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Alveolar macrophage cytokine response to air pollution particles: Oxidant mechanisms

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Abstract

Alveolar macrophages (AMs) primed with LPS and treated with concentrated ambient air particles (CAPs) showed enhanced release of tumor necrosis factor (TNF) and provide an in vitro model for the amplified effects of air pollution particles seen in people with preexisting lung disease. To investigate the mechanism(s) by which CAPs mediate TNF release in primed rat AMs, we first tested the effect of a panel of antioxidants. *N*-Acetyl-L-cysteine (20 mM), dimethyl thiourea (20 mM) and catalase (5 μM) significantly inhibited TNF release by primed AMs incubated with CAPs. Conversely, when LPS-primed AMs were treated with CAPs in the presence of exogenous oxidants (H_2O_2 generated by glucose oxidase, 10 μM/h), TNF release and cell toxicity was significantly increased. The soluble fraction of CAPs suspensions caused most of the increased bioactivity in the presence of exogenous H_2O_2. The metal chelator deferoxamine (DFO) strongly inhibited the interaction of the soluble fraction with H_2O_2 but had no effect on the bioactivity of the insoluble CAPs fraction. We conclude that CAPs can mediate their effects in primed AMs by acting on oxidant-sensitive cytokine release in at least two distinct ways. In the primed cell, insoluble components of PM mediate enhanced TNF production that is H_2O_2-dependent (catalase-sensitive) yet independent of iron (DFO-insensitive). In the presence of exogenous H_2O_2 released by AMs, PMNs, or other lung cells within an inflamed alveolar milieu, soluble iron released from air particles can also mediate cytokine release and cell toxicity.

Keywords: Alveolar macrophage; Cytokines; Air pollution; Particles; Oxidants

Introduction

The association between elevated concentrations of air pollution particles ($\leq PM_{2.5}$) and adverse health effects is especially prominent in people with inflammatory lung diseases such as bronchitis, asthma and pneumonia (Schwartz, 1994; Goldsmith and Kobzik, 1999; Atkinson et al., 2001). In both normal and diseased lungs, the alveolar macrophage (AM) is the first line of defense against inhaled particulate matter. Since people with pre-existing respiratory illness are especially affected by high PM levels, we have postulated that AMs residing within inflamed lungs are 'primed' for heightened pro-inflammatory (and injurious) responses to air particles. In the initial testing of this hypothesis, priming of AMs with LPS in vitro markedly enhanced their release of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF) and macrophage inflammatory protein (MIP-2) in response to suspensions of concentrated air pollution particles (CAPs) (Imrich et al., 1999). In contrast, when primed AMs were cultured with a relatively inert particle, TiO_2, there was no effect on release of cytokines. Since the same AM scavenger receptors mediate initial binding of both inert (e.g. TiO_2) and pathogenic (e.g., quartz, CAPs) particles (Kobzik, 1995), components unique to CAPs are most likely responsible for the enhanced cytokine release seen in primed AMs.

The question of which component(s) of particulate matter (PM) are active has been investigated using a variety of biologic outcome parameters (Tao et al., 2003). Data have been reported

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implicating transition metals (Ghio et al., 1999; Wilson et al., 2002), polyaromatic hydrocarbons (PAH) (Li et al., 2002a,2002b) and bacterial endotoxin (Becker et al., 1996; Ning et al., 2000) as bioactive components of PM. Many PM effects on normal lung cells in vitro and in vivo operate through oxidant-dependent mechanisms (Tao et al., 2003). However, much less is known about how particles mediate effects in primed or activated cells.

The studies reported here sought to directly test the hypothesis that oxidant mechanisms are necessary for the enhanced cytokine release seen in primed AMs upon culture with air pollution particles. We tested the postulate by examining the inhibitory effects of a panel of antioxidants added to the culture system, as well as the effect of exogenous oxidants produced by glucose oxidase. We also analyzed the contribution of soluble and insoluble fractions of CAPs, and the effect of metal-chelation with desferoxamine. The data support an oxidant (H_2O_2)-dependent mechanism for particle-mediated cytokine release by primed AMs, and identify novel differences in the bioactivity of metals (most likely iron) within soluble and insoluble fractions of air pollution particles.

