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A Toxicology Suite Adapted for Comparing Parallel Toxicity Responses of Model Human Lung Cells to Diesel Exhaust Particles and Their Extracts

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Epidemiological studies have shown that exposure to airborne particulate matter (PM) can be an important risk factor for some common respiratory diseases. While many studies have shown that PM exposures are associated with inflammatory reactions, the role of specific cellular responses in the manifestation of primary hypersensitivities and the progression of respiratory diseases remains unclear. In order to better understand mechanisms by which PM can exert adverse health effects, more robust approaches to support *in vitro* studies are warranted. In response to this need, a group of accepted toxicology assays was adapted to create an analytical suite for screening and evaluating the effects of important, ubiquitous atmospheric pollutants on two model human lung cell lines (epithelial and immature macrophage). To demonstrate the utility of this suite, responses to intact diesel exhaust particles (DEP) and mass-based equivalent doses of their organic extracts were examined. Results suggest that extracts have the potential to induce greater biological responses than those associated with their colloidal counterpart. Additionally, macrophage cells appear to be more susceptible to the cytotoxic effects of both intact DEP and their organic extract, than epithelial cells tested in parallel. As designed, the suite provided a more robust basis for characterizing toxicity mechanisms than the analysis of any individual assay. Findings suggest that cellular responses to PM are cell line dependent, and show that the collection and preparation of PM and/or their extracts have the potential to impact cellular responses relevant to screening fundamental elements of respiratory toxicity.

INTRODUCTION

Airborne particulate matter (PM) is gaining recognition as an important contributor to serious cardiovascular and pulmonary diseases (Bayra et al. 1998; Atkinson et al. 2001; Yun et al. 2009) and the exacerbation of allergies, asthma, and respiratory infections. Assessments by the World Health Organization (WHO) estimate that PM exposures may be responsible for up to 3.1 million deaths annually (WHO 2012). Current regulations are mass-based, separating PM into two size fractions: PM_{10} and $PM_{2.5}$ (particles with an aerodynamic diameter of less than 10 or 2.5 μm, respectively). Both fractions are made up of a complex mixture of organic and inorganic compounds, which vary in size, composition, and origin. The fine fraction, $PM_{2.5}$, is emerging as a serious public health concern because these particles may be carried deep into the lung where they can initiate critical changes in cellular functions during normal respiration (Valavanidis et al. 2008). Diesel engines are thought to be a major source of urban $PM_{2.5}$ and a majority of primary diesel particles are reported to be in a respirable size-range (Yun et al. 2009). In order to better understand the associations between PM exposure and respiratory disease developments, diesel exhaust particles (DEP) are often studied as a model faction of the greater PM pool that can induce negative biological responses (Li et al. 2003; Seagrave et al. 2007).

Airborne DEP is typically comprised of a complex mixture of polyaromatic hydrocarbons (PAHs), semi- and volatile organic compounds (VOCs), and metals entrained in, or otherwise associated with, various carbonaceous materials (Andrysik et al. 2011). Numerous *in vitro* studies have investigated cellular responses to DEP exposure by quantifying select categories of inflammatory biomarkers produced by mammalian lung cells (Bayra et al. 1998; Knebel et al. 2002; Okayama et al. 2006). A range of cellular responses has been reported, which appear to be influenced by the cell type used as well as on the age, source, and preparation of the DEP being studied (Bonvallot et al. 2001). In their comprehensive review, Schwartze and coworkers noted that freshly generated DEP has been typically found to be more potent than older, standardized samples of DEP (Schwarze et al. 2013). *In vitro* DEP studies focused on characterizing oxidative stress in

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mammalian cells, typically report expression thresholds of cyto- and chemokines in response to mass concentrations ranging between 50 and 1000 μg/ml using conventional biochemical assays (EPA 2002; Ma and Ma 2002).

A variety of studies on the potential respiratory toxicity of DEP have been conducted using organic DEP extracts as well (Li et al. 2002; Yun et al. 2009; Andrysik et al. 2011). Different extracts have been utilized for *in vitro* exposures as a conservative surrogate for the effect(s) intact particles can cause in lung tissues (Arimoto et al. 2007). The aim of these studies is to better elucidate mechanisms of DEP toxicity by liberating the compounds from DEP's carbonaceous surface and core, to enhance the potential bioavailability of its components. Because methods for DEP extraction are not standardized, however, different researchers use a variety of procedures ranging in complexity from sonication to soxhlet extraction (Bai et al. 2001; Masala et al. 2011). While extract-based research has yielded a more detailed set of toxicological information, the relationship and normalized comparison of the toxicity of DEP extracts, to that of intact DEP, has received relatively little attention, and the literature in this arena remains tenuous. Totlandsdal et al. and Bonvallot et al. conducted in-depth research that exposed human bronchial cells to intact DEP, its extracts, and carbonaceous particles; they evaluated classic cytotoxicity responses along with the expression of a wide range of inflammation-related biomarkers. Some studies report that exposure levels required to observe classic cytotoxic behavior are significantly higher than those required for observing inflammation markers (Holder et al. 2008), and note some differential effects in response to whole DEP particles and DEP extracts (Bonvallot et al. 2001; Totlandsdal et al. 2012).

Many respiratory toxicity studies on PM, however, utilize a single cell line (often not of human origin) and focus on the production of cytokines or chemokines (Li et al. 2002). While the release of these signaling proteins are an important part of the innate immune response, they can also be indicative of infection, inflammation, or other trauma; they do not specifically indicate the nature of the cellular damage that has led to cytokine release, nor can they be used quantitatively to predict toxicological endpoints (e.g., DNA damage). In this context, examining a solitary mechanism or biomolecule (e.g., cytokine) can potentially neglect important toxicity mechanisms and lead to limited views of downstream respiratory responses.

We report here, a suite of four complementary biochemical assays coordinated in a novel way to maximize their information yield on the toxicological effects of an exemplary aerosol (DEP), and its organic extract, on mammalian (human) respiratory cells. This approach is extended as a model for screening the potential for human lung tissue to respond to the stress presented by PM. The suite is comprised of a panel that can yield increased confidence in analysis of the classic toxicity modes PM can induce (cytotoxicity, genotoxicity, and oxidative damage). As a suite, the assays were adapted for formative and summative overlap, such that results from one assay offer independent potential to corroborate findings from others in the suite. This suite-based approach was designed such that, when viewed as a group, the results can provide converging lines of evidence for single-mode or multi-modal toxicity responses. Consideration of results from a set of four parallel assays increases the likelihood of detecting cellular responses occurring via a broader range of pathways and, additionally, improves the potential for recognizing toxic effects that may present as a result of simultaneous stresses.

