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# Inflammatory response of lung cells exposed to whole, filtered, and hydrocarbon denuded diesel exhaust

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**Abstract**

In vitro studies with the organic extracts of diesel particles have suggested that hydrocarbons such as PAH may play a role in an inflammatory response, but these have been limited by the possible artifacts introduced in the particle collection and processing. In this study, we avoid these artifacts and use an activated carbon denuder to remove hydrocarbons from the exhaust stream to investigate their role in the inflammatory response. Human bronchial epithelial cells (16HBE14o) were exposed at the air–cell interface to diluted and aged exhaust from a diesel generator operated at partial and no load conditions. When particles were removed with a filter before cell exposure, exhaust gases accounted for almost half of the response compared to the whole exhaust. Removal of gas phase and a portion of the particle phase hydrocarbons with the denuder decreased the interleukin-8 (IL-8) secretion to unexposed levels.
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## 1. Introduction

Diesel exhaust particles (DEP) have been implicated in adverse health effects in humans (Lighty et al., 2000). While there is ample evidence that combustion-derived particles induce morbidity and mortality, the biological mechanisms responsible for health effects are still unclear. DEP have been shown to cause an inflammatory response in the airways of healthy volunteers (Salvi et al., 2000). Bronchial epithelial cells exposed to DEP increased production of inflammatory mediators (Boland et al., 1999). Therefore, it is assumed that inflammation is central to the known adverse health effects of DEP. The cytokine/chemokine IL-8 plays a major role in neutrophil chemotaxis and has been widely used as a biomarker of inflammation of airborne particles (Mills et al., 1999).

The specific characteristics of DEP are dependent on many different factors, such as engine loading, fuel composition, and exhaust after treatments, but the effects of particle composition, size, and morphology on health have not been fully resolved. The particles are typically composed of an elemental carbon core with trace amounts of metals and are coated with a layer of condensed hydrocarbons (Kittelson, 1998). DEP coexist with irritant exhaust gases such as $NO_2$, $SO_2$, and hydrocarbons such as aldehydes which may act separately or in a synergistic fashion with the particles (US EPA, 2002).

Some animal exposure studies and most in vitro studies use collected DEP that are subsequently suspended in an aqueous solution (Boland et al., 1999). DEP are not readily soluble in water and require a surfactant to solubilize the particles. The collection and solubilizing processes alter the size, morphology, and surface chemistry of the particles, and may result in toxicity substantially different from that of DEP in the atmosphere.

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The aerosol properties of DEP as well as the associated exhaust gases can be preserved by exposing cells directly to particle-laden gas at the air–cell interface. Several researchers used this approach to study the effects of tobacco smoke (Ritter et al., 2003), and diesel exhaust (Abe et al., 2000; Knebel et al., 2002; Cheng et al., 2003; Seagrave et al., 2005). Knebel et al. (2002) found that diesel exhaust decreased human bronchial epithelial cell viability and this effect was inversely proportional to the dilution ratio. Abe et al. (2000) observed an increase in expression of pro-inflammatory cytokines in human bronchial epithelial cells exposed to diesel exhaust. Cheng et al. (2003) investigated the effects of diesel and gasoline exhaust on human alveolar cells by measuring IL-8 release. Seagrave compared acute response to DEP at the air–cell interface of human alveolar cells with mouse inhalation exposures and found that similar estimated doses led to a similar expression of heme oxygenase-1, a possible indicator of oxidative stress (Seagrave et al., 2005).

Major research efforts have focused on separating the effects of the organic carbon fraction from that of the elemental carbon fraction of the particle, since the organic fraction often contains human carcinogens such as PAH. The organic fraction has been linked with toxicity in cell cultures. Kawasaki et al. (2001) observed that when human lung cells were exposed to diesel particles the inflammatory response was associated with organic compounds in the particles that could be extracted with benzene. Vogel et al. (2005) exposed macrophages to DEP organic extract or DEP stripped of organic components, and found that although both components elicited a response, different cytokines were increased.

