Published in final edited form as:
Toxicol In Vitro. 2008 March ; 22(2): 498–509. doi:10.1016/j.tiv.2007.10.011.

Effects of Cell Type and Culture Media on Interleukin-6 Secretion in Response to Environmental Particles

John M. Veranth, **N. Shane Cutler**, **Erin G. Kaser**, **Christopher A. Reilly**, and **Garold S. Yost**
Department of Pharmacology and Toxicology, 30 South 2000 East, University of Utah, Salt Lake City, Utah, 84112 USA

Abstract

Cultured lung cells provide an alternative to animal exposures for comparing the effects of different types of air pollution particles. Studies of particulate matter *in vitro* have reported proinflammatory cytokine signaling in response to many types of environmental particles, but there have been few studies comparing identical treatments in multiple cell types or identical cells with alternative cell culture protocols. We compared soil-derived, diesel, coal fly ash, titanium dioxide, and kaolin particles along with soluble vanadium and lipopolysaccharide, applied to airway-derived cells grown in submerged culture. Cell types included A549, BEAS-2B, RAW 264.7, and primary macrophages. The cell culture models (specific combinations of cell types and culture conditions) were reproducibly different in the cytokine signaling responses to the suite of treatments. Further, Interleukin-6 (IL-6) response to the treatments changed when the same cells, BEAS-2B, were grown in KGM versus LHC-9 media or in media containing bovine serum. The effect of changing media composition was reversible over multiple changes of media type. Other variables tested included culture well size and degree of confluence. The observation that sensitivity of a cell type to environmental agonists can be manipulated by modifying culture conditions suggests a novel approach for studying biochemical mechanisms of particle toxicity.

Introduction

Elucidating the toxicological mechanisms that link specific components of inhaled air pollution particles with the adverse health effects observed by epidemiology studies is an active area of research. *In vitro* experiments with immortalized and normal cells have been widely used to study the relationship between many forms of particulate air pollution and the induction of proinflammatory cytokine signaling, as reviewed by Allen (Allen 2006), Fubini and Aust et al. (Fubini, Aust et al. 1998). and Oberdörster, Maynard et al. (Oberdorster, Maynard et al. 2006). For example, cytokine release by lung epithelial cells has been used to study responses

Author to whom correspondence should be sent: John M. Veranth, Research Associate Professor, Department of Pharmacology and Toxicology, 30 South 2000 East, University of Utah, Salt Lake City, Utah, 84112 USA email: John.Veranth@utah.edu, Phone 801-581-3789.

Conflict of Interest Statement
The authors declare that they have no competing financial interests.

Authors' Contributions
JMV designed the study, analyzed data, and drafted the manuscript. EK performed all the cell culture experiments. NSC and CAR provided assistance on experimental techniques and helped analyze data. GSY provided infrastructure and advice on study design. All authors have read and approved the final manuscript.

to charged particles (Veronesi, de Haar et al. 2002), metal-rich particles (Quay, Reed et al. 1998), urban dust (Smith and Aust 1997), diesel particulate (Steerenberg, Zonnenberg et al. 1998), carbon nanotubes (Pulskamp, Diabate et al. 2007), endotoxin (Schulz, Farkas et al. 2002), mineral dusts (Hetland, Refsnes et al. 2000; Øvrevik, Myran et al. 2005), and soil-derived dusts (Veranth, Moss et al. 2006). The immortalized cell lines are especially useful for mechanistic studies of particle toxicology because they allow experimental replication over extended time periods, and the cells can be manipulated by molecular biology techniques. However, proliferating immortalized cells may not model the responses of normal, terminally differentiated cells in tissues. The genetic alterations that allow these cells to be grown in perpetuity can alter other cell functions as well. Also, structural features such as tight junctions, distinct basal and apical surfaces, and cilia that characterize airway epithelial tissue are absent in non-confluent growing cells.

In a typical experiment, cells are grown submerged in culture media in dishes or wells, a particle suspension or extract is placed on the cells, and responses such as secretion of cytokines or changes in transcription of inflammation- and oxidative stress-relevant genes are measured. These *in vitro* experiments are used both to obtain descriptive data about specific environmental and occupational particles and to study mechanistic pathways linking particle exposure to biochemical responses. *In vitro* studies in the particle literature have used a wide range of cell models. In this paper the term “cell model” means a specific combination of cell type and cell culture protocol including, but not limited to, the specific source or subpopulation of cells, the culture media formulation, the method of passaging, the time from seeding to treatment; and the confluence of the cells.

Many hypotheses regarding the health effects of ambient particles involve lung inflammation as a central process mediating both pulmonary and cardiovascular pathologies (Frampton 2006; HEI 2002). As a result, *in vitro* studies of particle-induced response mechanisms have often measured cytokines that are associated with the induction and resolution of inflammation. For example, studies have measured the release of Interleukin-6 (IL-6) by airway cells due to the multiple roles of this cytokine in the induction and resolution of inflammation (Zitnik and Elias 1993) and its association with clinical disease (Chung 2001; Kanda and Takahashi 2004). Other particle and fiber studies have focused on genes controlled by the NF-κb transcriptional factor such as the cytokines IL-8, TNF-α and GM-CSF and other molecules such as ICAM-1 and iNOS as reviewed by Donaldson and Tran (Donaldson and Tran 2002). Studies of particulate matter *in vitro* have documented proinflammatory cytokine signaling in response to many particle types, but there have been few studies comparing treatments in multiple cell types or alternative cell culture protocols. Table 1a gives examples of studies that have used *in vitro* cell models to study the relative potency of different types of particles to induce the release of IL-6 and Table 1b gives examples of studies that have compared the IL-6 response to the same particle using more than one cell type.

This study was motivated by our initial difficulties in replicating published experiments that described induction of cytokines by BEAS-2B, A549 and RAW264.7 cells in response to treatment with various metal-containing agonists such as combustion ash and soluble vanadium. Specifically, our attempts to use soluble vanadium or synthetic residual oil fly ash mixtures (Samet, Stonehuerner et al. 1997) as a positive control in our studies of transition metals in coal fly ash and other types of particulate matter produced confusing results even though these materials had been reported to induce proinflammatory signaling in BEAS-2B (Kennedy, Ghio et al. 1998; Quay, Reed et al. 1998; Samet, Stonehuerner et al. 1997; Veronesi, de Haar et al. 2002) and NHBE cells (Carter, Ghio et al. 1997) and systemic responses in animals (Costa and Dreher 1997; Nurkiewicz, Porter et al. 2004; Prichard, Ghio et al. 1996). Our preliminary experiments measuring both IL-6 and IL-8 suggested that some cell culture models appeared sensitive to metals and resistant to lipopolysaccharide (LPS) while others

showed the reverse pattern. Discussions with the authors of the cited papers suggested cell culture protocol differences could be important, and this could be tested by using a consistent set of environmentally relevant agonists to treat different cell culture models.

Study Overview

This study tested the hypothesis that *in vitro* IL-6 responses to previously studied particle types vary in a characteristic manner with the cell model used. Many *in vitro* studies have compared the potency of different materials in one or two cell types. The objective of this study was to provide insight regarding the particle response differences between the various cell types and cell culture protocol conditions that have been used in prior work. A set of identical treatments was tested in various cell culture models found in the literature and follow-up experiments further quantified the effects of important variables. The treatments are listed in Table 2. Relevant prior work on these treatments includes: crustal or soil-derived dust (Becher, Hetland et al. 2001; Hetland, Refsnes et al. 2000; Veranth, Moss et al. 2006; Veranth, Reilly et al. 2004), soluble vanadium (Carter, Ghio et al. 1997; Quay, Reed et al. 1998; Veronesi, de Haar et al. 2002), coal fly ash (Aust, Ball et al. 2002; Veronesi, de Haar et al. 2002), diesel particles (Dybdahl, Risom et al. 2004; Steerenberg, Zonnenberg et al. 1998), lipopolysaccharide (Schulz, Farkas et al. 2002), titanium dioxide (Steerenberg, Zonnenberg et al. 1998), and kaolin clay (Gao, Keane et al. 2000). Vanadium and LPS are soluble in aqueous solution, but are included as particle surrogates since these materials are associated with particulate matter in the atmosphere. The cell types included BEAS-2 and A549 immortalized cells, RAW264.7 mouse cells, and primary rat macrophages. BEAS-2B cells are an SV40-transformed human bronchial epithelial cell line. Since the study was motivated by our difficulty in reproducing published results with BEAS-2B cells we specifically tested the BEAS-2B cells maintained our own laboratory, a sample of the cells maintained by the US EPA Human Studies Division, and cells purchased from American Type Culture Collection (ATCC) at the start of the study. A549 is a carcinoma-derived cell with characteristics of Type II alveolar epithelial cells. RAW264.7 is an immortalized mouse-derived monocyte-macrophage cell line, and primary rat macrophages were harvested as described in the methods. Cell culture conditions were based on protocols from the literature, but included alternative media and serum concentrations. Due to the number of experimental combinations, we focused on a single endpoint: IL-6 concentration in the media at fixed one day after treatment.

Material & Methods

Tables 2 and 3 identify the cell types, culture media, and particle treatments used in this study and list the abbreviation codes. The experimental designs used in different phases of this study are described below followed by specifics of materials and methods common to all experiment phases.