To further expand the information yield that can be provided by a suite, the assays are carried out on human cell lines selected as models for fundamentally distinct functions in the respiratory system. The first cell line, human phagocytic pre-macrophage monocytes (GDM-1), was chosen as a model for alveolar macrophage cells. This cell type is a part of the immune system that actively engulfs foreign particles in the human lung, and is thought to be the immune system's first line of defense against PM carried into the respiratory system. The second cell line, human alveolar epithelial lung cells (A549), was chosen to represent responses from the alveolar lining, which creates the barrier between the respiratory and circulatory systems. By executing parallel investigation of the responses from two functionally different respiratory cell types after exposure to a toxic agent, a more complete assessment of cumulative respiratory responses can be observed. These cells were selected because they are human lines whose relative ease of culturing, cost-effectiveness, and intact p53 response, made them good candidates for this suite-style screening approach.

This toxicological suite is comprised of four complementary assays: the first assay quantifies cell death, and can differentiate apoptotic from necrotic cytotoxicity (Annexin V assay). A second assay of cell cycle distributions is carried out in parallel to quantify a toxic agent's effects on DNA synthesis and division in a population of cells (Collins et al. 1997). Alterations to cell cycle DNA distributions are assessed to identify potential types of cellular damage (Nunez 2001). A third assay detects accumulation of the tumor suppressor protein p53, which accumulates in response to stress and genetic damage (Levine 1997). A final assay quantified intracellular pools of reactive oxygen species (ROS). Due to the overlapping nature of these four assays, the suite viewed as a whole provides a more robust view of toxicity and includes *de facto* validation of key endpoints (e.g., both cell cycle and p53 can suggest DNA damage and both Annexin V and cell cycle can detect apoptosis).

As a screening tool, the following demonstrates the utility of a suite-based analysis of toxicological endpoints carried out on human respiratory cell lines, which offers the following advantages. First, cellular responses occurring via a range of pathways can be detected. Second, this method has the potential to reveal toxic effects that may manifest from cellular damage, which occurs simultaneously. Last, the assays are

carried out on two human cell lines in parallel, which model disparate functions of the human respiratory system, providing a more comprehensive view of respiratory response. This work aims to provide a template for the development of more advanced *in vitro* screening of airborne pollutants and is the result of collaboration between toxicologists, biologists, and air quality engineers.

MATERIALS AND METHODS

DEP, DEP Extracts, and Controls

Standard reference materials (SRM) 2975 and 1975 were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). SRM 2975 is comprised of intact DEP collected and physico-chemically characterized by NIST (NIST 2009a). SRM 1975 is an organic extract of SRM 2975. In order to prepare SRM 1975, NIST set aside a portion of the homogenized SRM 2975 particles and subsequently extracted the particles for 24 h in dichloromethane (NIST 2009b). The extract was then concentrated by evaporation under nitrogen. Mass-based equivalency values for SRM 1975 were provided by NIST. In preparation for *in vitro* assays, DEP extracts were transferred to the biocompatible solvent dimethyl sulfoxide (DMSO) following widely accepted protocols (Lewtas and Gallagher 1990; de Kok et al. 2005).

For intact DEP exposures, concentrations reported were achieved by adding particles and 0.5% DMSO to growth media and sonicating the mixture for 5 min before adding to cells. For DEP extract exposures, appropriate volumes of DEP extract in DMSO were added to growth media. Because extracts were delivered to cells in DMSO, 0.5% DMSO was added to intact DEP solutions in order to make comparisons between intact DEP and extract exposures without concern for solvent effects. In all samples, final DMSO content was at or below 0.5%. DEP and DEP extracts were prepared in serum-containing growth media, which was selected in order to keep cells in optimal growth conditions for the 24 and 48 h exposure times (Hsiao and Huang 2013)

Each DEP and DEP extract exposure scenario included an untreated control population and a vehicle control population (cells exposed to only 0.5% DMSO). In all cases, vehicle controls exhibited no significant differences when compared to untreated controls (data not shown). Data reported as "control" herein represent responses of vehicle control populations.

Cell Culture

All cell culture media and assay reagents were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise noted. Human pulmonary type II epithelial adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) and cultured in Ham's F-12 nutrient mixture with 10% FBS including 1% antibiotic-antimycotic solution that contains penicillin, streptomycin, and fungizone (Life Technologies catalog # 15240–062). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. For assays and continuous cell propagation, adherent monolayers in exponential growth phase (70–80% confluence) were harvested in 0.05% Trypsin following standard protocols (Eisenbrand et al. 2002). The phagocytic human pre-macrophage monocyte cell line GDM-1 was obtained from the *German* Collection of Microorganisms and *Cell Cultures* (DSMZ, Braunschweig, Germany). Cells were cultured in RPMI 1640 nutrient mixture with 10% FBS including 1% antibiotic-antimycotic solution described above. Cells were harvested by centrifugation (1800 rpm, 5 min) in the logarithmic growth phase with a viability exceeding 90% as determined by trypan blue dye exclusion (Carero et al. 2001).

Apoptotic and Necrotic Cytotoxicity

Externalized phophatidylserine was detected using AlexaFluor488®-conjugated Annexin V in order to quantify apoptosis in a population of cells. Propidium iodide (PI) staining was performed concomitantly to differentiate necrotic cells from early apoptotic cells. Cytometric quadrant analysis, comparing single cell interrogation from fluorophore specific fluorescent detectors, was used to distinguish between apoptotic and necrotic cells (Vermes et al. 1995). A549 and GDM-1 cells were seeded into 25 cm^2 culture flasks and stimulated with a range of DEP (and extract) concentrations. After 24 h exposures, cells were washed with PBS and re-suspended in Annexin V buffer according to manufacturer's recommendations. A total of 100 μl of the cell suspension was mixed with 5 μl of Annexin V-FITC (proprietary solution included in kit and 3 μl of PI [100 μg/ml]). The mixture was incubated in the dark for 15 min at room temperature after which an additional 400 μl of Annexin V buffer was added. Samples were then placed on ice and analyzed by flow cytometry within 1 h. Assays were carried out in triplicate and a minimum number of 10,000 cell events were collected for each analysis. Population means were aligned via the use of single color controls and compensation by the analytical software.