The previous studies of the effects of the organic component of particles either exposed cells to collected particles that were processed beforehand or instilled particle suspensions. In contrast, we investigate freshly produced particles present as an aerosol, minimizing any artifacts arising from the collection or processing procedures. In this study, we measure the pro-inflammatory response of human lung cells when exposed at the air–cell interface to diesel exhaust. We examined the effects of the organic components in the gas phase and adsorbed onto particles by passing the exhaust through a hydrocarbon denuder that removes organic carbon from the gas phase and a portion of the hydrocarbons adsorbed on the surface of the soot. The contributions of the gas phase species are also investigated by filtering the particles from the exhaust stream.

## 2. Experimental methods

### 2.1. Cell culture

The human bronchial epithelial cell line 16HBE14o (immortalized by SV40 transformation) used for the exposure experiments was provided by Dieter Gruenert at California Pacific Medical Center Research Institute. Cells were grown in collagen-coated flasks in minimum essential medium (MEM) substituted with 10% fetal bovine serum (Gibco), glutamine, and antibiotics (100 U $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin); they were maintained in the logarithmic phase of growth. For an air–cell exposure, cells were grown in dual-chamber Transwells (Costar), which consist of an insert with a collagen-coated Teflon membrane growth surface and a well containing the culture medium. With this configuration, the upper cell surface is directly exposed to particles and gases. Both 6 and 24 well plates were used in these experiments, with 4.7 and 0.33 $cm^2$ growth area, respectively. The cells were incubated at 37 °C in a 5% $CO_2$ atmosphere until a confluent layer of cells was achieved. Immediately before the exposure, cells were prepared by supplying 1 and 0.25 ml fresh LHC medium (Biosource) to the bottom chamber, and 100 and 10 μl saline above the cells to reduce drying, for the 6 and 24 well plates, respectively. The response of the cells to the exhaust was quantified by measuring IL-8 secretion into the medium with an enzyme-linked immunosorbent assay (ELISA) kit (Biosource). Cell viability was measured with an MTT assay (Sigma).

### 2.2. Particle generation

A four-stroke direct injection diesel generator (Acme Motori model ADX-300) with a 301 $cm^2$ displacement volume operating on California #2 diesel fuel was used as an exhaust source. The generator was operated at 3600 rpm and loaded at 0 or 1.5 kW (4 kW full load).

An instrumented environmental chamber shown in Fig. 1 and described in Wagner et al. (2004) was used to age and dilute the diesel exhaust. The chamber is a room measuring 2.2 m × 2.4 m × 4.6 m with a controlled ventilation system that supplies HEPA filtered outside air into the chamber. Species in the chamber have a 20 min residence time in which the exhaust species equilibrate and the particles coagulate. The background particle concentration within the chamber was reduced by a separate HEPA filtration unit within the chamber for several hours before the beginning of an exposure. The ventilation flow rate, temperature, and relative humidity within the chamber were measured during an exposure. A recirculation pump (Fuji Electric Co. VFC063A) was used to draw air from the environmental chamber to dilute a sample of the diesel exhaust (11 l $min^{-1}$) and supply the mixture back into the chamber at a flow rate of 311 l $min^{-1}$. A fan inside the chamber enhanced the mixing of the exhaust with the dilution air within the chamber.

A TSI scanning mobility particle sizer (SMPS) Model 3071 and TSI condensation particle counter (CPC) Model 3025 were used to measure the particle size distribution within the chamber. A Horiba portable gas analyzer (Model PG-250) was used to measure the concentration of $NO_x$, CO, and $SO_2$ inside the chamber. At the start of an exposure the recirculation pump and diesel generator were turned on and operated for at least 1 h to stabilize the particle concentration within the chamber. Steady state