Methods

Preparation of particle suspensions. The urban air sample SRM 1649 was collected in Washington, DC (UAP) and was purchased from the National Bureau of Standards (Washington, DC; May et al., 1992). UAP was used as a positive control particle and contains both $PM_{2.5}$ and larger non-respirable particles including pollen, fungal fragments, etc. TiO_2 (~1 μm diameter) was generously provided by Dr. J. Brain. Both particles were suspended in analytical grade water (OPTIMA, Fisher Scientific) at 10 mg/ml and stored frozen. The CAPs samples were collected on a daily basis from Boston air as previously described (Imrich et al., 1999, 2000) and comprised particles of ≤2.5 μM diameter, corresponding to respirable size particles that AMs would likely encounter in the alveolar space. CAPs samples from different days vary in biological potency (Imrich et al., 1999, 2000), and are available in relatively small amounts, making initial experiments with UAP especially useful. CAPs were collected either onto Teflon filters (Teflon 47 mM, 2 μM; Gelman Sciences, Ann Arbor, MI) or onto polyurethane filters (Kavouras and Koutrakis, 2001). Particles were released from Teflon filters into water (OPTIMA) by brief sonication (model W-200P, Ultrasonics, Plainview, NY; setting 4) as previously described (Imrich et al., 2000). With the sponge-like PUF filters, particles were released by immersion in water followed by probe sonication for 1 min. This was done for three consecutive aliquots of water, followed by pooling of the water samples containing released CAPs in suspension. The concentration of particles or components released into water was determined by weighing an aliquot of the suspension (temperature of 68 °F, relative humidity of 41%; CAHN model C-31, Mettler-Toledo, Columbus, OH), as previously described (Imrich et al., 2000). In some experiments, CAPs suspensions were separated into soluble and insoluble fractions. To achieve this, 200 μl of CAPs suspension was centrifuged at 13,000×*g* for 5 min. The supernatant or soluble fraction (CAPs-s) was removed and the pellet or insoluble fraction (CAPs-i) was re-suspended in 200 μl of water. Both fractions were assayed together with the original particle suspension. Iron content of soluble CAPs fraction samples was measured by a modified ferrozine method (Voelker and Sulzberger, 1996).

Reagents. *N*-Acetyl-L-cysteine (NAC) was prepared in RPMI–1% FBS (R1%) and the pH adjusted to 7.4 prior to use (final concentration of 2–20 mM). Dimethylthiourea (DMTU) was suspended in R1% and used at final concentrations of 2–20 mM. Bovine liver catalase (Fluka, Milwaukee, WI) was suspended in R1%, sterile filtered and used at final concentrations of 1–10 μM. The molar activity of the catalase solution was verified to be accurate by measuring its ability to remove H_2O_2 that was added to R1% (H_2O_2 concentration was quantified by measuring its optical density OD_{240}). Desferoxamine (DFO) mesylate is a metal chelator that binds iron, copper and zinc, although its affinity for iron is several logs of magnitude stronger than for other metals (Halliwell and Gutteridge, 1999). Particle suspensions were pre-incubated with DFO (stock made in OPTIMA water) for 30 min prior to use. Glucose oxidase (GO) is an enzyme that uses glucose (contained in culture media) as a substrate to produce hydrogen peroxide. The activity of GO used in this study was determined by measuring the optical density of H_2O_2 (OD_{240}), allowing conversion of GO concentration into μM of H_2O_2 produced per hour. Using the GO reagent at 0.25 μg/ml yielded 10 μM of H_2O_2/h in RPMI media. All reagents not otherwise specified were purchased from Sigma (St. Louis, MO).