Cell Cycle Analysis

To determine distribution of cells in each cell cycle stage, cells were cultured in 25 cm^2 flask and were exposed to a range of concentration of DEP for indicated times. After exposure, cells were harvested, washed in PBS, and then incubated in a hypotonic solution containing PI, according to the methods of Krishan et al (Krishan 1975). Cells were incubated for a minimum of 24 h incubation at 5°C in the dark (Nunez 2001) and then analyzed by flow cytometry. Cell stage was determined by analysis of fluorescence intensity histograms with FloJo analytical software (Tree Star Inc., Ashland, OR,

USA) and utilizing the Dean-Jett curve-fitting algorithm when appropriate. Assays were carried out in triplicate and a minimum number of 10,000 cell events were collected for each analysis.

p53 Accumulation

In order to examine accumulation of the p53 protein, an immunoblot analysis was performed according to widely practiced protocols previously outlined by Donner et al. (Donner et al. 2007). Cells were cultured in 25 cm^2 flasks and treated with a range of DEP concentrations for 24 h. DNA damage was induced in positive controls by the addition of 10 uM Nutlin. After exposure to DEP, DMSO, or Nutlin, cells were washed twice with cold PBS and solubilized in RIPA extraction buffer, which was then sonicated and centrifuged at 14,000 g for 10 min. Cell lysates in supernatant were collected and protein concentrations were determined by bicinchoninic acid (BCA) protein assay according to manufacturer's recommendations (Pierce BCA Kit, Thermo Scientific). Western blotting was utilized to determine p53 expression in the exposed populations of cells compared to controls. Equal amounts of cellular protein from each sample were boiled for 5 min in SDS sample loading buffer (Invitrogen), and then separated on a 7.5% polyacrylamide gels (Biorad, Hercules, CA, USA). Proteins were then electroblotted onto a polyvinylidene difluoride (PVDF) membrane using transfer buffer (Biorad, Hercules, CA, USA). Membranes were pretreated (blocked to reduce non-specific binding) for 1 h with 5% non-fat milk protein followed by incubation with monoclonal primary antibodies that have high affinity for select oligopeptide structures of p53 (p53: catalog # ab80645, Abcam, Cambridge, MA, USA; nucleolin [loading control] catalog # sc8031, Santa Cruz Biotechnology, Dallas, TX, USA) for 18 h at 5°C. Membranes were then rinsed with wash buffer and incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (p53: catalog # ab97245, Abcam, Cambridge, MA, USA; nucleolin: catalog # sc-2005; Santa Cruz Biotechnology, Dallas, TX, USA). Finally, membranes were rinsed with wash buffer and treated with an enhanced chemi-luminescence kit according to vendor instructions (SuperSignal West PicoLuminescence Kit, Fisher Scientific) for detection of chemiluminescence by Image Quant LAS4000.

Reactive Oxygen Species

Intracellular ROS formation was measured using the cell-permeant fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Cat. D-399, Invitrogen) following published protocols (Landreman et al. 2008). Cells were loaded with 10 uM H_2DCFDA and incubated for 45 min at 37°C in the dark. After incubation, cells were treated with a range of DEP concentrations for 150 min. Intracellular ROS content was then measured by flow cytometry. Log-normalized mean fluorescence intensity (MFI) was detected by acquisition of fluorescence of channel 1 (FL1). Assays were carried out in triplicate and a minimum number of 10,000 cell events were collected for each analysis.

Flow Cytometric Analysis

Samples, including DEP-only sample controls, were analyzed by CyAn ADP Flow Cytometric Analyzer (Beckman Coulter, Brea, CA, USA). Post-analysis, including compensation when appropriate, was performed using FloJo software (Treestar, Inc., Ashland, OR, USA).

Statistical Analysis

Results are presented as the mean ±SD from at least three independent experiments. Student *t*-test and one-way analysis of variance (ANOVA), followed by Tukey's multiple-range test when appropriate, was used to determine the statistical significance of the difference between means. A p-value of <0.05 was considered statistically significant.

RESULTS

Apoptotic and Necrotic Cytotoxicity

As judged by the Annexin V assay, A549 cells exposed to a range of DEP extract concentrations, but not intact DEP, led to significant increases in cell death (Figure 1). Statistical analysis of these data indicated no significant difference between controls and cell populations challenged with intact DEP concentrations ranging between 300 and 3000 μg/ml. Analysis of

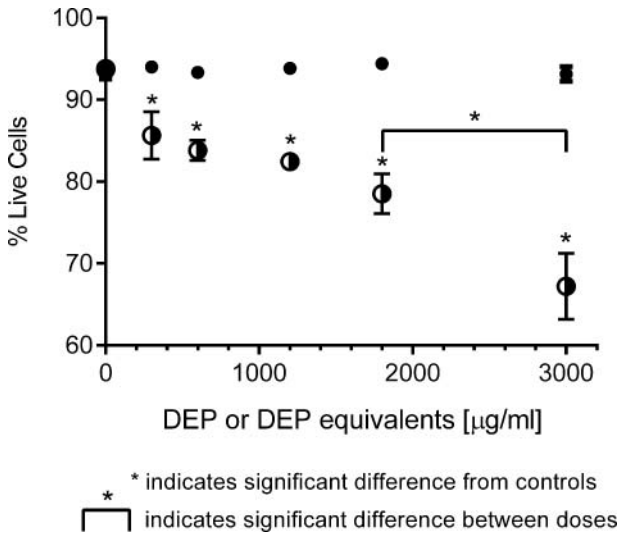

FIG. 1. Response of A549 epithelial cells exposed to increasing concentrations of DEP (●) and its organic extract (○) as judged by Annexin V and propidium iodide staining. Error bars represent pooled standard deviation of three independent trials, each of which include a minimum of 10^4 cells analyzed. *Indicates significant differences at a 95% confidence level.

cells exposed to an identical range of equivalent DEP doses of its extract, however, revealed significant increases in cell death when compared to controls for all concentrations examined (Figure 1). Cells exposed to 3000 μg/ml exhibited significantly higher levels of cell death when compared to cells exposed to 1800 μg/ml ($p < 0.001$).

In addition to distinguishing live from dead cells, the Annexin V assay also differentiates between the two primary pathways of cell death: apoptosis (programmed cell death) and necrosis (death as indicated by membrane permeability). For all concentrations analyzed, A549 cells exposed to DEP extract underwent significantly higher levels of both apoptotic and necrotic cell death when compared to controls, where no changes were observed in response to intact DEP (Figure 2).