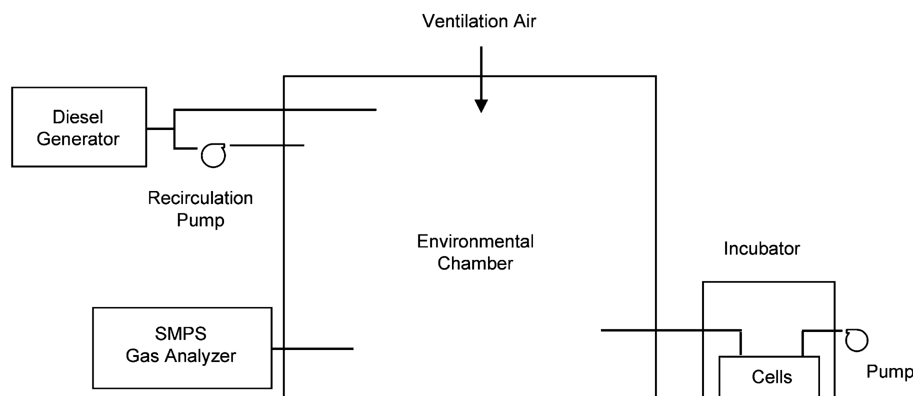


Fig. 1. Schematic of diesel generator, recirculation system, environmental chamber, instrumentation, and exposure enclosure.

is achieved when the particle size distribution in the chamber, as monitored by the SMPS, varies by 10% or less.

### 2.3. Cell exposure

The cells were contained within a sealed enclosure inside an incubator held at 37 °C. A sample from the environmental chamber was drawn through the enclosure at a constant flow rate, exposing the cells to the diluted exhaust. A control group of transwells was placed in another incubator during the exposure. The enclosure containing the transwell plates is a stainless steel box ($7.6 \times 14 \times 21.6\ cm^3$) that is sealed from the surroundings. Chamber air supplied by the sampling line was drawn in one end of the container at $1.3\ l\ min^{-1}$ by a pump (Thomas Industries 107CA14) controlled by a needle valve and monitored with a rotameter (Matheson 603). This enclosure design allows passive deposition of particles to the cell surface by diffusion and gravitational settling. After the exposure, 0-h measurements of viability were performed, and the remaining wells from each group received fresh medium and were incubated before other assays were performed.

### 2.4. Exhaust modification

Several different exhaust modifications were investigated; the number of replicate wells, the exposure duration, and the time incubated before performing assays for each condition are listed in Table 1. An increased dose was achieved by increasing the exposure duration; diffusion of exhaust gases and particles to the cell surface is constant over time since the system is at a steady state. Particles were removed from the exhaust stream with an Anodisc 0.02 μm pore filter (47 mm diameter) placed in the sample line upstream of the cell enclosure. A second sample line from the chamber allowed simultaneous exposure of whole exhaust to another set of cells. The final effect investigated was that of the organic carbon content in the exhaust using a hydrocarbon denuder in the sample line. The denuder consists of layers of activated carbon sheets (Tang et al., 1994) that remove volatile and semi-volatile organic gases. Removal of gas phase semi-volatiles in the denuder shifts the equilibrium, driving semi-volatiles from the particle surface, which are then removed in the denuder. Compounds with lower vapor pressure, such as the larger PAH (greater than six rings), will remain on the particle as it passes through the denuder. The denuded exposure was performed with an unloaded engine.

Table 1
Summary of exposure conditions

| Engine load (kW) | Exhaust modification | Exposure duration (h) | Number of wells | Incubation time (h) |
|---|---|---|---|---|
| 1.5 | Timed | 1, 2, 4 | 3 | 24, 48 |
| 1.5 | Filtered | 6 | 4 | 6, 20 |
| 0 | Denuded | 4 | 3 | 6, 20 |

## 3. Results and discussion

### 3.1. Exposure conditions

A summary of the chamber measurements for each exposure condition is listed in Table 2. The baseline particle count in the chamber was approximately $10^2\ cm^{-3}$ when measured before the diesel generator and recirculation system were turned on. A stable particle size distribution in the chamber was typically achieved after running the recirculation system about 1 h. The concentration of $NO_x$ was approximately 7 ppm for the loaded engine and 5 ppm for the unloaded, and $SO_2$ was below the detection limit of the analyzer for all conditions (4 ppm). Assuming that all of the sulfur in the fuel (approximately 400 ppm wt.) is oxidized to $SO_2$ the expected concentration in the chamber is 0.2 ppm.