Experimental Designs

Factorial Study—A three-variable, two-level, full-factorial experimental design tested for the effects of BEAS-2B cell source, cell culture media, and protocol for culture plate cell seeding on the IL-6 response to three agonists: soil dust S and soluble vanadium V at 80 μg/cm^2, and LPS at 1000 EU/mL. BEAS-2B cells, types E and U, were cultured for one passage in either KGM or LHC-9 and kept in the same media for treatment. The cell seeding protocol was designed to vary cell confluence by seeding either 20,000 cells/cm^2 and treating after one day or seeding 35,000 cells/cm^2 and allowing the cells to grow for three days before particle treatment. All conditions were run in triplicate.

Cell and Treatment Matrix—The basic experimental design was a dose-response experiment using five logarithmically spaced particle concentrations with a matrix consisting of seven environmentally relevant particle treatments and eleven lung cell models, of which only six are discussed in this paper. The treatments were soil-derived dust, soluble vanadium, coal fly ash, diesel particulate, LPS, titanium dioxide, and kaolin clay. Doses ranged from the maximum concentrations listed in the caption of Table 2 down to 0.01 of the maximum plus an untreated control. Cell models tested included BEAS-2B (A, E, and U stocks as indicated in Table 3), in LHC-9 and KGM media, and A549, RAW264.7, and primary macrophages in the media listed below. All the combinations of treatments and concentrations were run N = 1 on a single 48-well plate, and the experiment with each cell model was replicated with at least three independent cell passages.

Media Composition Study—The design consisted of a concentration-response design using an untreated and agonist-treated cells seeded in culture wells with variable compositions of the media. The experiments used a series of mixtures of the commercial LHC-9 and KGM media and increasing amounts of bovine serum added to the commercial media formulation.

Well Size Study—The four sizes of wells were all seeded at 35,000 per cm^2 with cells from the same passage. The treatments were applied with constant media depth and constant mass concentration per well area. The design was N = 6 wells per condition, and the experiment was conducted twice.

Reversibility Study—This was a time-series design where the variable was alternating LHC-9 and KGM media with each passage. To start, cells that had been maintained in the same media (e.g. KGM) for an extended time were passaged and a cell culture plate containing the current growth media was seeded for particle treatment while the remaining cells were seeded in a culture flask containing the other media (e.g. LHC-9). After cells had attached the media in the culture flask was replaced once to dilute any residual old media, and cells were feed thereafter nominally every 2 days. After 3 to 5 days the culture flask was ready for passaging to seed the next culture plate in the new media (e.g. LHC-9) and a new flask representing another media change (e.g. back to KGM). At each step in the time series a single flask of cells was used to seed an experiment N = 5 wells per condition. The time series study was replicated starting with new frozen stocks of cells.

Cell Types

BEAS-2B cells (type A) were American Type Culture Collection number CRL-9609 (www.atcc.org, Manassas, VA). BEAS-2B cells (type E) were an aliquot from the frozen stocks maintained at the US EPA Human Studies Division. The BEAS-2B cells (type U) were originally obtained from NIH (Reddel, Yang et al. 1989) and were maintained at the University of Utah. A549 were our working stock originally obtained from ATCC. RAW 264.7 were from ATCC. Primary rat macrophages were obtained by bronchoalveolar lavage during necropsy of Sprague-Dawley rats under a protocol approved by the University of Utah Institutional Animal Care and Use Committee. A cannula was inserted into the airway and ice cold phosphate buffered saline was repeatedly injected and withdrawn from the lung. The cells were separated from the recovered fluid by centrifugation and then resuspended and seeded into cell culture plates.

Cell Culture Media

BEAS-2B cells were grown in either LHC-9 or KGM. Lechner and Laveck media, LHC-9 was prepared from LHC-8 media (Biosource #141–500, Invitrogen, Carlsbad, CA) by adding retinoic acid (33 nM) and epinephrine (2.75 μM). KGM media was prepared from KBM basal media CC-3101 with the CC-4131 bullet kit additives (Cambrex/Lonza, East Rutherford, NJ).

A549 were grown in DMEM/F12 mixture (Gibco #11330, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Invitrogen) added. RAW 264.7 cells were grown in DMEM media (ATCC #20–2002) with 10% fetal bovine serum (Invitrogen) added. Rat macrophages were grown in RPMI 1640 media (Gibco #11835–030) with 1% penicillin/streptomycin added.

Cell Culture Conditions

All cells were maintained in T-75 culture flasks at 37 °C and 6% CO_2, and seeded in 48-well plates for treatment. Media was replaced every second day, and cells were passaged when approximately 85% confluent. BEAS-2B (stocks A and U) were grown in culture flasks (Costar, Thermo Fisher Scientific, Waltham, MA) and multi-well plates precoated with LHC-basal media containing bovine serum albumin (100 μg/mL), collagen (30 μg/mL), and fibronectin (10 μg/mL) and were passaged by washing with Ca- and Mg-free phosphate buffered saline (PBS) and dislodging with 0.05% trypsin. BEAS-2B (stock E) were grown in uncoated flasks, and residual trypsin was removed from the passaged cells by adding soybean trypsin inhibitor, centrifuging, decanting, and resuspending in media. The standard protocol for the BEAS-2B (E) cells was based on the method used by US EPA and involved seeding at 35,000/cm^2 and treating with the agonists three days after seeding, which resulted in cell confluence. The standard protocol for the A and U BEAS-2B cells did not include the trypsin inhibitor step, cell were seeded at 20,000/cm^2 and treated after one day when the cells were still proliferating. RAW264.7 cells and primary macrophages were seeded at 35,000/cm^2 and treated one day after seeding. Primary macrophages harvested by bronchioalveolar lavage, centrifuged at 200 × g, resuspended in fresh media, seeded in the multiwell plates at 80,000 cm^2, aspirated after 6 hr to remove non-attached cells, and incubated in fresh media overnight. Cell densities at treatment were similar for the two macrophage-related types since RAW 264.7 double in less than 24 hours but primary macrophages do not divide. Table 4 summarizes the culture and passaging conditions for the different cell models.

PM Types

The experiments were conducted with a fixed set of particle samples which included soil-derived dust (S), soluble vanadium (V), coal fly ash (C), diesel particulate (D), lipopolysaccharide (L), titanium dioxide (T), and kaolin (K). The $PM_{2.5}$-enriched soil dust and coal fly ash were prepared from the authors' field samples by mechanical tumbling to resuspend the particles followed by aerodynamic separation of the dust cloud using a cascade impactor (Veranth, Smith et al. 2000). Treatment S is identical with sample DD in (Veranth, Reilly et al. 2004) and sample 18 in (Veranth, Moss et al. 2006). Composition is dominated by calcium (19.3%) and organic carbon (8.1%). The coal fly ash was derived from a commercial power plant burning Utah bituminous coal and is identical with sample 29 in (Veranth, Moss et al. 2006) and was also used in (Smith, Veranth et al. 2006). Composition of the coal fly ash is dominated by silicon (12.4%), aluminum (4.9%), calcium (5.6%), iron (4.2%), elemental carbon (3.2%), and organic carbon (1.1%). The soluble vanadium was prepared from $VOSO_4$ (Catalog #22354, Alfa Aesar, Ward Hill, MA). The diesel particles were tailpipe particles collected by the authors from a high emitting (black smoker) on-road light duty truck. The LPS was *Pseudomonas aeruginosa* lipopolysaccharide (Catalog # L9143, Sigma, St Louis, MO) and we previously compared potency of different bacterial LPS formulations in BEAS-2B cells (Veranth, Kaser et al. 2007). The titanium dioxide was 1.0 –2.0 μm aerodynamic particle size powder (Alfa Aesar, stock #43047). The kaolin was commercial clay purchased from a pottery material supplier (Capitol Ceramics, Salt Lake City, UT). Table 2 lists the nitrogen adsorption surface area measurements. Detailed elemental and carbon fraction composition data for D appears in (Veranth, Reilly et al. 2004) and for C appears in (Smith, Veranth et al. 2006), but trace elements were not measured in D, T, or K.

Particle Exposure

To avoid biological contamination of the cell culture media the particle samples were sterilized with 70% ethanol (approximately 50 μL alcohol for 1– 10 mg of particles) and vacuum dried at low temperature. We have previously documented that this procedure does not significantly change semivolatile organic concentration (Veranth, Moss et al. 2006) as any material dissolved by the ethanol is presumably redeposited on the particles during drying. For maximum uniformity of treatments between experiments, all materials were weighed, sterilized, suspended in PBS at a concentration of 800 μg/mL, split into multiple test tubes, and stored frozen at −20 °C. As needed, the concentrated treatment stocks were thawed, dispersed by 5 minutes of agitation in an ultrasonic bath, and vortexed immediately before use. Previous scanning electron microscopy verified that this procedure disperses clusters but preserves the primary particles (Veranth, Reilly et al. 2004). Primary particles of soil-derived dust and of coal fly ash that have been dispersed by sonicating and vortexing are shown in Figure 1. The images were obtained from a previous study but the starting samples were from the same source as S and C and similar resuspension techniques were used. The soil sample shows irregular particles resulting from weathering and mechanical attrition while the coal fly ash sample shows the characteristic spherical particles formed from molten ash.

