2018;13:e0199772.
24 Chen H, Oliver BG, Pant A, Olivera A, Poronnik P, Pollock CA, Saad S: Particulate Matter, an Intrauterine Toxin Affecting Foetal Development and Beyond. Antioxidants (Basel) 2021;10
25 Costa LG, Cole TB, Dao K, Chang YC, Garrick JM: Developmental impact of air pollution on brain function. Neurochem Int 2019;131:104580.
26 Morris RH, Counsell SJ, McGonnell IM, Thornton C: Early life exposure to air pollution impacts neuronal and glial cell function leading to impaired neurodevelopment. Bioessays 2021;43:e2000288.
27 Roque PJ, Dao K, Costa LG: Microglia mediate diesel exhaust particle-induced cerebellar neuronal toxicity through neuroinflammatory mechanisms. Neurotoxicology 2016;56:204-214.
28 Levesque S, Taetzsch T, Lull ME, Johnson JA, McGraw C, Block ML: The role of MAC1 in diesel exhaust particle-induced microglial activation and loss of dopaminergic neuron function. J Neurochem 2013;125:756-765.
29 Levesque S, Taetzsch T, Lull ME, Kodavanti U, Stadler K, Wagner A, Johnson JA, Duke L, Kodavanti P, Surace MJ, Block ML: Diesel exhaust activates and primes microglia: air pollution, neuroinflammation, and regulation of dopaminergic neurotoxicity. Environmental health perspectives 2011;119:1149-1155.
30 Chen X, Liu S, Zhang W, Wu C, Liu H, Zhang F, Lu Z, Ding W: Nrf2 deficiency exacerbates PM2.5-induced olfactory bulb injury. Biochem Biophys Res Commun 2018;505:1154-1160.
31 Davis DA, Akopian G, Walsh JP, Sioutas C, Morgan TE, Finch CE: Urban air pollutants reduce synaptic function of CA1 neurons via an NMDA/NO pathway in vitro. J Neurochem 2013;127:509-519.
32 Di Domenico M, Benevenuto SGM, Tomasini PP, Yariwake VY, de Oliveira Alves N, Rahmeier FL, da Cruz Fernandes M, Moura DJ, Nascimento Saldiva PH, Veras MM: Concentrated ambient fine particulate matter (PM2.5) exposure induce brain damage in pre and postnatal exposed mice. Neurotoxicology 2020;79:127-141.