The average size distribution for a loaded engine is shown in Fig. 2. The particles exhibit a lognormal distribution with a median diameter of 110 nm and a number concentration of $8.9 \times 10^5\ cm^{-3}$. In previous experiments with this engine, it was observed that when the engine is unloaded, the number concentration and the mean diameter were reduced approximately in half, which results in an

Table 2
Summary of chamber measurements for each exposure condition

| | Temperature (°C) | Relative humidity (%) | Ventilation flow ($l\,min^{-1}$) | Dilution ratio | $NO_x$ (ppm) | Number concentration ($cm^{-3}$) | Median diameter (nm) |
|---|---|---|---|---|---|---|---|
| Timed | 20 | 44 | 1250 | 1:114 | 7.2 | –[a] | –[a] |
| Filtered | 24 | 56 | 1210 | 1:110 | 6.3 | $8.9 \times 10^5$ | 110 |
| Denuded | 20[b] | 60[b] | 1160 | 1:106 | 5.0 | –[a] | –[a] |

[a] No SMPS measurement for this exposure.
[b] Values estimated from atmospheric data.

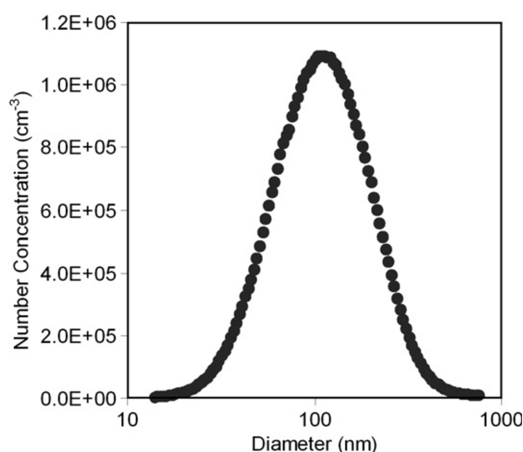


Fig. 2. Particle size distribution inside the environmental chamber for a loaded engine. The total number concentration is $8.9 \times 10^5\ cm^{-3}$ with a median diameter of 110 nm.

order of magnitude lower mass of particles in the exhaust compared to the loaded case. The chemical composition of the exhaust is also affected by engine loading, with the gas phase hydrocarbons and the organic carbon content of the particles increasing when the engine is unloaded (Damm et al., 2002).

The size distributions observed in the environmental chamber are comparable to ambient urban conditions. Ambient number concentration can vary greatly depending on time and location; for example, at a bus interchange in Singapore the number concentration varied from $5 \times 10^3$ (non-operating hours) to $5 \times 10^5\ cm^{-3}$ (operating hours) (See et al., 2006). The deposition on the cell layer is a fraction of the chamber concentration that may be different from the fraction that deposits in the bronchial region of the lung. However, the size distribution deposited is more relevant to human exposures than particles collected on a filter and suspended in growth medium, which skews the size distribution towards larger particles. Because the particle composition can vary by size, the composition might also differ to some extent between in vitro and in vivo exposure.

### 3.2. Cell response to diesel exhaust

Viability and IL-8 assays for each exposure condition were compared to the corresponding unexposed group to account for differences in the preparation and incubation time. Viabilities were measured from a limited sample immediately after the exposure and after a period of incubation. Measurements of IL-8 secretion were made either immediately after the exposure to assess IL-8 release during the exposure period or after the indicated incubation time. The IL-8 release is presented as the incremental change for each incubation time to make it easier to compare the time response for each exposure group.

Measurements of IL-8 secretion for cells exposed to exhaust for varying exposure time are shown in Fig. 3. After 24 h of incubation, cells exposed to 4 h of whole diesel exhaust had the highest IL-8 secretion (190% of the unexposed value) followed by the 2 h exposure (140% of the unexposed) (Fig. 3a). The IL-8 secretion showed a dose response increasing with increasing exposure duration. After 24 h of incubation the medium was removed, fresh medium was added, and incubation was continued for another 24 h. The IL-8 released in this 24–48 h incubation period was approximately the same for the exposed and unexposed cells (Fig. 3b). This suggests that the effects of the exhaust are transient and last <24 h after the exposure. Subsequent exposures were performed with incubation times <24 h to resolve when the maximum response occurs. It was later observed that the maximum IL-8 release occurs from 0 to 6 h after exposure (Fig. 4d).