To allow using identical agonist treatment in different cell culture media the 800 μg/mL stock of particle treatments and the soluble LPS stock were diluted in PBS to 10X the highest treatment concentration indicated in Table 2 and then aliquoted to prepare the maximum concentration suspension (1X) in each cell culture media. At the time of agonist treatment the media from multiwell plate cell seeding was aspirated from the wells and immediately replaced with media containing the treatment suspension. The various agonist concentrations used for the dose-response experiments were prepared by 2/decade serial dilution from the 1X stock. After agonist treatment the multiwell plates were returned to the incubator overnight.

ELISA Assay

The media was harvested from the wells 20–22 hrs after particle treatment. The concentration of IL-6 in the cell culture media was determined using a sandwich ELISA assay. For IL-6, we used plates prepared with anti-human IL-6, biotin-conjugated anti-human IL-6, and avidin-horseradish peroxidase from eBioscience (San Diego CA). Absorbances were read on a Molecular Devices Model SpectraMax 250 plate reader with Softmax Pro and the concentrations were expressed as pg/mL based on the standard curve obtained for each plate. All IL-6 values were quantified using an R&D Systems recombinant human IL-6 standard.

Cytotoxicity Assay

To determine if cell death was the cause of low cytokine response, cell viability was assessed on all culture wells. Immediately after harvesting the media for cytokine analysis we refed the cells with culture media containing 4% of the WST-8 reagent (Cell Counting Kit CCK-8, Dojindo Laboratories, Gaithersberg MD) and incubated the cells for 2 h before reading absorbance. This assay measures metabolic activity by mitochondrial reduction of the reagent to form a colored product, and results are reported relative to blank-corrected untreated control.

Statistics

Multiple comparisons to control used Dunnett's test in the JMP statistical package (SAS Institute) to determine the statistical significance ($P < 0.05$). Factorial experiments used Student's t-test and multivariate modeling in JMP.

Results

Factorial Experiment

We used a three-variable, two-level, full-factorial experimental design to test for the effects of BEAS-2B cell subtype, cell culture media, and time from seeding of cells to particle treatment on the IL-6 protein secretion responses to soil-derived dust, soluble vanadium, and LPS. The factorial study indicated that all three variables reached statistical significance for at least one of the tested treatments. The effect of cell culture media on IL-6 achieved $p<0.01$ significance for both the soil dust and vanadium treatments. Increasing the time from cell seeding to particle treatment from 1 to 3 days resulted in a higher number of cells which increased absolute levels of cytokine in the media, but this variable showed a statistically significant difference in fold increase over control only for vanadium treatment. The subsequent dose-response experiments focused on the effects of cell type and media composition.

Viability

The treatment concentration ranges for the antagonists were set to achieve a robust cytokine response at the maximum concentration while minimizing confounding of the results by cell death during the treatment period. In general, the cell viability after agonist treatment was greater than 75% of control. The highest concentration of soluble vanadium resulted in less than 50% of control viability with all cell models, and the highest concentration of kaolin caused cell death in some cell models.

Matrix of treatments and cell models

Figure 2 compares the responses of different cell models to the set of treatments. The BEAS-2B (A) cells in LHC-9 media (panel a) show a response to the soil dust and LPS, a non-significant response to kaolin, and no response to vanadium. In contrast, the BEAS-2B (E) cells in KGM media (panel b) show a strong response to vanadium and soil dust but no response to LPS. Addition of serum to the BEAS-2B (U) cells in LHC-9 media (panel c) greatly raises the control level of IL-6 secretion, reducing response expressed as fold over control, but the BEAS-2B cells still have a statistically significant response to soil dust and LPS. A549 cells grown in DMEM/F12 media with serum show significant responses to soil dust and kaolin and no response to vanadium (panel d). The mouse macrophage line RAW 264.7 and the primary rat macrophages (panels e and f) both show strong response to LPS and little response to the other agonists. The data in Figure 2 are merged from three independent experiments. Data shown are for the maximum concentration (coded as 1.0). Figure 3 shows concentration-response results from a single experiment with BEAS-2B (A) cells in LHC-9 media and illustrates typical results observed in the matrix of treatment and cell models. The expected increase in cytokine secretion with increasing treatment concentration was observed for combinations of treatments and cell models that showed a strong response but not for combinations where all IL-6 responses were near control level. The full dose-response experiment was conducted on all combinations of cells and treatments, N=1 well/condition on a single culture plate, and replicated with three independent passages of cells.

Effect of culture media composition

The observed rank order of potency of different particle agonists can be changed by the choice of cell media. Further, the potency of a particular treatment expressed as fold over control differs between media because the control level of IL-6 secreted by BEAS-2B cells is consistently lower in KGM media compared to LHC-9 media.

Figure 4 shows that the IL-6 secretion of the cells could be titrated by using mixtures of LHC-9 and KGM cell culture media for both untreated control cells and for cells treated with 31 μg/

cm^2 of the S soil dust particles. The data in Figure 4 are the merged results from multiple experiments using BEAS-2B from the A, E, and U working stocks and the statistically significance trend suggests that any differences between the BEAS-2B stocks are small compared to the effect of media. The IL-6 concentration is highest for cells grown in 100% LHC-9 media, but the control (no PM treatment) level of IL-6 is lowest in 100% KGM media, and this effects results reported as fold increase over control.

The IL-6 response of BEAS-2B cells can also be titrated by varying the amount of serum. Figure 5 shows the effect of zero to 10% newborn calf serum added to the KGM media. Increasing serum results in increased IL-6 for both the control and soil dust treated conditions. The relative effect of serum on the no-particle-treatment cells versus soil dust treated cells again affects results expressed as fold increase. With zero serum the IL-6 response to soil dust was 5.7 fold over control but with 10 % serum the control and treatment IL-6 levels were equal.

Effect of Culture Well Size

Figure 6 shows the results of growing BEAS-2B in plasticware ranging from a 6-well plate (9.6 cm^2/well) to a 96-well plate (0.32 cm^2/well). For both the untreated control and for the TNF-α treated positive there was a trend toward increasing IL-6 with decreasing well size. The data are merged from two replicate experiments that both used KGM and LHC-9 media indicating that the well size effect is independent of the media composition effects. Again, the change in control level relative to treatment level affects the data reported as fold increase over control with TNF-α response going from 11 fold for 6-well plates to 3 fold for 96-well plates even though the highest absolute IL-6 concentration was measured with the 96-well plate.

Since cell seeding density, media depth, and agonist treatment per area of the culture well were all held constant these observations suggest that edge effects, such as the meniscus formed at the liquid-wall interface, are the cause of the change in IL-6 response. Raabe, Moyer et al. (Raabe, Moyer et al. 2004) reported that the meniscus formed during cell refeeding affected cell growth and survival resulting in an annular zone of cell death, and that this effect had more impact in smaller wells.

Reversibility of the Media Effect

The effect of media on the IL-6 response was reversible when media was changed between LHC-9 and KGM on successive passages. Figure 7 shows the results of four consecutive passages with cells grown and treated in alternating KGM and LHC-9 media. The responses to both LPS and to 31 μg/cm^2 of the S soil dust particles were significantly greater in LHC-9 versus KGM, consistent with the results in Figures 2 and 4. Data shown are for BEAS-2B (E) cells which have historically been grown in KGM media. A similar pattern of IL-6 response to LPS and soil dust was also seen in BEAS-2B (A) cells grown in alternating KGM and LHC-9 media (data not shown).

Discussion

This study showed that the IL-6 secretion response of lung-derived cells exposed to a standard set of environmentally relevant agonists varies with the cell type, the media, and the cell culture conditions. Differences between distinct cell types, for example bronchial epithelial versus macrophage-derived cells, are expected and have been previously documented (Hetland, Cassee et al. 2004; Riley, Boesewetter et al. 2005; Schulz, Farkas et al. 2002; Xu, Hoet et al. 2002). This study extends the comparisons to a larger matrix of cells and agonists, and further demonstrates that the cell culture protocol details can modify the response observed with a given cell type. In contrast to primary cells from varying donors, immortalized cell lines are usually considered stable and well defined. However the results presented here suggest that

manipulation of seemingly innocuous details, such as substituting a different commercial media, can have major effects on the cell phenotype.

Documenting the effects of cell culture protocol differences on *in vitro* responses to environmental particles supports the development of validated non-animal methods for toxicology. Further, the ability to manipulate cell phenotype *in vitro* has the potential to be a novel technique for elucidating the mechanisms of particle toxicology.