33 Chew S, Lampinen R, Saveleva L, Korhonen P, Mikhailov N, Grubman A, Polo JM, Wilson T, Komppula M, Ronkko T, Gu C, Mackay-Sim A, Malm T, White AR, Jalava P, Kanninen KM: Urban air particulate matter induces mitochondrial dysfunction in human olfactory mucosal cells. Part Fibre Toxicol 2020;17:18.
34 Guo Z, Hong Z, Dong W, Deng C, Zhao R, Xu J, Zhuang G, Zhang R: PM2.5-Induced Oxidative Stress and Mitochondrial Damage in the Nasal Mucosa of Rats. Int J Environ Res Public Health 2017;14
35 Araujo JE, Jorge S, Santos HM, Chiechi A, Galstyan A, Lodeiro C, Diniz M, Kleinman MT, Ljubimova JY, Capelo JL: Proteomic changes driven by urban pollution suggest particulate matter as a deregulator of energy metabolism, mitochondrial activity, and oxidative pathways in the rat brain. Sci Total Environ 2019;687:839-848.
36 Nephew BC, Nemeth A, Hudda N, Beamer G, Mann P, Petitto J, Cali R, Febo M, Kulkarni P, Poirier G, King J, Durant JL, Brugge D: Traffic-related particulate matter affects behavior, inflammation, and neural integrity in a developmental rodent model. Environ Res 2020;183:109242.
37 Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F: Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J Neuroimmunol 1990;27:229-237.
38 Baburamani AA, Hurling C, Stolp H, Sobotka K, Gressens P, Hagberg H, Thornton C: Mitochondrial Optic Atrophy (OPA) 1 Processing Is Altered in Response to Neonatal Hypoxic-Ischemic Brain Injury. International journal of molecular sciences 2015;16:22509-22526.
39 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-408.
40 Gomez-Budia M, Konttinen H, Saveleva L, Korhonen P, Jalava PI, Kanninen KM, Malm T: Glial smog: Interplay between air pollution and astrocyte-microglia interactions. Neurochem Int 2020;136:104715.
41 Milani C, Farina F, Botto L, Massimino L, Lonati E, Donzelli E, Ballarini E, Crippa L, Marmiroli P, Bulbarelli A, Palestini P: Systemic Exposure to Air Pollution Induces Oxidative Stress and Inflammation in Mouse Brain, Contributing to Neurodegeneration Onset. International journal of molecular sciences 2020;21
42 Mudway IS, Kelly FJ, Holgate ST: Oxidative stress in air pollution research. Free Radic Biol Med 2020;151:2-6.
43 Guerra R, Vera-Aguilar E, Uribe-Ramirez M, Gookin G, Camacho J, Osornio-Vargas AR, Mugica-Alvarez V, Angulo-Olais R, Campbell A, Froines J, Kleinman TM, De Vizcaya-Ruiz A: Exposure to inhaled particulate matter activates early markers of oxidative stress, inflammation and unfolded protein response in rat striatum. Toxicol Lett 2013;222:146-154.
44 Cole TB, Coburn J, Dao K, Roque P, Chang YC, Kalia V, Guilarte TR, Dziedzic J, Costa LG: Sex and genetic differences in the effects of acute diesel exhaust exposure on inflammation and oxidative stress in mouse brain. Toxicology 2016;374:1-9.
45 Balaban RS, Nemoto S, Finkel T: Mitochondria, oxidants, and aging. Cell 2005;120:483-495.
46 Block ML, Calderón-Garcidueñas L: Air pollution: mechanisms of neuroinflammation and CNS disease. Trends in neurosciences 2009;32:506-516.
47 Bolton JL, Huff NC, Smith SH, Mason SN, Foster WM, Auten RL, Bilbo SD: Maternal stress and effects of prenatal air pollution on offspring mental health outcomes in mice. Environmental health perspectives 2013;121:1075-1082.
48 Bolton JL, Marinero S, Hassanzadeh T, Natesan D, Le D, Belliveau C, Mason SN, Auten RL, Bilbo SD: Gestational Exposure to Air Pollution Alters Cortical Volume, Microglial Morphology, and Microglia-Neuron Interactions in a Sex-Specific Manner. Front Synaptic Neurosci 2017;9:10.
49 Bai KJ, Chuang KJ, Chen CL, Jhan MK, Hsiao TC, Cheng TJ, Chang LT, Chang TY, Chuang HC: Microglial activation and inflammation caused by traffic-related particulate matter. Chem Biol Interact 2019;311:108762.