We then proceeded to investigate the effects of the exhaust gases separate from the particles. The exhaust stream was filtered, removing most of the particles. The efficiency of the filter was measured by the SMPS as 99% for particles larger than 20 nm, decreasing to approximately 70% below this diameter. When compared with the unexposed group, the filtered exposure resulted in 86% 0-h viability and the whole exhaust exposure resulted in 67% 0-h viability (Fig. 4a). That is, gases alone contributed half of the toxicity observed with the whole mixture. The gases also had a large effect on IL-8 secretion after 6 h of incubation, with the filtered group producing 310% and the whole exhaust producing 430% of the unexposed group (Fig. 4b). The contribution of the exhaust gases is thus considerable, with approximately half of the response observed attributable to gases. This result is reasonable since the $NO_2$ concentration in the chamber is estimated to be 10% of the $NO_x$ level (Finlayson-Pitts and Pitts, 1986) (approximately 500 ppb), and is known to elicit IL-8 secretion in human bronchial epithelial cells (Ayyagari

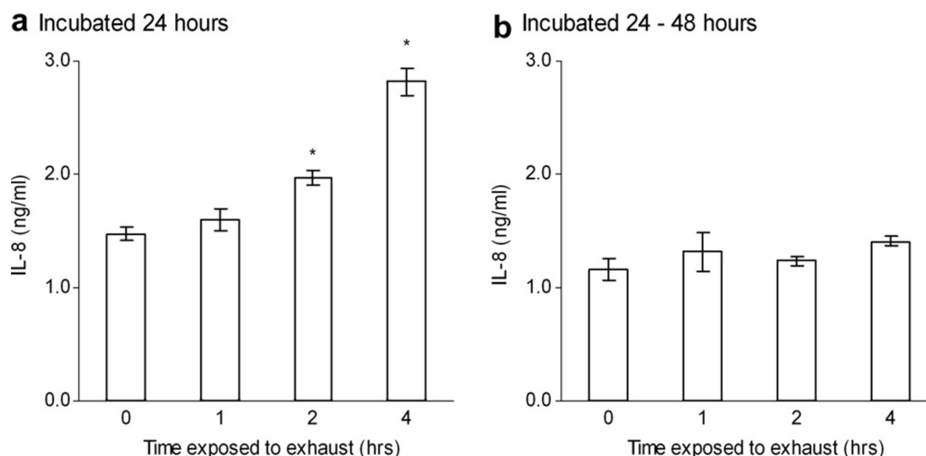


Fig. 3. IL-8 secretion for 0, 1, 2, and 4 h exposure to whole diesel exhaust: (a) incubated for 24 h after exposure and (b) incubated from 24 to 48 h after exposure. Cells were cultured on a 24 well plate ($2.3 \times 10^5$ cells $cm^{-2}$). Values are reported as mean ± standard error of mean, $^*P < 0.01$ compared to unexposed using student's $t$-test.