The observation that choice of commercial media formulation can alter the sensitivity of BEAS-2B cells to metals and LPS is an informative result when comparing and integrating studies in the environmental particle toxicology literature since investigators have used various protocols in published studies. A large number of studies using power plant residual oil fly ash, soluble extracts of filters from areas near metallurgical plants, and synthetic surrogates containing soluble vanadium and nickel have used BEAS-2B (E) cells grown in KGM (Carter, Ghio et al. 1997; Frampton, Ghio et al. 1999; Ghio, Carter et al. 1999; Kennedy, Ghio et al. 1998; Quay, Reed et al. 1998; Samet, Graves et al. 1998; Veronesi, de Haar et al. 2002). The cytokine secretion response to soluble vanadium treatment occurs reproducibly in BEAS-2B (E) cells grown in KGM media in accordance with the protocol used provided to the authors by the US EPA laboratory. However, the large fold increase in IL-6 in response to vanadium treatment is attenuated or absent in other cell models, including A549, rodent macrophages, and even in precisely the same BEAS-2B (E) cells grown in LHC-9 media. Likewise, BEAS-2B cells cultured in KGM are essentially nonresponsive to LPS but become sensitive when cultured in LHC-9. Although the factorial experiment found statistically significant differences between the three working stocks of BEAS-2B cells, the subsequent experiments showed that the A, E and U stocks of cells were reasonably similar and that the media composition was a more important variable. Having a sensitive *in vitro* model has contributed to recent advances in understanding the biochemical mechanisms associated with transition metals in atmospheric particulate (Ghio, Carter et al. 1999; Quay, Reed et al. 1998; Samet, Graves et al. 1998; Samet, Stonehuerner et al. 1997), but given the dependence of results on protocol details, toxicologists may question whether any one *in vitro* cell culture model is an appropriate surrogate for human responses or whether comparison of multiple models may be needed for *in vitro* studies to elucidate the true biochemical pathways linking air pollution to human disease.

The differences between cell culture models in their sensitivity to metals and other agonists reported here suggests a strength of *in vitro* studies of environmental agonists in that isolated cells can be manipulated in many ways to enhance response. A sensitive *in vitro* model that responds with reproducible, statistically significant changes is needed before undertaking mechanistic studies using receptor agonists or molecular biology techniques. However caution is needed as the mechanisms observed may be unique to a specific cell model. Our results show that different cell culture models are suited for studying the same proinflammatory response to different irritants. These models are useful for understanding the mechanisms of these responses *in vivo* but clearly these mechanisms cannot be assumed to be universally present in all lung cells, *in vitro* and *in vivo*.

Studies using *in vitro* assays to screen or compare different particle types may be misleading if the ranking depends on a single cell model, and several papers have reported poor correlation between *in vitro* and *in vivo* potency (Sayes, Reed et al. 2007; Seagrave, Mauderly et al. 2003). A study with stone dust particles showed a correlation between *in vitro* cytokine responses and neutrophil count in the BAL fluid of rats, but different rankings of *in vitro* potency were found with type 2 cells and alveolar macrophages (Becher, Hetland et al. 2001). The cell model differences reported here help explain the observed poor correlation. Our results suggest that future development of *in vitro* methods for studying environmental

particles should consider the combined data from multiple cell models to improve predictive power.

The mechanisms responsible for the observed differences between disparate lots of BEAS-2B cells available in different laboratories and between the same BEAS-2B cells grown under different conditions remain unknown, but we can speculate on plausible reasons. The original BEAS-2B cells were a mixed population, not a clone derived from a single cell (Reddel, Yang et al. 1989) so differences between the three BEAS-2B sources tested could be the result of culture conditions selectively enriching specific cell types in the mixed population. This effect can be minimized by maintaining archive stocks of early passages. A related issue of increasing concern is authentication of cell lines used for research due to possible contamination by another cell type (Masters, Thomson et al. 2001). However, the demonstration that the sensitivity of BEAS-2B cells to agonists can be reversibly modified within one passage suggests that the sensitivity to an environmentally relevant treatment is due either to cofactors in the media or changes in receptor populations, but does not involve selecting genetically different subpopulations of cells.

The method of cell passaging, the exact growth factors in the media, and the changes that occur as cells become confluent and start to differentiate are all likely to affect both the populations of receptors on the cell surface and the intracellular level of many signal transduction intermediates. The immediate changes in response that occur with a media change could be due to a media component that is an essential coactivator for receptor response to the particles, or it could be the result of a media factor that induces changes in the expression of different response elements in the cell and on the cell membrane. KGM and LHC-9 media appear to differ in epidermal growth factor, retinoic acid, and 3, 3′5-triiodothyronine. Unfortunately, we were unable to make a quantitative comparison of all potentially significant media factors since both KGM and LHC-9 are proprietary formulations and both contain incompletely defined additives, such as bovine pituitary extract.

Changes in cell-surface populations of specific receptors may explain the variable sensitivity to the agonists. Recently our research group showed that both cytotoxicity and induction of IL-6 in response to the TRPV-1 receptor agonist nonivamide was modulated by short-term drug pretreatments through a mechanism involving translocation of receptors from the endoplasmic reticulum to cell surface (Johansen, Reilly et al. 2006). Alternatively, the cell response could depend on the availability of receptor cofactors. For example, the response of Toll-like receptors to LPS depends on the presence of soluble CD14 protein (Schulz, Farkas et al. 2002). Either changes in subcellular protein localization or the concentration of essential cofactors could account for rapid and reversible changes in the responses of cells to various environmental agonists.

The ability to manipulate cell phenotype by varying culture conditions suggests a potentially powerful approach for elucidating mechanisms of cell-particle interaction by exploiting the versatility of *in vitro* cell models. A major unanswered question in the toxicology of inhaled ambient particles is the precise identification of the proximal molecular receptors in the airway cells that ultimately trigger the physiological responses. Systematic studies comparing protein expression in cell lines where response is modified by manipulating culture conditions would be a novel approach that may lead to identification of receptor and signal transduction targets involved in responses to particles. The precise formulations of the commercial media used in this study are proprietary, so it may be worthwhile to study the effect of media composition by supplementing a fully defined basal media with specific factors. Measurements of the levels of key cell membrane receptors and signal transduction proteins between different cell subpopulations or between the same cell source grown in different media are a logical extension of this work.

Conclusions

This study showed that the IL-6 response of different *in vitro* cell models varies with both the cell type and the cell culture conditions. BEAS-2B cells grown in LHC-9 media are sensitive to the soil-derived dust and LPS, but this response is attenuated when the cells are grown in KGM media, and these changes are reversible over multiple media changes. Observations of this type suggest that published reports of specific particles being either potent or benign for induction of cytokine signaling need to be evaluated with caution. However, the fact that rank order of particle potency varies with cell type and cell culture conditions does not detract from the utility of *in vitro* models for studying mechanisms such as receptor activation, signal transduction, and gene regulation. Cell culture responses to particles can be manipulated, as demonstrated by the effects of media composition, so there is the potential to exploit these reproducible differences between *in vitro* cell models to elucidate toxicological mechanisms.

Acknowledgements

Training on the EPA cell culture protocol and a sample of BEAS-2B cells were generously provided by Robert Devlin and Lisa Dailey at US EPA. Bellina Veronesi, Andy Ghio, Jan Powell, Katherine Squibb, Ann Aust, and Kevin Smith provided information regarding protocols for particle studies. Technical help for this study was provided by Cassandra Deering, Diane Lanza, Tyler Moss, Mike Koch, and Martha Veranth. Gauri Seshadri produced the SEM images as part of previous project. Financial support for this work was provided by NIEHS K25 ES0011281, US EPA STAR RD 8317230, and SCERP A-04-04. This manuscript has not been submitted for agency review. Study design, data analysis, and opinions expressed are the responsibility of the authors.

References

Alfaro-Moreno E, Martinez L, Garcia-Cuellar C, Bonner JC, Murray JC, Rosas I, Rosales SPdL, Osornio-Vargas Á. Biologic effects induced in vitro by PM10 from three different zones of Mexico City. Environmental Health Perspectives 2002;112(7):715–720. [PubMed: 12117649]

Allen, CB. In vitro models for lung toxicology, in Toxicology of the Lung. 4. Gardner, DE., editor. Boca Raton: Taylor and Francis; 2006.

Aust, AE.; Ball, JC.; Hu, A.; Lighty, JS.; Smith, KR.; Straccia, AM.; Veranth, JM.; Young, WC. Research Report 110. Health Effects Institute; 2002. Particle characteristics responsible for effects on human lung epithelial cells.

Becher R, Hetland RB, Refsnes M, Dahl JE, Dahlman HJ, Schwarze PE. Rat lung inflammatory responses after *in vivo* and *in vitro* exposure to various stone particles. Inhalation Toxicology 2001;13(9):789–805. [PubMed: 11498806]

Becker S, Dailey LA, Soukup JM, Grambow SC, Devlin RB, Huang YC. Seasonal variations in air pollution particle-induced inflammatory mediator release and oxidative stress. Environmental Health Perspectives 2005;113(8):1032–1038. [PubMed: 16079075]

Carter JD, Ghio AJ, Samet JM, Devlin RB. Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. Toxicol Appl Pharmacol 1997;146(2):180–188. [PubMed: 9344885]

Chung KF. Cytokines in chronic obstructive pulmonary disease. Eur Respir J 2001;18(Suppl 34):50s–59s.