50 Xu MX, Zhu YF, Chang HF, Liang Y: Nanoceria restrains PM2.5-induced metabolic disorder and hypothalamus inflammation by inhibition of astrocytes activation related NF-kappaB pathway in Nrf2 deficient mice. Free Radic Biol Med 2016;99:259-272.
51 Onoda A, Umezawa M, Takeda K, Ihara T, Sugamata M: Effects of maternal exposure to ultrafine carbon black on brain perivascular macrophages and surrounding astrocytes in offspring mice. PLoS One 2014;9:e94336.
52 Andrade-Oliva MD, Aztatzi-Aguilar OG, Garcia-Sierra F, De Vizcaya-Ruiz A, Arias-Montano JA: Effect of in vivo exposure to ambient fine particles (PM2.5) on the density of dopamine D2-like receptors and dopamine-induced [(35)S]-GTPgammaS binding in rat prefrontal cortex and striatum membranes. Environ Toxicol Pharmacol 2018;60:58-65.
53 Connor M, Lamorie-Foote K, Liu Q, Shkirkova K, Baertsch H, Sioutas C, Morgan TE, Finch CE, Mack WJ: Nanoparticulate matter exposure results in white matter damage and an inflammatory microglial response in an experimental murine model. PLoS One 2021;16:e0253766.
54 Carletti B, Piemonte F, Rossi F: Neuroprotection: the emerging concept of restorative neural stem cell biology for the treatment of neurodegenerative diseases. Curr Neuropharmacol 2011;9:313-317.
55 Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY: Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat Biotechnol 2002;20:1103-1110.
56 Bacigaluppi M, Pluchino S, Peruzzotti-Jametti L, Kilic E, Kilic U, Salani G, Brambilla E, West MJ, Comi G, Martino G, Hermann DM: Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. Brain 2009;132:2239-2251.
57 Zhang T, Zheng X, Wang X, Zhao H, Wang T, Zhang H, Li W, Shen H, Yu L: Maternal Exposure to PM2.5 during Pregnancy Induces Impaired Development of Cerebral Cortex in Mice Offspring. International journal of molecular sciences 2018;19
58 Imbriani G, Panico A, Grassi T, Idolo A, Serio F, Bagordo F, De Filippis G, De Giorgi D, Antonucci G, Piscitelli P, Colangelo M, Peccarisi L, Tumolo MR, De Masi R, Miani A, De Donno A: Early-Life Exposure to Environmental Air Pollution and Autism Spectrum Disorder: A Review of Available Evidence. Int J Environ Res Public Health 2021;18
59 Chun H, Leung C, Wen SW, McDonald J, Shin HH: Maternal exposure to air pollution and risk of autism in children: A systematic review and meta-analysis. Environ Pollut 2020;256:113307.
60 Blomgren K, Hagberg H: Free radicals, mitochondria, and hypoxia-ischemia in the developing brain. Free Radic Biol Med 2006;40:388-397.
61 Sotty J, Kluza J, De Sousa C, Tardivel M, Antherieu S, Alleman LY, Canivet L, Perdrix E, Loyens A, Marchetti P, Lo Guidice JM, Garcon G: Mitochondrial alterations triggered by repeated exposure to fine (PM2.5-0.18) and quasi-ultrafine (PM0.18) fractions of ambient particulate matter. Environ Int 2020;142:105830.
62 Salvi A, Liu H, Salim S: Involvement of oxidative stress and mitochondrial mechanisms in air pollution-related neurobiological impairments. Neurobiol Stress 2020;12:100205.
63 Frye RE, Cakir J, Rose S, Delhey L, Bennuri SC, Tippett M, Melnyk S, James SJ, Palmer RF, Austin C, Curtin P, Arora M: Prenatal air pollution influences neurodevelopment and behavior in autism spectrum disorder by modulating mitochondrial physiology. Mol Psychiatry 2021;26:1561-1577.
64 Grevendonk L, Janssen BG, Vanpoucke C, Lefebvre W, Hoxha M, Bollati V, Nawrot TS: Mitochondrial oxidative DNA damage and exposure to particulate air pollution in mother-newborn pairs. Environ Health 2016;15:10.
65 Yan Q, Liew Z, Uppal K, Cui X, Ling C, Heck JE, von Ehrenstein OS, Wu J, Walker DI, Jones DP, Ritz B: Maternal serum metabolome and traffic-related air pollution exposure in pregnancy. Environ Int 2019;130:104872.

66 Kim JH, Yan Q, Uppal K, Cui X, Ling C, Walker DI, Heck JE, von Ehrenstein OS, Jones DP, Ritz B: Metabolomics analysis of maternal serum exposed to high air pollution during pregnancy and risk of autism spectrum disorder in offspring. Environ Res 2021;196:110823.

Figure Legends

Fig. 1. Exposure to high concentrations of UPM adversely alters BV2 cell health. (A) Increased LDH release, indicating cytotoxicity, was observed following BV2 exposure to 30µg/ml and 100µg/ml UPM for 24h. (B) Increased cytotoxicity correlated with BV2 cell number as measured by Hoechst staining of live cells (inset). (C) No significant increase in C17.2 cytotoxicity or cell death (D) was observed. Data were analysed by one-way ANOVA followed by Dunnett's *post hoc* test where appropriate. Biological replicates are as indicated on the graphs (n=3-6) and expressed as mean ± SEM. *p<0.05; **** p<0.0001. Scale bar indicates 100µm.