Cell-free assay for oxidant activity of particles. The oxidant-reactive, fluorescent reporter molecule, DCFH (OxyBurst-BSA; Molecular Probes, Eugene, OR) was used to measure oxidant generation by CAPs suspensions. The reagent was suspended in OPTIMA water and used at 10 μg/ml. Oxidant activity of particle suspensions was measured in triplicate wells of a 96-well plate in the presence or absence of 10 μM H_2O_2. The chelator DFO (0.05 mM) was used to inhibit iron-mediated secondary oxidant generation. After 1-h incubation at 37 °C, the green fluorescent reaction product was measured in a fluorescence plate reader using 485 nm excitation and 535 nm emission wavelengths (Tecan SpectraFluor Plus, Research Triangle Park, NC). Data are reported as relative fluorescence units (RFU).

Macrophage isolation and culture. Adult, female CD rats (12–14 weeks old; VAF, Harlan Sprague Dawley Inc., Indianapolis, IN) were housed in a barrier facility prior to use. Animals were euthanized with an intraperitoneal injection of sodium pentobarbital and bronchoalveolar lavage (BAL) was performed. A phosphate-buffered saline was used for bronchoalveolar lavage (PBS, BioWhittaker, Walkersville, MD). BAL cells were centrifuged at 250×*g* for 10 min and resuspended in buffer consisting of RPMI-1640 (BioWhittaker) supplemented with 1% heat inactivated fetal bovine serum (Gemini Bioproducts, Woodland, CA), 0.075% bovine serum albumin (BSA), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (R1%). Cells recovered from lavage were greater than 95% viable as measured by Trypan blue exclusion. Cytocentrifuge preparations were stained with Diff-Quik, a modified Wright–Giemsa stain (VWR, Boston, MA) to allow differential analysis. All samples contained greater than 95% AMs.

All cell incubations were carried out under non-adherent conditions (to facilitate cell harvesting for analyses at the end of experiments) using Ultra Low Attachment Cluster plates (Costar, Cambridge MA) at 37 °C in humid 5% CO_2. Lavaged rat AMs were suspended at 10^6 cells/ml in R1% and treated with bacterial lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8, 250 ng/ml) for 3 h. After this priming period, cells were washed 3 times with cold PBS containing 0.075% BSA and adjusted to 2.5×10^6 cells/ml R1%. In all experiments, primed AMs, oxidant treatments and particle suspensions were dispensed together (80 μl each per well, 240 μl total volume, 2×10^5 cells/well) into 96-well plates. Supernatants were collected after 18–20 h of particle incubation and stored frozen at −70 °C. The cells were placed on ice and wells were filled with 200 μl of cold PBS containing propidium iodide (20 μg/ml). AMs were easily removed from the low attachment plates and cell viability quantified by flow cytometry (see below).

Flow cytometric viability assay. A Coulter ELITE flow cytometer (Coulter Corporation, Miami, FL) equipped with a 15-mW 488 nm emitting air-cooled argon laser (Cyonics/Uniphase) was used. Propidium iodide, a red fluorescent, DNA binding dye, allowed separation of live cells (red-negative) from those without nuclear membrane integrity (red-positive). The percent cell death was defined as the percent of red-positive events within a forward scatter versus red fluorescence bivariate plot. A total of 3000 live cells were analyzed from each sample. Free particles and cellular debris were excluded from the analysis.

Cytokine and nitrite assays. Cell supernatants from rat AMs were assayed for TNF bioactivity using the TNF-sensitive WEHI 164 clone 13 tumor cell line in a previously described micro-plate assay with a lower limit of detection of 5 pg/ml (Imrich et al., 1998). All samples were analyzed in triplicate wells. Briefly, adherent WEHI cells were incubated with AM culture media or recombinant rat TNF standard (Endogen Inc., Woburn, MA). TNF-mediated cell death was

measured using PI (see above) and red fluorescence was quantified in a fluorescence plate reader (excitation 535 nm, emission 595 nm; Spectrafluor Plus, Tecan, Research Triangle Park, NC). TNF values of AM culture media were calculated using a 4-parameter curve fit (soft MAX software, Molecular Devices, Menlo Park, CA). The three antioxidants NAC, DMTU and catalase were tested for non-specific effects on TNF-mediated WeHi cell death. There were no changes in the bioactivity of TNF standard in the presence of these cell treatments (data not shown).