Costa DL, Dreher KL. Bioavailable transition metals in particulate matter mediate cardiopulmonary injury in healthy and compromised animal models. Environmental Health Perspectives 1997;105 (Suppl 5):1053–1060. [PubMed: 9400700]

Donaldson K, Tran CL. Inflammation caused by particles and fibers. Inhalation Toxicology 2002;14:5–27. [PubMed: 12122558]

Dybdahl M, Risom L, Bornholdt J, Autrup H, Loft S, Wallin H. Inflammatory and genotoxic effects of diesel particles in vitro and in vivo. Mutation Research 2004;562(1–2):119–131. [PubMed: 15279835]

Frampton MW. Inflammation and airborne particles. Clin Occup Environ Med 2006;5(4):797–815. [PubMed: 17110293]

Frampton MW, Ghio AJ, Samet JM, Carson JL, Carter JD, Devlin RB. Effects of aqueous extracts of PM10 filters from the Utah Valley on human airway epithelial cells. American Journal of Physiology, Lung Cellular and Molecular Physiology 1999;21:L960–L967.

Fubini B, Aust AE, Bolton RE, Borm PJ, Bruch J, Ciapett G, Donaldson K, Elias Z, Gold J, Jaurand MC, Kane AB, Lison D, Muhle H. Non-Animal Tests for Evaluating the Toxicity of Solid Xenobiotics. Alternatives to Laboratory Animals 1998;26:579–615.

Gao N, Keane M, Ong T, Wallace W. Effects of simulated pulmonary surfactant on the cytotoxicity and DNA-damaging activity of respirable quartz and kaolin. J Toxicol Environ Health A 2000;60(3): 153–167. [PubMed: 10884165]

Ghio AJ, Carter JD, Dailey LA, Devlin RB, Samet JM. Respiratory epithelial cells demonstrate lactoferritin receptors that increase after metal exposure. Am J Physiol Lung Cell Mol Physiol 1999;276(6 Pt 1):L933–940.

HEI. Understanding the Health Effects of Components of the Particulate Matter Mix: Progress and Next Steps. Health Effects Institute, HEI Perspectives; 2002 April 2002.

Hetland RB, Cassee FR, Refsnes M, Schwarze PE, Lag M, Boere AJF, Dybing E. Release of inflammatory cytokines, cell toxicity, and apoptosis in epithelial lung cells after exposure to ambient air particles of different size fractions. Toxicology in Vitro 2004;18:203–212. [PubMed: 14757111]

Hetland RB, Refsnes M, Myran T, Johansen BV, Uthus N, Schwartz PE. Mineral and/or metal content as critical determinants of particle-induced release of IL-6 and IL-8 from A549 cells. J Toxicol Environ Health A 2000;60(1):47–65. [PubMed: 10832617]

Johansen ME, Reilly CA, Yost GS. TRPV1 antagonists elevate cell surface populations of receptor protein and exacerbate TRPV1-mediated toxicities in human lung epithelial cells. Toxicological Sciences 2006;89(1):278–286. [PubMed: 16120755]

Kanda T, Takahashi T. Interleukin-6 and cardiovascular disease. Japanese Heart Journal 2004;42(2):183–193. [PubMed: 15090695]

Kennedy T, Ghio AJ, Reed W, Samet J, Zagorski J, Quay J, Carter J, Dailey L, Hoidal JR, Devlin RB. Copper-dependent inflammation and nuclear factor -kB activation by particulate air pollution. Am J Respir Cell Mol Biol 1998;19:1–14. [PubMed: 9651173]

Masters J, Thomson J, Daly-Burns B, Reid Y, Dirks W, Packer P, Toji L, Ohno T, Tanabe H, Arlett C, Kelland L, Harrison M, Virmani A, Ward T, Ayres K, Debenham P. Short tandem repeat profiling provides an international reference standard for human cell lines. Proc Natl Acad Sci U S A 2001;98 (14):8012–8017. [PubMed: 11416159]

Nurkiewicz TR, Porter DW, Barger M, Castranova V, Boegehold MA. Particulate matter exposure impairs systematic microvacular endothelium-dependent dialation. Environmental Health Perspectives 2004;112(13):1299–1306. [PubMed: 15345343]

Oberdorster G, AMaynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B, Kreyling W, Lai D, Olin S, Monteiro-Riviere N, Warhei DYH. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Particle and Fibre Toxicology 2006;2(8)

Øvrevik J, Myran T, Refsnes M, Lag M, Becher R, Hetland RB, Schwarze PE. Mineral particles of varying composition induce differential chemokine release from epithelial lung cells: Importance of physico-chemical characteristics. Annals of Occupational Hygine Advance. 2005

Pascal LE, Tessier DM. Cytoxtoxicity of chromium and manganese to lung epithelial cells in vitro. Toxicology Letters 2004;147:143–151. [PubMed: 14757318]

Prichard RJ, Ghio AJ, Lehmann JR, Witsett DW, Tepper JS, Park P, Gilmour MI, Dreher KL, Costa DL. Oxidant generation and lung injury after particulate air pollutant exposure increase with the concentrations of associated metals. Inhalation Toxicology 1996;8(5):457–477.

Pulskamp K, Diabate S, Krug H. Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. Toxicol Lett 2007;168(1):58–74. [PubMed: 17141434]

Quay JL, Reed W, Samet J, Devlin RB. Air pollution particles induce IL-6 gene expression in human airway epithelial cells via NF-kappaB activation. Am J Respir Cell Mol Biol 1998;19(1):98–106. [PubMed: 9651185]

Raabe, H.; Moyer, G.; Mun, G.; Clear, M.; Harbell, JW. Induction of a zone of cell death in multi-well plates by refeeding. Society of Toxicology Annual Meeting; Baltimore, MD, Poster. 2004.

Reddel, RR.; Yang, K.; Rhim, JS.; Brash, D.; Su, RT.; Lechner, JF.; Gerwin, BI.; Harris, CC.; Amstad, P. Immortalized human bronchial epithelial mesothelial cell lines. USA: Department of Health and Human Services; 1989.

Riley MR, Boesewetter DE, Turner RA, Kim AM, Collier JM, Hamilton A. Comparision of the sensitivity of thre lung derived cell line to metals from combustion derived particulate matter. Toxicology in Vitro 2005;19(411–419)

Romberger DJ, Bodlack V, Von Essen SG, Mathisen T, Wyatt TA. Hog barn dust extract stimulates IL-8 and IL-6 release in human bronchial epithelial cells via PKC activation. J Appl Physiol 2002;93:289–296. [PubMed: 12070216]

Salonen RO, Halinen AI, Pennanen AS, Hirovenen MR, Sillanpaa M, Hillamo R, Shi T, Borm PJ, Sandell E, Koskentalo T, Aarnio P. Chemical and in vitro toxicologic characterization of wintertime and springtime urban-air particles with an aerodynamic diameter below 10 microm in Helsinki. Scand J Work Environmental Health 2004;30(Suppl 2):80–90.

Samet JM, Graves LM, Quay J, Dailey LA, Devlin RB, Ghio AJ, Wu W, Bromberg PA, Reed W. Activation of MAPKs in human bronchial epithelial cells exposed to metals. Am J Physiol (Lung Cell Mol Physiol) 1998;275:L551–L558.

Samet JM, Stonehuerner J, Reed W, Devlin RB, Dailey LA, Kennedy TP, Bromberg PA, Ghio AJ. Disruption of protein tyrosine phosphate homeostasis in bronchial epithelial cells exposed to oil fly ash. Am J Physiol Lung Cell Mol Physiol 1997;272:L426–L432.

Sayes CM, Reed KL, Warheit DB. Assessing toxicity of fine and nanoparticles: Comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicological Sciences. 2007Epub ahead of print

Schulz C, Farkas L, Wolf K, Krätzel K, Eissner G, Pfeifer M. Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). Scan J Immunol 2002;56:294–302.

Seagrave J, Mauderly JL, Seilkop SK. In vitro relative toxicity screening of combined particulate and semivolatile organic fractions of gasoline and diesel engine emissions. J Toxicol Environ Health A 2003;66(12):1113–1132. [PubMed: 12854533]

Smith KR, Aust AE. Mobilization of iron from urban particulates leads to generation of reactive oxygen species *in vitro* and induction of ferritin synthesis in human lung epithelial cells. Chemical Research in Toxicology 1997;10(7):828–834. [PubMed: 9250418]

Smith KR, Veranth JM, Kodavanti UP, Aust AE, Pinkerton KE. Acute pulmonary and systemic effects of inhaled coal fly ash in rats: comparison to ambient particles. Toxicological Sciences 2006;93(2): 390–399. [PubMed: 16840564]

Steerenberg PA, Zonnenberg JA, Dormans JA, Joon PN, Wouters IM, van Bree L, Scheepers PT, Van Loveren H. Diesel exhaust particles induced release of interleukin 6 and 8 by (primed) human bronchial epithelial cells (BEAS 2B) in vitro. Exp Lung Res 1998;24(1):85–100. [PubMed: 9457471]

Veranth JM, Kaser EG, Veranth MM, Koch M, Yost GS. Cytokine responses of human lung cells (BEAS-2B) treated with oxide micron-sized and nanoparticles compared to soil dusts. Particle and Fibre Toxicology 2007;4(2)

Veranth JM, Moss TA, Chow JC, Labban R, Nichols WK, Walton JC, Watson JG, Yost GS. Correlation of in vitro cytokine responses with the chemical composition of soil-derived particulate matter. Environmental Health Perspectives 2006;114(3):342–349.