Fig. 2. UPM exposure evokes a concentration-dependent inflammatory response that is more profound in BV2 cells. RNA was prepared from UPM-exposed cells and qRT-PCR performed using primers specific for the genes shown. All values are compared against GAPDH and an experimental control. Increased expression of IL-6 (A), TNFα (B) and IL-1β (C) was observed for BV2 cells compared with C17.2 cells following UPM exposure with no significant difference in anti-inflammatory IL-10 gene expression in either cell type. Data were analysed by two-way ANOVA followed by Sidak's *post hoc* test where appropriate. Biological replicates are as indicated on the graphs (n=3-6) and expressed as mean ± SEM. *p<0.05; **p<0.01;**** p<0.0001.

Fig. 3. Exposure to high concentrations of UPM increases ROS production. Alterations in ROS production (DHE) were determined after UPM exposure in BV2 (A) and C17.2 (B) cells. UPM treatment significantly increased ROS production in BV2 cells (A) following exposure to 30 µg/ml and 100 µg/ml UPM, but no change was observed in C17.2 cells (B). Data were analysed by one-way ANOVA followed by Dunnett's *post hoc* test where appropriate. Biological replicates are as indicated on the graphs (n=3-6) and expressed as mean ± SEM. **p<0.01. Scale bar indicates 100µm (C) qRT-PCR was performed on C17.2 and BV2 RNA as previously described to determine the expression of NRF2. Increased expression was observed in C17.2 cells compared with BV2 cells particularly at higher concentrations of UPM exposure. Data were analysed by two-way ANOVA followed by Dunnett's *post hoc* test (n= 4-5, **p<0.01)

Fig. 4. Exposure to UPM decreases mitochondrial health. (A) ROS production was evaluated after UPM exposure in BV2 cells pretreated with mitochondrially targeted antioxidant mitoTEMPO (M, grey bars), compared with cells that did not receive pretreatment. MitoTEMPO prevented the UPM-mediated increase in DHE fluorescence. (B) BV2 cells were exposed to UPM at the concentrations shown and then labelled with TMRM. A decrease in TMRM fluorescence was observed (inset) which reached significance at the highest UPM concentration indicating decreased mitochondrial

membrane potential. Scale bar indicates 75µm (C) BV2 cells were pretreated with mitoTEMPO (M, grey bars) and cell number determined following UPM exposure. For (A) and (C), data were analysed by two-way ANOVA followed by Sidak's *post hoc* test. Biological replicates are as indicated on the graphs (n=3) and expressed as mean ± SEM. *p<0.05; **p<0.01. For (B) data were analysed by one-way ANOVA followed by Dunnett's *post hoc* test. Biological replicates are as indicated on the graphs (n=3) and expressed as mean ± SEM. *p<0.05.