In contrast, analysis of phagocytic GDM-1 cells exposed to both intact DEP and DEP extract for 24 h resulted in dose-dependent increases in cell death (Figure 3). Statistical analysis of the data indicated significant differences between controls and cell populations challenged with intact DEP concentrations of 1800 and 3000 μg/ml (p values 0.015 and 0.0001, respectively). GDM-1 cells exposed to an identical range of equivalent doses of the DEP extract displayed significant increases in cell death when compared to controls for all concentrations greater than or equal to 1200 μg/ml (p values < 0.0001). Significant increases in cell death were also observed between each increasing dose (p values <0.0001).

As illustrated in Figure 4a, exposure of GDM-1 cells to a range of intact DEP concentrations led to significant increases in apoptotic cell death for populations exposed to 1800 and 3000 μg/ml. GDM-1 populations exposed to DEP extracts displayed significant increases (over controls) in both apoptotic and necrotic cell death for all concentrations greater than or equal to 1200 μg/ml (Figure 4b). Discrimination of necrotic from apoptotic cell death demonstrated that the cytotoxicity initiated in GDM-1 cells by both intact DEP and DEP extract can lead to both types of cell death; however, apoptosis appeared to be the dominant pathway.

Taken together, results of the four sets of Annexin V cytotoxicity assays demonstrate that intact DEP has the potential to induce distinctly different levels of cell death depending on the cell line selected. Further, results suggest that the preparation of the DEP has the potential to significantly impact not only the magnitude of cell death, but also the mode of cell death after exposure *in vitro*. Exposure of A549 cell populations to DEP extract led to increases in both apoptotic and necrotic cell death. Results suggest that for GDM-1 cells exposed to both intact DEP and its extract, apoptosis is the dominant path to cell death. Of the two cell lines evaluated, GDM-1 cells displayed a greater sensitivity to both preparations of DEP.

Cell Cycle

Cells exposed to intact DEP and DEP extract were examined for changes in cell cycle distributions. DNA content profiles indicated that A549 cells exposed to intact DEP resulted in no significant changes in their cell cycle distributions (Figure 5a). Analysis of A549 cells exposed to increasing concentrations of DEP extract, however, led to increases in the portion of cells that failed to complete cell division (the sum of all cells in the S, G2, and mitosis stages; Figure 5b); however, these increases were not statistically significant. The alteration of this distribution suggests that cells may be being halted at the S or G2 checkpoints, which serve to prevent cells with DNA or spindle damage from entering into mitosis. A portion of A549 cells exposed to DEP extract also presented

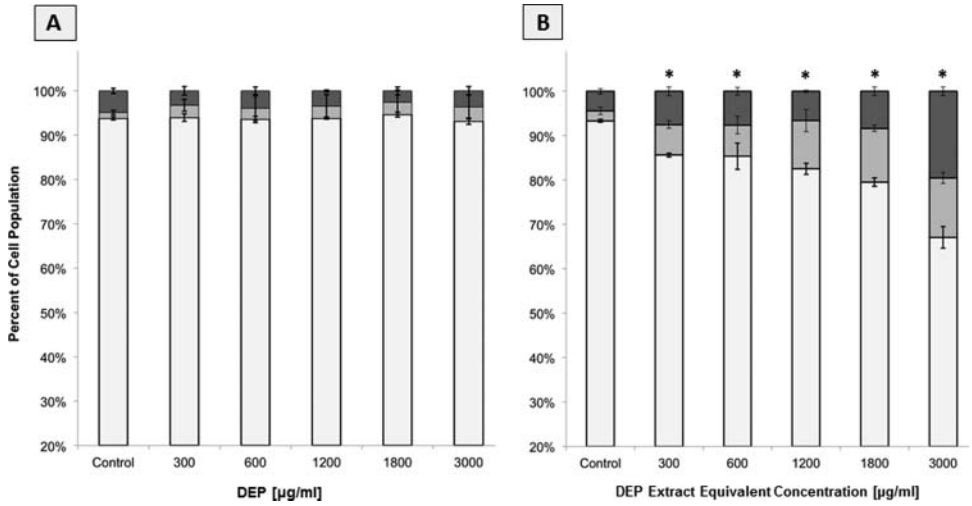

FIG. 2. Flow cytometric analysis of apoptosis and necrosis in A549 cell populations as judged by Annexin and PI staining. Columns represent percent of cell population existing as live (□), apoptotic (■), or necrotic (■). Error bars represent pooled standard deviation of three independent trials; each includes a minimum of 10^4 cell analyzed. *Indicates significant differences at a 95% confidence level. (a) A549 cells after 24 h exposure to a range of DEP concentrations. (b) A549 cells after 24 h exposure to a range of DEP extract concentrations.

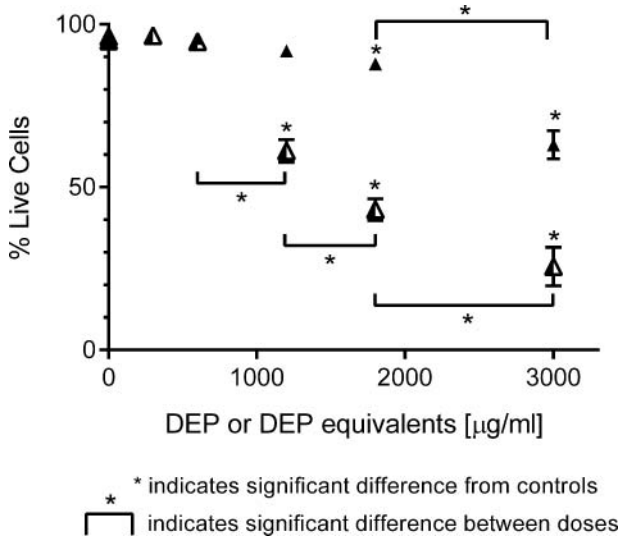

FIG. 3. Response of GDM-1 immature macrophage cells exposed to increasing concentrations of DEP (▲) and its organic extract (Δ) as judged by Annexin V and propidium iodide staining. Error bars represent pooled standard deviation of three independent trials, each of which include a minimum of 10^4 cell analyzed. *Indicates significant differences at a 95% confidence level.

changes in DNA content indicative of apoptosis (data not shown), further supporting findings from cytotoxicity assays.