Veranth JM, Reilly CA, Veranth MM, Moss TA, Langelier CR, Lanza DL, Yost GS. Inflammatory cytokines and cell death in BEAS-2B lung cells treated with soil dust, lipopolysaccharide, and surface-modified particles. Toxicological Sciences 2004;82:88–96. [PubMed: 15310859]

Veranth JM, Smith KR, Aust AE, Dansie SL, Griffith JB, Hu AA, Huggins ML, Lighty JS. Coal fly ash and mineral dust for toxicology and particle characterization studies: Equipment and methods for PM2.5- and PM1-enriched samples. Aerosol Science and Technology 2000;32(2):127–141.

Veronesi B, de Haar C, Lee L, Oortgiesen M. The surface charge of visible particulate matter predicts biological activation in human bronchial epithelial cells. Toxicology and Applied Pharmacology 2002;178:144–154. [PubMed: 11858730]

Xu H, Hoet PHM, Nemery B. In vitro toxicity assessment of polyvinyl chloride particles and comparisons of six cellular systems. Journal of Toxicology and Environmental Health Part A 2002;65:1141–1159. [PubMed: 12167213]

Zitnik, RJ.; Elias, JA. Interleukin-6 and the Lung, in Cytokines of the Lung. Jason, K., editor. New York: Marcel Dekker; 1993. p. 229-280.

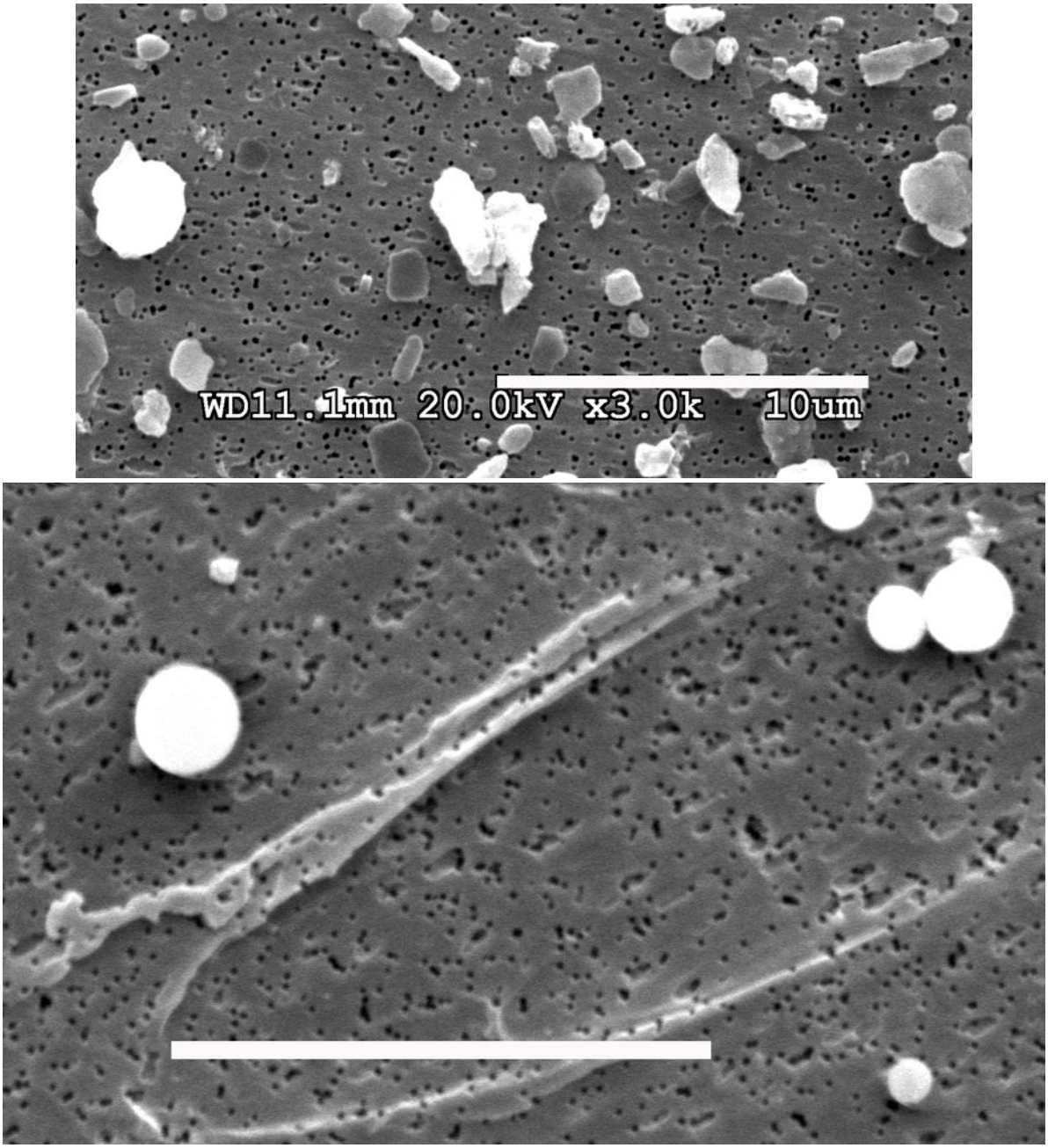

Figure 1.
SEM images of Treatment S soil-derived dust (top) and Treatment C coal fly ash (bottom) that have been resuspended by sonicating and vortexing similar to the methods used to prepare the particle samples for this study. Black marks are pores in the polycarbonate membrane. White scale bar is 10 μm in both images. Images by G. Seshadri.

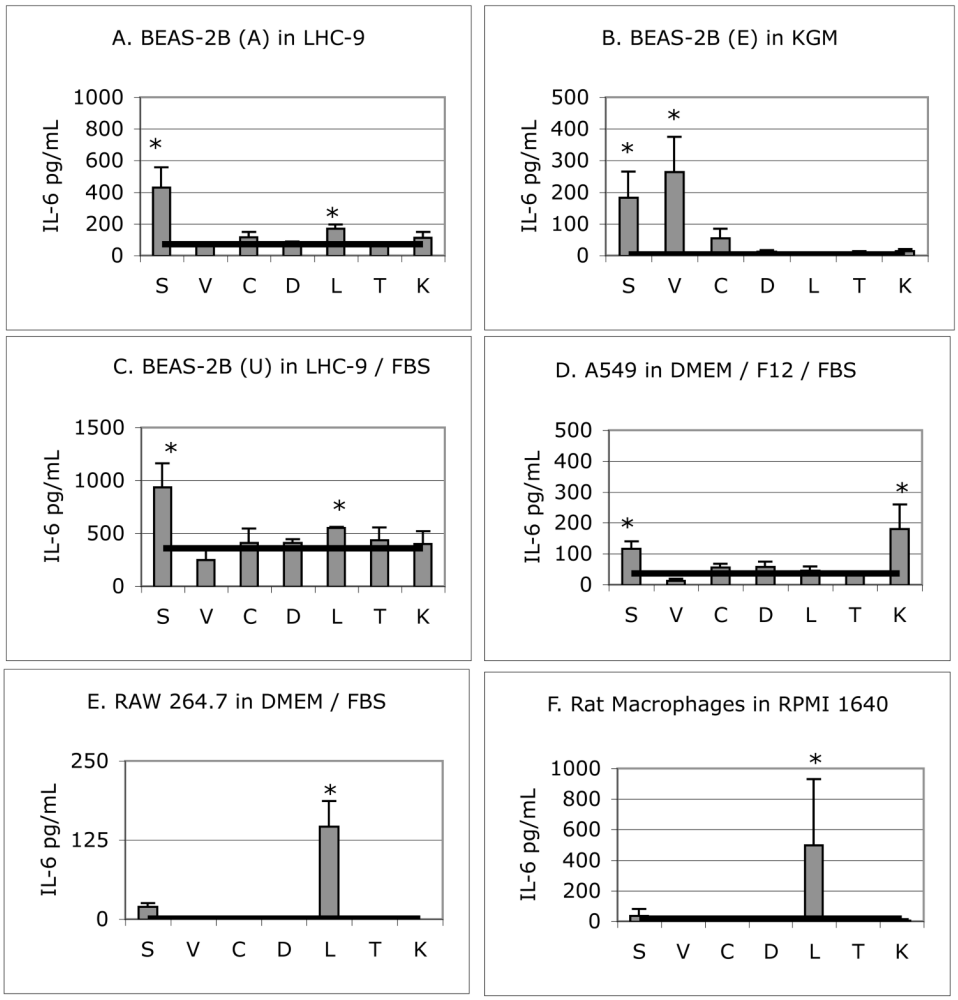

Figure 2.

The various cell models show characteristic patterns of response to the suite of identical treatments. Data are IL-6 secretion in pg/mL in response to the maximum concentration for each treatment. Y-axis varies based on response level, mean and s.d. from three independent experiments, * designates statistically greater than the control which is shown by the solid line. Treatment codes are in Table 2.