Rat macrophage inflammatory protein (MIP-2) was detected using a previously described sandwich ELISA with a lower limit of detection of 30 pg/ml (Imrich et al., 1999). All samples were analyzed in triplicate wells. Optical density of the ELISA reaction product (450 nM) was quantified by a microplate spectrophotometer (vMax, Molecular Devices) and MIP-2 levels were calculated using a 4-parameter polynomial curve fit (soft MAX software, Molecular Devices).

Priming of rat AMs with LPS causes expression of the inducible form of nitric oxide synthase (iNOS). This enzyme generates large amounts of nitric oxide (NO) and nitrite, the final measurement used as an indicator of iNOS activity. The Griess method was employed for detection of nitrite in cell supernatants (Schmidt and Kelm, 1996) and absolute amounts were calculated from a sodium nitrite standard curve using a 4-parameter curve fit.

Analysis of transferrin receptor mRNA levels in AMs. Total cellular RNA was isolated by using RNA Bee (TelTest, Freindswood, TX). Northern blot analysis was performed as described previously (Knutson et al., 2001). Randomly primed ^{32}P-labeled DNA probes were prepared using cDNA fragments excised from plasmids pT7T3D-Pac-rat transferrin receptor (GenBank Accession No. AI454017; Resgen, Huntsville, AL) and pBluescriptSK(−)-human β-actin (GenBank Accession No. AA173249; ATCC, Manassas, VA). Signal intensities were determined by phosphorimaging (Personal FX, Bio-Rad, Hercules, CA), and values obtained for transferrin receptor transcripts were normalized to those obtained for β-actin.

Statistical analysis. Descriptive statistics (means and standard deviations) were calculated for all outcomes, for each treatment combination. For correlation analyses, to satisfy model assumptions of normality and homoskedastic variance, TNF was log-transformed, whereas MIP and cell death were left untransformed. Multi-way ANOVA techniques were used to detect differences among treatment and dose groups. Statistical significance for all models was based on an alpha of 0.05. Bonferonni's method was used to control the Type I error rate in the presence of multiple comparisons. To assess the dose–response associations between H_2O_2 and each outcome, linear regression models that specified a piecewise linear dose–response with a cut-point specified at 10 μM/h H_2O_2 were run. This model equates to specifying two linear dose–response relationships between H_2O_2 and an outcome, one below and one above 10 μM/h of H_2O_2. Interactions between these slopes and particle treatment were built into the regression models to assess whether the dose–response slopes differed by particle treatment for each outcome. All statistical analyses were performed using PROC GLM in SAS version 8 (SAS Institute, Cary, NC).

Results

Antioxidants inhibit AM response to particles

Initial experiments used the positive control particle UAP to perform dose–response analysis in the presence of the antioxidants dimethylthiourea (DMTU at 2–20 mM) and *N*-acetyl-L-cysteine (NAC at 2–20 mM). After 3 h of LPS priming, the effect of antioxidants on AM release of TNF and nitrite, and cell death were measured. Similar to previous observations (Imrich et al., 1999, 2000), the results presented in Table 1 show that in the absence of antioxidants: 1) UAP significantly increased TNF release by primed AMs; 2) UAP toxic effects include increased cell death; 3) the relatively high baseline production of nitrite (due to LPS-priming) was not affected by UAP. The highest concentration of DMTU (20 mM) was effective at diminishing TNF release by primed AMs in both control and UAP-treated groups (Table 1) but did not cause any significant changes in cell death. The antioxidant NAC was a potent inhibitor of nitrite release and moderately inhibited TNF production (Table 1), but only when used at the highest concentration (20 mM) (not statistically significant). To test the hypothesis that H_2O_2 is one specific oxidant species required for UAP-mediated TNF release, primed AMs were treated with catalase in the presence or absence of UAP. Catalase significantly inhibited both control and UAP-mediated TNF production (Table 2). Similar to DMTU, catalase inhibited TNF to the same degree in both control and particle-treated groups.