Analysis of the DNA content profiles of phagocytic GDM-1 cells exposed to DEP and DEP extract showed dose-dependent changes to the distribution of cells in cell cycle phases. GMD-1 cells challenged with intact DEP resulted in increases in the fraction of cells failing to complete cell division (S, G2, Mitosis) after exposure to 600 and 1200 μg/ml (Figure 6a). This trend, however, was not observed for cells exposed to 1800 and 3000 μg/ml, due to variability in experimental replicates. Similar to findings for A549 cells, increases in the fraction of immature macrophage cells failing to complete division (S, G2, Mitosis) were observed with increasing doses of DEP extract (Figure 6b). Increases in cells failing to complete division were significantly greater than controls after exposure to 1800 and 3000 μg/ml. Data show that cell cycle disturbances were greatest in cells exposed to DEP extract and overall, GDM-1 cells displayed greater sensitivity to DEP as intact particles or extracts. A portion of GDM-1 cells exposed to both DEP and DEP extract also presented changes in DNA content indicative of apoptosis (data not shown). These findings were also consistent with results of cytotoxicity assays.

Accumulation of p53

p53 accumulation in each cell line was judged by standard Western blotting, after exposing log-phase cells to a range of doses of DEP and DEP extract. As judged by optical volume (Figure 7a), exposure to intact DEP induced no significant increases to the intercellular pools of p53 in A549 cells. In contrast, A549 cells accumulated increased levels of p53 (compared to control) after exposure to the entire range of DEP extract concentrations tested. GDM-1 cells exposed to intact DEP exhibited detectable increases in p53 content, but presented dose-dependent increases when exposed to DEP extract (Figure 7b). In addition to findings from cell cycle, accumulation of p53 in cells further suggests cell stress responses, which may include DNA damage in cells exposed to DEP extracts.

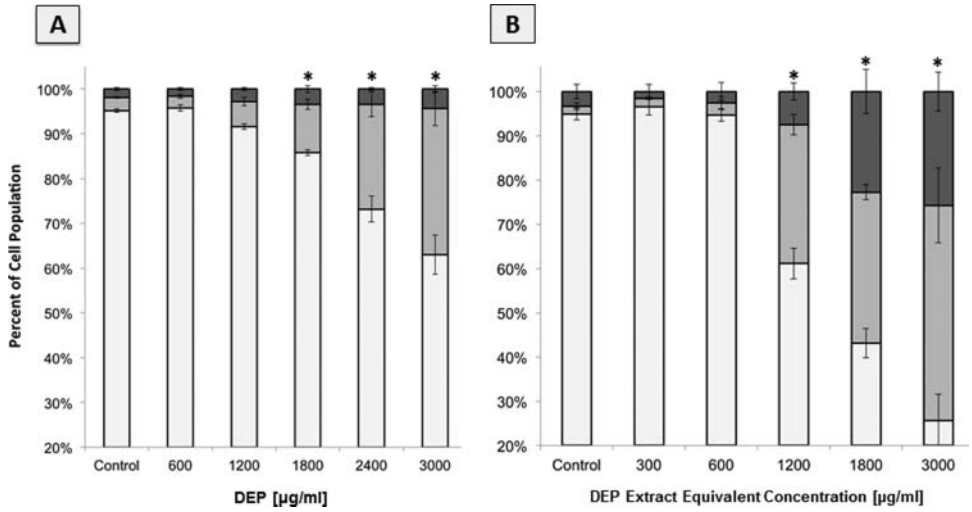

FIG. 4. Flow cytometric analysis of apoptosis and necrosis in GDM-1 cell populations as judged by Annexin and PI staining. Columns represent percent of cell population existing as live (□), apoptotic (■), or necrotic (■). Error bars represent pooled standard deviation of three independent trials, each of which include a minimum of 10^4 cell analyzed. *Indicates significant differences at a 95% confidence level. (a) GDM-1 cells after 24 h exposure to a range of DEP concentrations. (b) GDM-1 cells after 24 h exposure to a range of DEP extract concentrations.

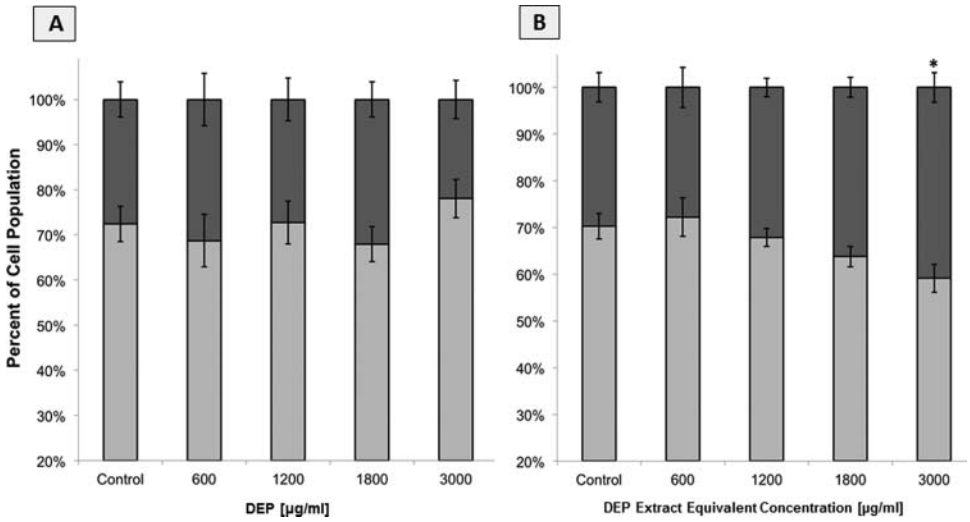

FIG. 5. Flow cytometric analysis of A549 population cell cycle distributions based on DNA content histograms, following 24 h of exposure to DEP or extract. Columns represent percent of cell population in the following cell cycle phases: the sum of S+G2+Mitotic (■) and G1 (■). Error bars represent pooled standard deviation of three independent trials, each of which include a minimum of 10^4 cell analyzed. *Indicates significant differences at a 95% confidence level. (a) DNA content histogram following 24 h exposure to intact DEP. (b) DNA content histogram following 24 h exposure to DEP extract.

Accumulation of Reactive Oxygen Species

A549 cells stimulated by intact DEP resulted in a weak induction of dose-dependent ROS increases (Figure 8a). Cell populations exposed to 1800 and 3000 μg/ml were found to have significantly higher concentrations of intracellular ROS when compared to controls. By comparison, A549 cells exposed to DEP extract exhibited much larger dose-dependent increases in ROS, as high as ten-fold that of intact DEP (Figure 8a). Statistical analysis showed significant increases over controls for all concentrations examined. Additionally, increases in response to DEP extract concentrations above 600 μg/ml led to significant increases in ROS production when compared to all lower doses. These data suggest that equivalent doses of DEP extracts have much higher potential to induce ROS production in epithelial cells.