Figure 1

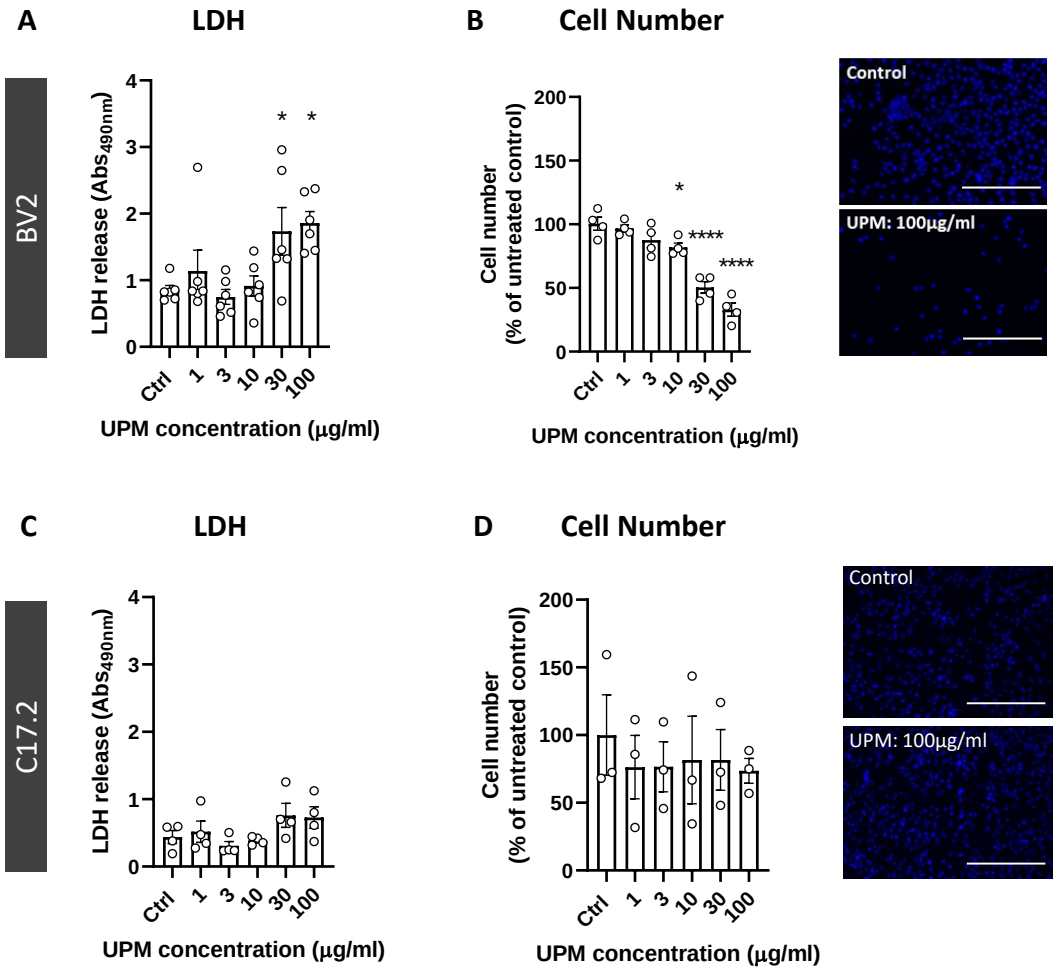

A
LDH
B
Cell Number
BV2
LDH release (Abs490nm)
Cell number (% of untreated control)
Control
UPM: 100µg/ml
Ctrl
1
3
10
30
100
UPM concentration (µg/ml)
C
LDH
D
Cell Number
C17.2
LDH release (Abs490nm)
Cell number (% of untreated control)
Control
UPM: 100µg/ml
UPM concentration (µg/ml)