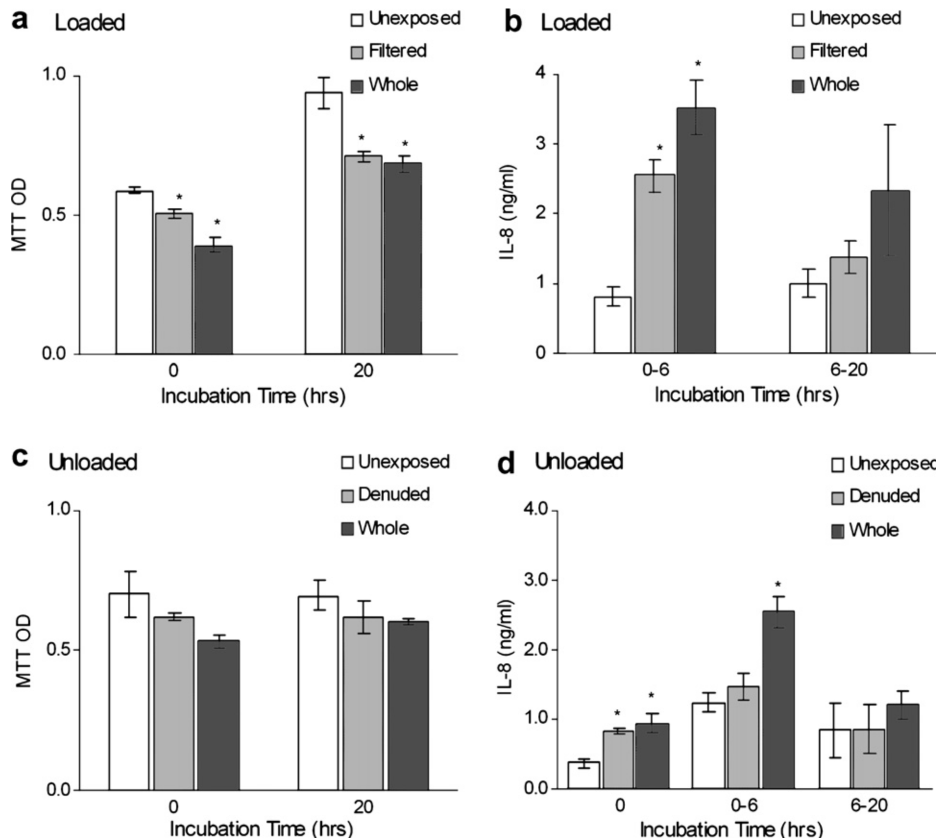


Fig. 4. Effect of filtration and denuding of whole diesel exhaust on viability and IL-8 release. (a) Viability (24 well plate, $1.5 \times 10^5$ cells $cm^{-2}$), (b) IL-8 release (6 well plate, $1.1 \times 10^5$ cells $cm^{-2}$) for filtered versus whole diesel, (c) Viability (24 well plate, $1.6 \times 10^5$ cells $cm^{-2}$), and (d) IL-8 release (6 well plate, $1.1 \times 10^5$ cells $cm^{-2}$) for denuded versus whole diesel. Viability is reported as optical density (OD) of the MTT solution at 570 nm. $^*P < 0.01$ compared to unexposed.

et al., 2004). Furthermore, most of the sulfur in the fuel is converted to $SO_2$, a known irritant gas that has been associated with an inflammatory response in human lung cells (Kienast et al., 1994). The high concentration of these irritant gases in controlled laboratory exposures may contribute to a large portion of the observed inflammatory response, which limits the generalization of these results to ambient conditions where the concentrations of these gases are much lower. For example, ambient measurements made in the San Francisco bay area on a spring day were two orders of magnitude lower than the levels within the chamber, with the peak 1 h average $NO_2$ at 20 ppb, NO

at 30 ppb, and $SO_2$ at 5 ppb (California Air Resources Board, 2006).

Organic constituents extracted from particles have been linked to toxicity in both in vitro and in vivo exposures. However, the usual extraction procedure only collects particle-bound organic content and does not allow for investigation of the toxicity of gas phase and semi-volatile organic compounds. By using a denuder to remove organic content from the exhaust, we were able to examine the effects of the gas phase and semi-volatile components on cell viability and IL-8 release. Furthermore, this exposure was done with exhaust from an unloaded engine, which results in a greater amount of hydrocarbons in gas phase and is expected to contribute to a larger amount of adsorbed PAH and other organic carbon compounds on the particle surface (Kittelson, 1998).