Phagocytic GDM-1 cells exposed to intact DEP and DEP extract responded with increased concentrations of intracellular ROS (Figure 8b). Statistical analysis indicated significant

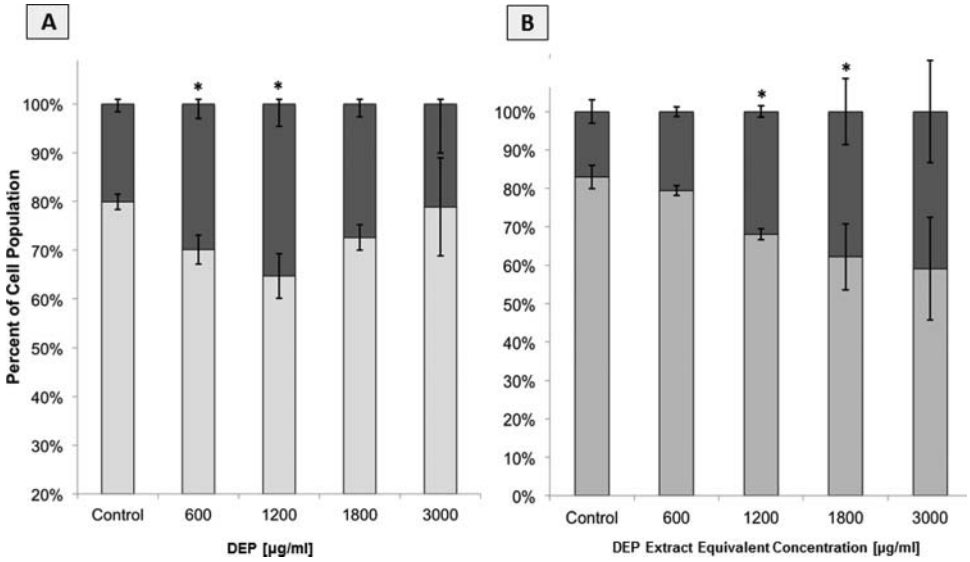

FIG. 6. Flow cytometric analysis of GDM-1 population cell cycle distributions based on DNA content histograms. Columns represent percent of cell population in the following cell cycle phases: the sum of S+G2+Mitotic (■) and G1 (■). Error bars represent pooled standard deviation of three independent trials, each of which include a minimum of 10^4 cell analyzed. *Indicates significant differences at a 95% confidence level. (a) DNA historgrams following 24 h of exposure to DEP. (b) DNA historgrams following 24 h of exposure to DEP extracts.

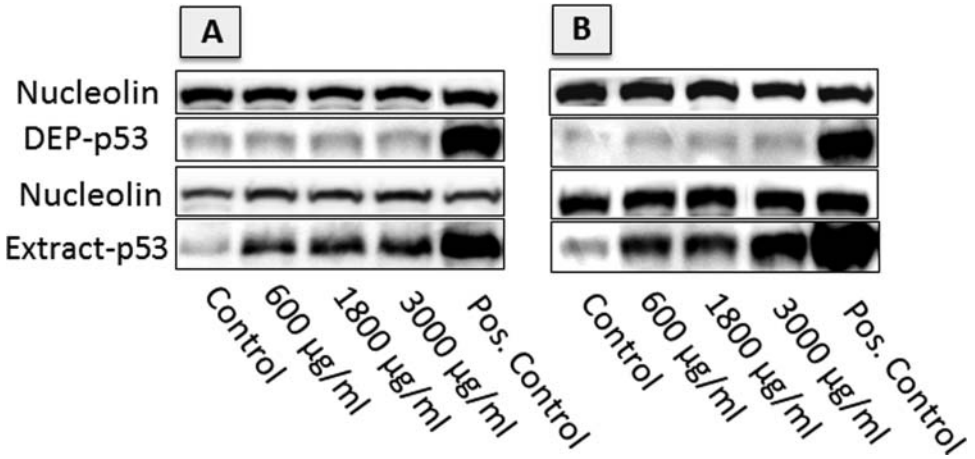

FIG. 7. Image of western blots showing p53 accumulation in cells exposed to DEP and DEP extracts at different concentrations, following 24 h exposures. Loading was normalized by nucleolin, and response was positively controlled by inducing DNA damage with 10 μM Nutlin (positive control). (a) A549 cells; (b) GDM-1 cells.

increases over controls within cell populations exposed to intact DEP concentrations greater than or equal to 600 μg/ml. Each increase in concentration over 600 μg/ml also led to significant increases in ROS production when compared to lower doses. In comparison to their epithelial A549 counterparts, GDM-1 cells exposed to intact DEP accumulated ROS concentrations ranging from two- to ten-fold greater. GDM-1 cells (and other phagocytic cell types) actively produce ROS after engulfing particles, in a process called oxidative burst, that likely accounts for the higher concentrations of ROS in GDM-1 cells exposed to intact DEP. GDM-1 cell populations exposed to DEP extract displayed significant increases in ROS production for all concentrations examined (Figure 8b). Further, each increase in concentration examined led to significantly higher concentrations of ROS compared to lower doses. Trends were similar between the two preparations of DEP for GDM-1 cells; however, exposure to DEP extract resulted in two-fold increases in ROS concentrations compared to those measured in cells challenged with intact particles. In comparison to their epithelial A549 counterparts, GDM-1 cells exposed to DEP extract accumulated ROS concentrations ranging from two- to three-fold greater. DEP extracts are free from particles and therefore this difference cannot be accounted for by ROS production during phagocytosis. ROS generated in GDM-1 cells after exposure to DEP extract suggests that GDM-1 cells have greater sensitivity to DEP extracts.