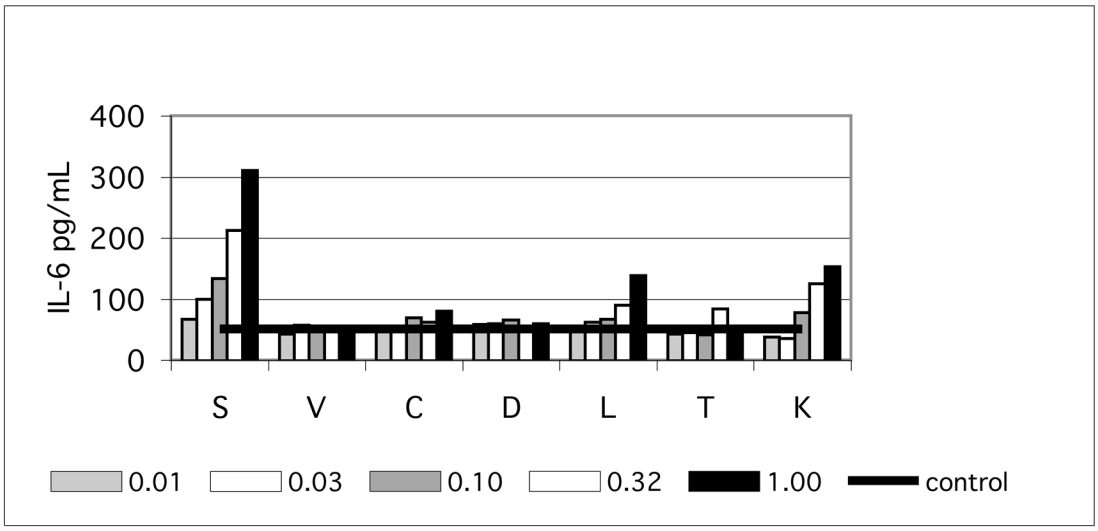

Figure 3.

Typical concentration-response data from a single cell culture plate experiment with BEAS-2B (A) cells, N=1. Treatment codes are in Table 2. Maximum concentrations: S, V, C, T, K, 100 μg/cm2; D, 32 μg/cm2, L, 1000 EU/mL. Treatments were applied as two per decade serial dilutions at 1.0, 0.31, 0.1, 0.031, and 0.01 of the maximum, plus a blank media control.

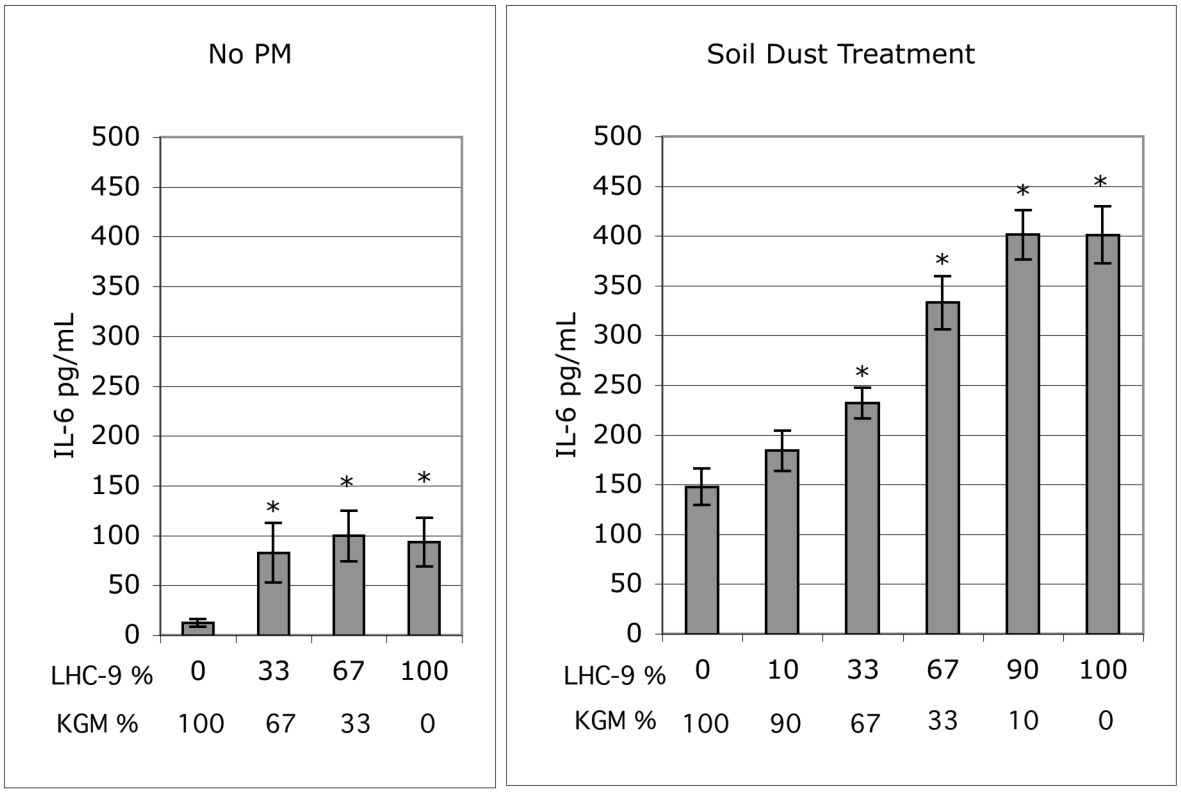

Figure 4.

The IL-6 response of BEAS-2B cells can be titrated by varying media composition from pure LHC-9 to pure KGM. Data are merged results from multiple independent experiments, mean ± standard error of the mean, N = 16 −24, * designates statistically different from the 100 % KGM media condition.

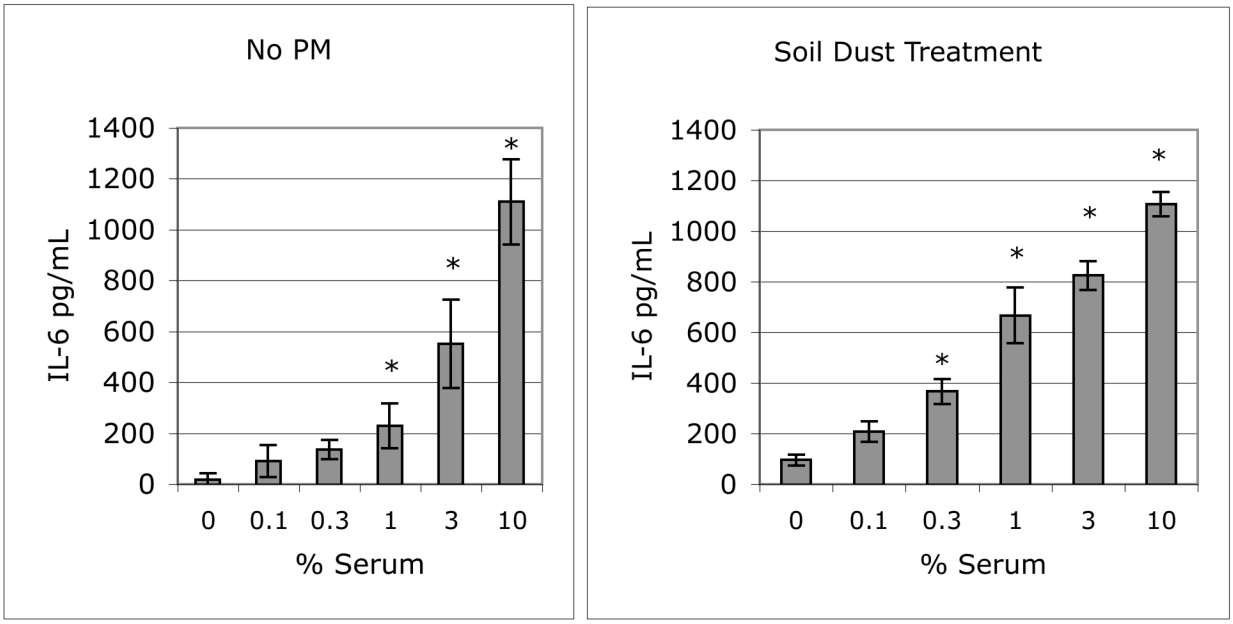

Figure 5.
The IL-6 response of BEAS-2B cells can be titrated by increasing the amount of serum added to KGM media. Mean ± s.d., N = 4, * designates statistically greater than the serum-free condition. Similar results were seen in LHC-9 media.

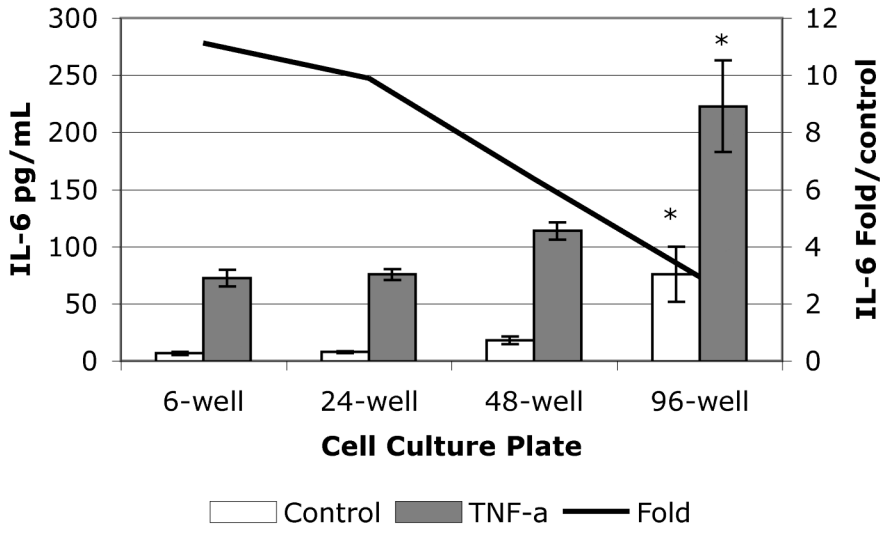

Figure 6.