Figure 2

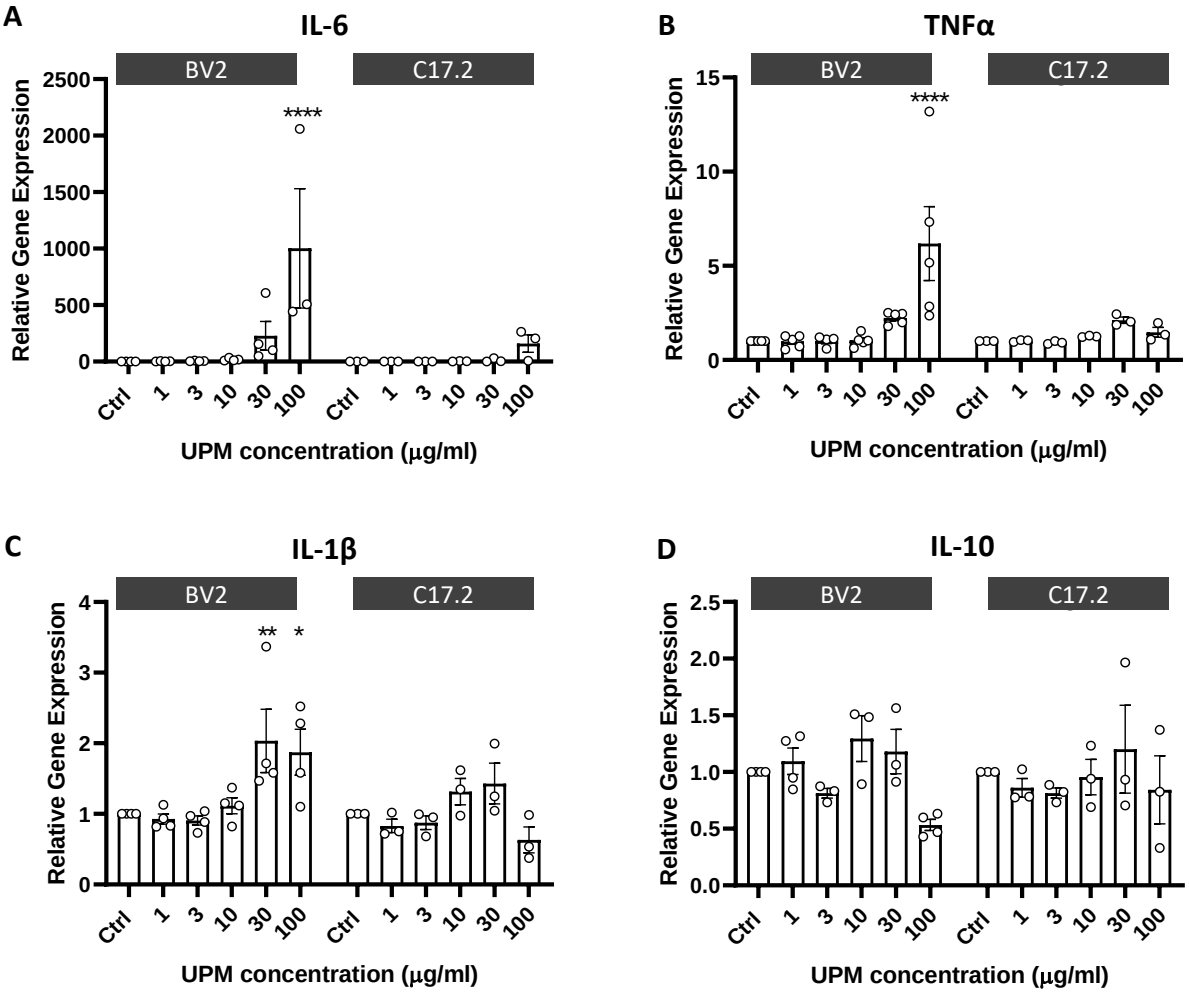

A
IL-6
BV2
C17.2
Relative Gene Expression
2500
2000
1500
1000
500
0
****
Ctrl
1
3
10
30
100
UPM concentration (μg/ml)
B
TNFα
15
10
5
0
C
IL-1β
4
3
2
1
**
*
D
IL-10
2.5
2.0
1.5
1.0
0.5
0.0