DISCUSSION

Improving our understanding of how DEP and its components have the potential to result in adverse health effects is an important step towards the development of more targeted strategies in air pollution mitigation and regulation. DEP is often

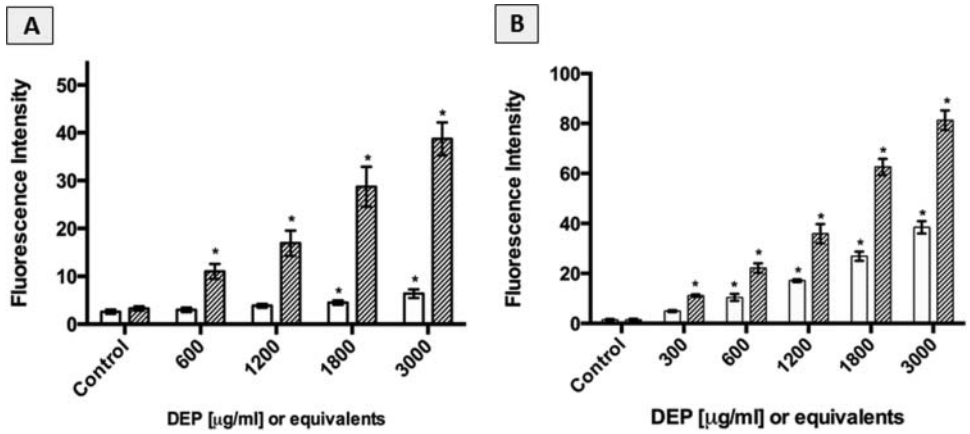

FIG. 8. Intracellular ROS response of cells exposed to increasing concentrations of DEP (□) and its organic extract (▨) as indicated by fluorescence intensity. Error bars represent pooled standard deviation of three independent trials, each of which include a minimum of 10^4 cell analyzed. *Indicates significant differences from respective controls at a 95% confidence level. (a) A549 cell responses; (b) GDM-1 cell responses.

cited as one of the most common and harmful air pollutants in the biosphere; yet, despite numerous toxicity studies, the underlying mechanisms driving DEP toxicity are only partially understood. Better resolution of the different modes in which DEP and other types of PM can induce cellular stresses can provide new opportunities for preventative measures to reduce risks for developing respiratory diseases caused and exacerbated by PM.

For this research, we exposed distinct cell lines to two PM preparations and systematically evaluated differences in cellular responses via a panel of four complementary assays. Our findings provide a useful model for this suite-based approach, and we demonstrate here that the cell type selected for *in vitro* studies, as well as the preparation of PM, can lead to significant differences in fundamental biological stress responses observed. While it has been shown that extractable organic species can be a more potent indicator than the residual DEP nuclei for health effects (Bonvallot et al. 2001), relatively few *in vitro* studies comparing the effects of intact DEP and organic DEP extracts have been reported (Totlandsdal et al. 2012), whereas the use of various extracts is common.

PM has been shown in many studies (Bayra et al. 1998; Li et al. 2000; Schwarze et al. 2013) to lead to decreases in the viability of cells exposed to it; however, few studies have obtained converging lines of independent evidence to implicate modal stress responses in a common cytometric platform (e.g., cyto-, geno-, and oxidative toxicity). Nor have such results been organized as a cohort matrix with analytical overlap designed to associate independent assays with a dominant mode. For the purposes of this project, a separate platform for the detection of p53 accumulation was used to independently validate results; however, in future studies, p53 could be assessed via cytometry in order to make this a true, single platform approach (Zamai et al. 2002).

Cytotoxicity challenges (Annexin V assay) were designed to reveal both qualitative and quantitative differences in how cells progress to their death under DEP stresses. This response varied significantly depending on the cell type challenged, as well as the preparation of the DEP. For GDM-1 cells, equivalent DEP doses delivered in extract resulted in significantly higher cellular mortality and enhanced levels of apoptosis when compared to responses of their epithelial counterparts. Cell death via apoptosis is a highly regulated biochemical process, which can be initiated by DNA damage. In addition to cytotoxicity findings, apoptotic indicators were also observed in evaluations of the cell cycle (an analysis of intracellular DNA content), and further supported by the accumulation of the p53 protein. These results exemplify how assays selected for this suite can provide independent lines of complementary evidence, which support (cyto)toxicity findings.

One potential limitation of these results is that cells were exposed to diesel preparations in serum-containing media. Research has shown that fetal bovine serum can interact with some components of PM (Okeson et al. 2004). However, because serum is important for proper cell growth and protein production (Hsiao and Huang 2013), serum-contained media was opted for, despite the potential for a decrease in the observed toxicity of PM.

Cell cycle analyses were conducted to evaluate potential genotoxicity of DEP and its standardized extract. The cell cycle is composed of discrete phases that are regulated by a series of biochemical checkpoints (Sclafani and Holzen 2007). Cells stalled at different points in the replication process can be detected by measuring the quantity of DNA within individual cells as they move through cell cycle phases. The accumulation of cells within specific phases can indicate the type of damage caused by a toxic agent, and, when analyzed in tandem with other toxicity biomarkers, can improve confidence in distinguishing toxicological mode(s). Under the exposure conditions reported here, each cell line displayed markedly different DNA profiles from independent DNA preparations. As with the cytotoxicity experiments, cell cycle analyses suggest that GDM-1 cells are more vulnerable to intact DEP than A549 cells. Exposure to DEP extract led to increases in the number of cells failing to complete cell division for both cell types; however, only changes in GDM-1 cells reached statistical significance. After exposure, these cells displayed an accumulation of cells in the S, G2, and M phases. These accumulations occur when cells initiate DNA synthesis, but fail to complete cell division. This type of disturbance to the cell cycle has been reported as indicative of spindle or DNA damage (Speirs et al. 2010). As indicated in the cytotoxicity results, exposed cells died predominately via apoptosis. By juxtaposing the distribution of cell cycle phases where DEP and DEP extract interfere with reproduction, this second and independent analytical line of analysis serves to provide more evidence toward the pathway that leads to the observed apoptotic cell death. In this context, these results suggest that the exclusive use of an organic extract as a surrogate for particles may limit interpretations of the cellular mechanisms involved in respiratory responses to DEP.

The third assay examined the accumulation of the p53 protein. This protein is widely conserved and acts as a transcription factor that plays an important role in maintaining the integrity of the genome and in the subsequent elimination of damaged or tumorigenic cells (Vousden 2007). Because p53 is involved in regulation of the cell cycle, as well as the initiation of apoptosis, the results of this assay add another line of independent evidence, which further aids in elucidating the cellular stress pathways leading to cell death. These analyses indicated that p53 accumulates in both cell types after exposure to DEP extract. Cells exposed to intact DEP, however, did not accumulate detectable amounts of p53. Findings are consistent with the increases in (primarily apoptotic) death observed in cytotoxicity studies. And p53 results also complement findings from cell cycle analyses (which implicate DNA damage) by illustrating that cell groups exhibiting the highest levels of cell death also displayed the highest

levels of p53 protein, which is primarily expressed in response to DNA damage.