Left side axis scale: The IL-6 release of BEAS-2B cells increases with decreasing well size. Data are merged from two experiments, mean ± standard error of mean, N = 12, * designates statistically different than the 48-well culture plate condition. Right side axis scale: The solid line illustrates the effect of changing control level on results expressed as fold increase.

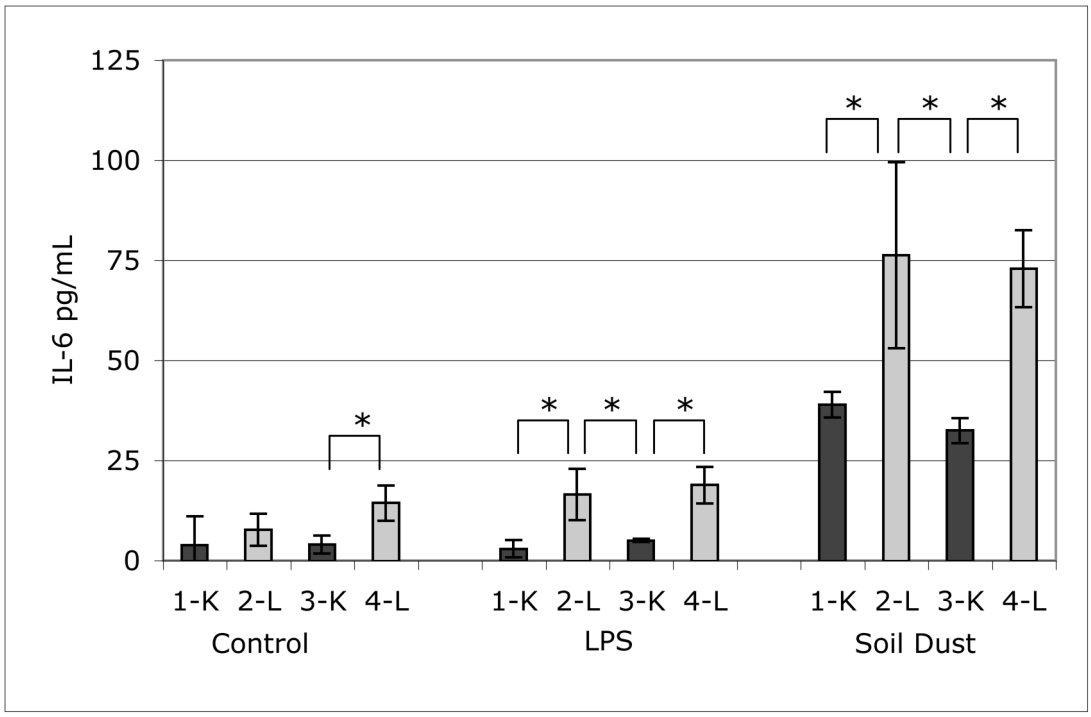

Figure 7.
The effect of media on IL-6 response is reversible as shown by an experiment series where media was changed with each passage as described in the text. Bars are coded with the passage sequence and media, KGM (K) and LHC-9 (L). Data are mean ± s.d., N=5, * denotes statistically different from the adjacent member of the sequence.

Table 1

Examples of *in vitro* studies comparing the potency of different particles to induce IL-6 release into the media

1a. Studies measuring IL-6 response to different particle types.

Cells	Particles	Conclusions	Citation
BEAS-2B	Provo urban PM Cu2+	Effects of urban PM could be replicated by copper ion.	(Kennedy, Ghio et al. 1998)
BEAS-2B	Diesel PM SiO_2 TiO_2	Rank order SiO_2 > Diesel > TiO_2.	(Steerenberg, Zonnenberg et al. 1998)
BEAS-2B	Welding fume metals	Cytotoxicity and cytokine release in response to Cr and Mn but not Ni.	(Pascal and Tessier 2004)
BEAS-2B	St. Louis urban PM Ottawa urban PM Mt. St. Helens ash Oil fly ash Coal fly ash Woodstove	IL-6 release correlated with surface charge (zeta potential) of the particles.	(Veronesi, de Haar et al. 2002)
BEAS-2B	Soil and road surface $PM_{2.5}$	Potency correlated with low volatility carbon fractions.	(Veranth, Moss et al. 2006)
BEAS-2B	Hog barn dust	Treatment to remove endotoxin did not remove potency.	(Romberger, Bodlack et al. 2002)
A549	Road tunnel dust	Coarse fraction > fine or ultrafine.	(Hetland, Cassee et al. 2004)
A549	Stone quarry mineral dusts	Quartz and amphibole > plagioclase.	(Hetland, Refsnes et al. 2000)
NHBE	Chapel Hill urban PM	Coarse fraction > fine or ultrafine. Seasonal variation with maximum in October. Correlated with Fe and Si.	(Becker, Dailey et al. 2005)
Rat lung fibroblasts	Mexico City PM	Differences between collection sites.	(Alfaro-Moreno, Martinez et al. 2002)
RAW264.7	Helsinki urban PM	Spring PM_{10} > winter PM_{10}.	(Salonen, Halinen et al. 2004)

1b. Studies comparing cytotoxicity and/or IL-6 response in multiple cell types.

Cells	Particles	Conclusions	Citation
Rat RLE-6TN Rat NR8383 A549	Combustion metals	Metals much more toxic to macrophages than to epithelial cells.	(Riley, Boesewetter et al. 2005)
A549 Primary rat type II	Road tunnel dust	Cytokine induction in A549 but not in primary rat cells.	(Hetland, Cassee et al. 2004)
BEAS-2B A549	LPS	A549 response is soluble CD14- dependent but not the BEAS-2B response.	(Schulz, Farkas et al. 2002)
A459 Rat macrophages Primary human	Polyvinyl chloride SiO_2	Rat alveolar macrophages most sensitive, A549 least sensitive for cytotoxicity.	(Xu, Hoet et al. 2002)

Table 2

Agonist Treatments

Code	Name	Source	Nominal size	BET surface m^2/g	Maximum treatment concentration
S	Desert dust	Field collection	$PM_{2.5}$-enriched	6.2	100 µg/cm^2
V	Soluble vanadium	$VOSO_4$, Alfa Aesar	Soluble		100 µg/cm^2
C	Coal fly ash	Field collection	$PM_{2.5}$-enriched	5.4	100 µg/cm^2
D	Diesel particulate matter	Field collection	Aggregates of submicron particles	Not determined	32 µg/cm^2
L	Lipopolysaccharide	Sigma	Soluble		1000 EU/mL
T	Titanium dioxide	Alfa Aesar	1–2 µm	3.5	100 µg/cm^2
K	Kaolin	Ceramic clay	< 200 mesh	24	100 µg/cm^2

Table 3

Abbreviations for Cell Types, Particle Treatments, and Doses

Cells		
BEAS-2B (A)	Human bronchial epithelial cells	ATCC # CRL-9609 Starting passage 44
BEAS-2B (E)	Human bronchial epithelial cells	US EPA Human Studies Division Passage 76–87
BEAS-2B (U)	Human bronchial epithelial cells	U of U sample. Passage 89–97
A549	Human alveolar epithelial cells	U of U sample Starting passage 84
RAW 264.7	Mouse macrophage line	ATCC # TIB-71
1° Macrophages	Rat lung lavage	Primary cells
Culture media		
LHC-9	Lechner and LaVeck medium	Invitrogen
KGM	Keratinocyte growth medium	Lonza
DMEM/F12	50% Dulbecco's modification of Eagle's medium, 50% Ham's F12 medium	Gibco
FBS	Fetal bovine serum (added to media formulations)	Invitrogen

Table 4

Culture and Passaging Conditions

Cell Model	Culture Media	Precoat	Passaging	Seeding density	Time to treatment
BEAS-2B (A & U)	LHC-9 Note 1	albumin/collegen/fibronectin	wash with PBS, 0.05% trypsin 1 min, aspirate trypsin, incubate 5 min, dislodge with media	20 K/cm^2	1 da
BEAS-2B (E)	KGM Note 1	none	aspirate old media, apply0.05% trypsin, incubate 6 min, trypsin inhibitor, dislodge, centrifuge, resuspend in media	35 K/cm^2	3 da
A549	DMEM/F12 with 10% serum	none	wash with PBS, 0.05% trypsin 1 min, incubate 3–4 min, dislodge with media	20K/cm^2	1 da
RAW 264.7	DMEM with 10% serum	none	dislodge by scraping	35 K/cm^2	1 da
Primary rat macrophages	RPMI 1640	none	centrifuge BALF, replace media, seed treatment plate, replace media after 6 hr	80 K/cm^2	1 da

Notes.

[1]The standard growth media is indicated. Cells were also grown in the alternative media and in media mixtures as indicated in the text.