Figure 3

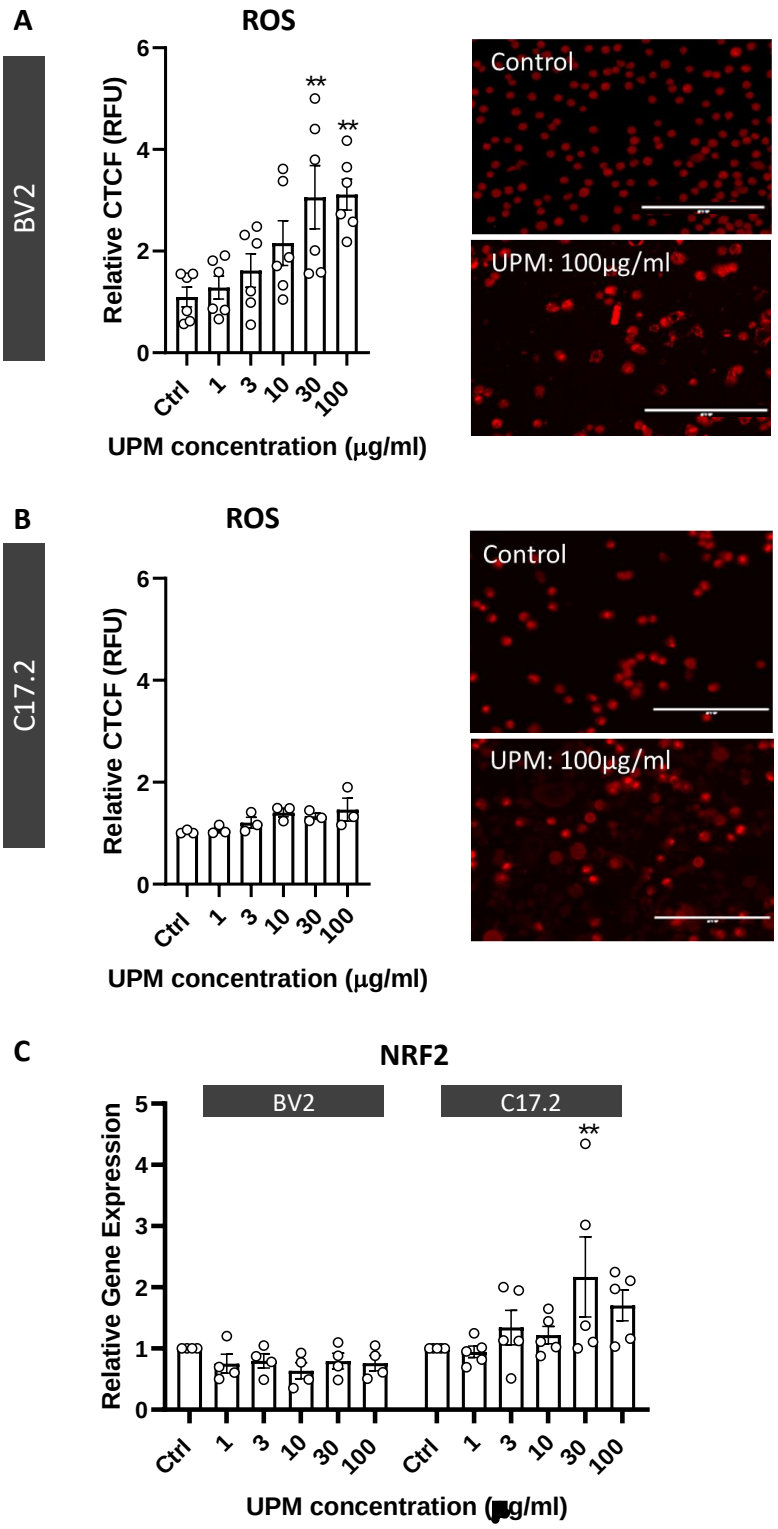
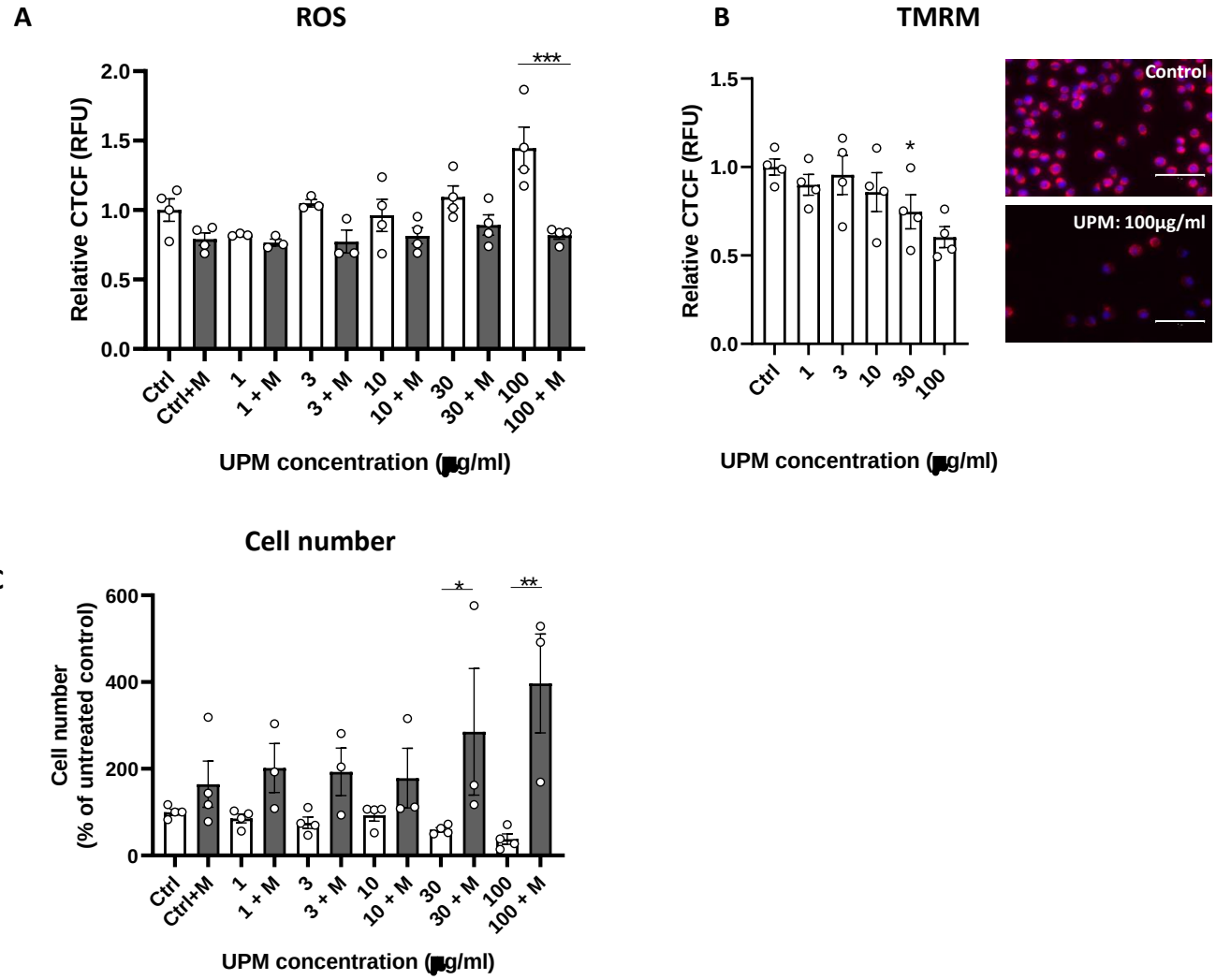

A
ROS
BV2
Relative CTCF (RFU)
Control
UPM: 100µg/ml
Ctrl
1
3
10
30
100
UPM concentration (µg/ml)
B
ROS
C17.2
Relative CTCF (RFU)
Control
UPM: 100µg/ml
UPM concentration (µg/ml)
C
NRF2
BV2
C17.2
Relative Gene Expression
UPM concentration (µg/ml)

Figure 4


A
ROS
Relative CTCF (RFU)
2.0
1.5
1.0
0.5
0.0
***
Ctrl
Ctrl+M
1
1 + M
3
3 + M
10
10 + M
30
30 + M
100
100 + M
UPM concentration (µg/ml)
B
TMRM
Relative CTCF (RFU)
1.5
1.0
0.5
0.0
*
Ctrl
1
3
10
30
100
UPM concentration (µg/ml)
Control
UPM: 100µg/ml
C
Cell number
Cell number
(% of untreated control)
600
400
200
0
*
**
Ctrl
Ctrl+M
1
1 + M
3
3 + M
10
10 + M
30
30 + M
100
100 + M
UPM concentration (µg/ml)