The last assay examined the accumulation of ROS. ROS are produced in low levels during normal metabolism, and are present in all healthy cells. However, particulate pollutants can be potent inducers of oxidative stress (Ball et al. 2000), which can lead to the intracellular accumulation of ROS well above normal background levels. This type of oxidative stress can lead to a host of types of cellular damage as a result of oxygen species' probability of reacting with other cellular biopolymers including DNA, key lipids, and proteins (Li et al. 2002). Oxidative stress has also been shown to lead to macroscale pro-inflammatory effects in the respiratory system and has been linked with the development of asthma (Li et al. 2003). The ability of PM to cause oxidative stress is typically attributed to two general mechanisms: first, the primary oxidant-generating properties of the compounds present, which can lead to imbalances in redox cycling and the production of high levels of ROS within a cell. Second, PM exposure can stimulate intra-organelle generation of ROS (e.g., oxidative burst during phagocytosis in macrophage and other immune cells; Landreman et al. 2008). The two cell lines selected for this study were chosen, in part, to address and compare both of these mechanisms. The GDM-1 cells generate and sequester ROS in organelles after engulfing particles, while the A549 epithelial cells do not carry out this function. Therefore, the ROS concentrations in A549 cells after exposure to DEP provide a useful representation of the primary oxidant-generating capacity of the intact DEP. Alternately, ROS concentrations observed in GDM-1 cells (which produce ROS during phagocytosis) exposed to intact DEP can provide information about the sum of both primary oxidant-generating capacity and cellular generation of ROS. Because of this, juxtaposing ROS assay on these two cell lines in parallel has the potential to provide additional insights into ROS generation.

Quantitation of intracellular ROS reported here revealed dose-dependent increases in both cell lines after exposure to both DEP and DEP extract, with significantly greater responses to the extract. Differences between the two preparations were most pronounced in A549 cells, with DEP extract generating as much as ten-fold greater ROS concentrations in cells than equivalent doses of intact DEP. These increases in ROS are likely an expression of the primary oxidant-generating potential of the extract. The results from our ROS experiments suggest that many of the organic compounds rendered bioavailable by extraction may be less available for stimulating ROS generation when they are sorbed to carbonaceous cores of intact particles. Intracellular ROS concentrations generated by GDM-1 cells after challenges were generally two-fold higher for cells exposed to DEP extract. The detection of greater ROS accumulation after exposure to particle-free extracts is somewhat surprising due to the fact that GDM-1 cells are phagocytic and were expected to actively produce internal ROS upon engulfing intact DEP. Our findings clearly indicated that model phagocytic cells exposed to DEP extract generated significantly more ROS than cells exposed to intact DEP. Overall, ROS levels were consistent with increases in apoptosis, supporting previous findings illustrating that increasing levels of ROS induce a hierarchy of biological effects, the highest being cytotoxicity (Li et al. 2003). Additionally, this work provided unique insights into the variability of responses of epithelial and phagocytic cells when exposed to different preparations of PM.

While future research is needed to reveal the explicit mechanisms by which DEP and associated ROS initiates apoptosis and affects the cell cycle, this style of analysis serves to narrow the scope of subsequent investigations (e.g., quantification of protein kinases and accumulation of s15p53; Andrysik et al. 2011; Longhin et al. 2013).

When analyzed as a suite of complementary assays, these findings highlighted that the use of organic extracts alone may limit observations of important cellular mechanisms involved with DEP exposure. Further, our results underscore that cell line selection for toxicological analyses of DEP (or other PM) can lead to differences in both the magnitude and mode of toxicological responses observed. These results suggest that the parallel use of multiple cell lines provide important insights into the range of responses of the cellular components that make up the respiratory system and that there is a need for more systematic comparisons among commonly used cell lines. The DEP literature includes results, which both align (Yang et al. 1997; Bonvallot et al. 2001; Inoue et al. 2007; Totlandsdal et al. 2012) and are inconsistent with findings reported here (Li et al. 2002). These (dis)agreements may be expected given the variety of different cell lines used in past research and differences in source, collection, storage, and exposure methods, and thus suggest the need for the details presented in this research.

This suite of assays was selected as a useful and informative compromise between the more detailed levels of toxicology testing available (Bonvallot et al. 2001; Andrysik et al. 2011) and limited, single cell line studies that are common in the air quality engineering field (Landreman et al. 2008; de Bruijne et al. 2009; Lichtveld et al. 2012). Because this suite is intended for use as a screening tool, it has inherent limitations. These include the use of only one type of epithelial and one type of phagocytic cell, the use of a non-differentiated pre-macrophage monocyte to model alveolar macrophage cells, the inclusion of serum in exposures, and a liquid exposure scenario. These compromises were made to ensure that this suite was kept as simple and cost-effective as possible, increasing the likelihood that it could be used as a screening tool by researchers who are also involved in the collection of environmental PM. The suite-style screening offers a means of reducing the innumerable types of responses that cells can experience after an environmental insult, and provides a significantly narrowed list of likely toxicity mechanisms for further examination. In future research, this suite could be

adapted for use with air–liquid interface systems and/or other appropriate cell lines of interest.

For the present study, a commonly utilized source of DEP (SRM 2975) was selected to highlight some potential issues and differences that can arise when studying DEP and DEP extracts using *in vitro* assays. While this DEP source was collected from outdated diesel technology with respect to representing environmental PM in many developed countries, it serves as a successful demonstration to enhance comparative perspectives from the formidable amount of research that already exists in this arena. Indeed, the DEP used here was an older SRM and the collection and storage requirements of this material were not intended to preserve volatile and semi-volatile components. This likely accounts for the larger doses that were required in this study, compared with experiments that used fresher DEP samples (Schwarze et al. 2013).

Advancements in PM research have revealed the importance of several factors, which were not considered in the PM toxicity studies of the past. For example, the significance of semi-volatile components, as illustrated by Seagrave et al. (2003), and the development of innovative air–liquid interface exposure systems (Cooney and Hickey 2011; Kooter et al. 2013). These are some of the advancements that have highlighted the need for scientists and engineers, who collect environmental PM, to work more closely with toxicologists in evaluating the pollution potential. This study demonstrates how classic biochemical assays could be leveraged and compared across a common platform, for the purpose of creating a matrix that can provide higher confidence in observing dominant toxicological modes and providing toxicologists with relevant information for further research. While applied to DEP as a demonstration, this suite-based screening approach can be extended to include other classes of aerosol particles of immediate public health concern, including, but not limited to, bioaerosols and the study of the potential for synergistic effects of PM mixtures relevant to the polluted atmosphere.

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