The viability and IL-8 secretion of unexposed cells and cells exposed to whole or denuded exhaust are shown in Fig. 4c and d. Immediately after exposure the viability was highest for the unexposed, less for the denuded exhaust (89% of unexposed), and lowest for the whole exhaust (76% of unexposed). There was little difference in the IL-8 release during the 4-h exposure (0-h incubation time) between the whole and denuded exhaust exposures (denuded = 230% and whole = 260% of the unexposed). However, after a 6-h incubation period the denuded group recovered and secreted approximately the same amount as the unexposed group (118% of the unexposed), unlike the whole exhaust group which remained elevated (205% of the unexposed). After 20 h of incubation time, the same trend was apparent, with the IL-8 secretion of the denuded group similar to the unexposed group and the whole exhaust group elevated from both. The lower IL-8 secretion of the denuded group compared with whole exhaust group suggests that compounds removed by the denuder are responsible for the inflammatory response observed in cells exposed to whole exhaust. Similar results were also found in studies with extracts of organic compounds from DEP. Benzene-extracted compounds from DEP were most important in stimulating IL-8 release from human bronchial epithelial cells (Kawasaki et al., 2001). Boland et al. (1999) compared DEP produced from vehicles with and without an oxidation catalyst and found that the catalyst reduced PAH by 60% and reduced release of granulocyte macrophage – colony stimulating factor (an inflammatory cytokine) from human bronchial epithelial cells. Our results showed that denuding particles reduced toxicity and IL-8 release, and results in almost no difference from the unexposed group when the cells were incubated after exposure. We expect that many of the higher vapor pressure semi-volatile compounds are removed in the denuder; however, we have not quantified in detail how denuding affects the exhaust composition. These semi-volatile compounds are frequently vaporized during sampling and may not be present in the extracted material from collected particles that have been used in these other studies. Our results indicate that the more volatile organic compounds removed in the denuder are also important in causing inflammation. This result may be quite different for a loaded engine, which will have a much lower level of gas and particle phase hydrocarbons.

The effects of exhaust from a loaded and unloaded engine can be assessed by comparing the 4-h whole exhaust exposure from the denuded experiment (unloaded) with the 4-h whole exhaust exposure in the timed experiment (loaded). Although slightly different protocols were followed, the viability and the IL-8 secretion can be compared when normalized to the unexposed group for each case. The 0-h viability of the unloaded was 76% and the loaded was 63% of the unexposed for each experiment. The IL-8 secretion increased 170% (20-h incubation) and 190% (24-hour incubation) above the unexposed for the unloaded and loaded groups respectively. As the exhaust gases and particle concentrations were reduced in the unloaded condition (the $NO_x$ concentration was 20% lower and the particle concentration was lower by an order of magnitude from the loaded case), it is not surprising that this resulted in increased viability and decreased IL-8 secretion. Accompanying changes in the chemical composition of the particles produced under each loading condition are likely to affect the response as well. This outcome was observed by Madden et al. (2003) who exposed cultured bronchial epithelial cells to exhaust extracts (particles and water-soluble gases) collected at varying engine loads. The observed IL-8 secretion was greater for a high load when compared to a low load of the same mass, which the authors concluded was due to variation in the chemical composition at the two engine conditions. While we cannot isolate the effects of changes in the chemical composition of the particles from the differences in the concentration, we can conclude that cell toxicity from an unloaded engine is less than that from a loaded engine.

The air–cell interface exposure simulates in vivo conditions better than conventional in vitro exposures as artifacts from particle collection and resuspension are avoided, and a simultaneous exposure to exhaust gases is evaluated. This is not feasible for conventional cell exposures to particles collected and resuspended in cell culture medium. As with all types of particle exposure experiments, it is difficult to quantify the dose of particles. In our system, the dose is governed by the particle deposition to the cell surface, which is currently unknown. Quantitative measurements of dose are vital to determine an accurate representation of the particle interaction with the cell.

## 4. Conclusion

When we exposed human bronchial epithelial cells at the air–cell interface to diluted diesel exhaust viability decreased and IL-8 secretion increased. The increased production and secretion of IL-8 in response to diesel exhaust exposure indicates that the exposure causes inflammation and can have health consequences, especially in individuals with pre-existing pulmonary disease. The IL-8 secretion is highest early after exposure and diminishes with increasing

incubation. The exhaust gases are responsible for nearly half of the inflammatory response. Removal of volatile and semi-volatile organic compounds from the exhaust resulted in minimal differences in viability from the whole exhaust, but greatly reduced the IL-8 release, implying that PAH and other organic compounds play a major role in the inflammation caused by diesel exhaust. Exhaust from a loaded engine resulted in a larger toxic and inflammatory response than from an unloaded engine, which may be due to the increased particle mass loading and exhaust gas concentrations from that of an unloaded engine.

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