To investigate AM responses using more realistic $PM_{2.5}$ ambient particles, primed cells were treated with 3 different samples of CAPs in the presence or absence of NAC (20 mM), DMTU (20 mM) or catalase (5 μM). Results presented in Fig. 1 show all three antioxidants significantly inhibited TNF release by primed AMs. Similar to effects seen with UAP, both DMTU and catalase inhibited TNF release to the same degree in both control and CAPs-treated groups. In contrast to effects seen with UAP, NAC caused greater inhibition of CAPs-mediated TNF production compared to inhibition of TNF production in cells not treated with CAPs (Fig. 1).

Table 1
Optimization of antioxidant concentration and inhibition of AM response to particles

	TNF (ng/ml)		Nitrite (μM)		Cell death (%)	
	Control	UAP	Control	UAP	Control	UAP
Control	0.93±0.42	9.3±4**	16.6±1.4	15.7±3.6	13±3	26±5**
NAC 2 mM	0.81±0.39	8.2±4.6	8.5±0.5*	10.2±3.5	12±3	19±8*
NAC 10 mM	0.62±0.28	6.1±3.4	3.5±0.4*	4.6±1.9*	12±3	23±5
NAC 20 mM	0.71±0.45	5.6±2.7	1.7±0.3*	2.1±1.1*	12±3	19±5*
DMTU 2 mM	0.92±0.37	8.2±3.8	12±0.5	12.8±2.6	11±3	24±4
DMTU 10 mM	0.58±0.33	4.1±2.4	8.6±0.3*	10.5±2.2	9±3	22±4
DMTU 20 mM	0.04±0.05*	0.3±0.2*	6±0.5*	8.4±1.1*	11±3	22±4

Rat AMs were primed for 3 h then cultured with or without UAP (100 μg/ml) in the presence or absence of antioxidants NAC and DMTU for 20 h (n=3). A total of 19 comparisons were made within and across groups and significance was accepted when $p \leq 0.00263$. Significant increases were noted (**) when comparing UAP versus control without antioxidants. Significant inhibition was noted (*) when comparing cells±antioxidants within control or UAP groups.

Table 2
Effect of catalase on particle mediated TNF release

	TNF (ng/ml)		Cell death (%)	
	Control	UAP	Control	UAP
Control	2.2±0.5	13.5±2.3**	8.3±2.1	17.2±3.0**
CAT 1 μM	1.8±0.4	8.9±1.7*	11.3±2.1	18.4±2.7
CAT 5 μM	1.5±0.5*	6.7±1.3*	11.2±1.6	15.4±2.7
CAT 10 μM	1.5±0.5*	6.4±1.1*	8.9±1.2	15.3±2.7

Primed AMs were incubated with or without UAP (100 μg/ml) and three concentrations of catalase (CAT), $n=4$. UAP-mediated TNF release and cell toxicity were measured and 10 comparisons were made. Significance was accepted when $p \leq 0.005$. Significant increases were noted (**) when comparing UAP versus control without CAT. Significant inhibition was noted (*) when comparing cells±antioxidants within control or UAP groups.

Exogenous H_2O_2 enhances particle effects

Data using antioxidants suggest that H_2O_2 is important for CAPs-mediated cytokine release. We investigated this hypothesis by adding exogenous H_2O_2, testing the prediction that increased H_2O_2 will further enhance AM responses to particles. The effect of glucose oxidase-generated H_2O_2 was measured on primed AMs in the presence or absence of the positive control particle UAP. Analysis of the slopes of the lines plotted from 0 to 10 μM H_2O_2/h showed significant interactions between UAP and exogenous H_2O_2 for each of the three parameters measured $p \leq 0.0167$, as shown in Fig. 2. Exogenous H_2O_2 and UAP (100 μg/ml) together caused marked enhancement of AM release of TNF. Levels of MIP-2, another inflammatory cytokine, were also significantly increased by the interactions of H_2O_2 and UAP (50 μg/ml). Finally, the combination of UAP (100 μg/ml) and H_2O_2 caused toxic effects, as noted by the increased cell death measured at the end of the 20-h incubation period (Fig. 2).

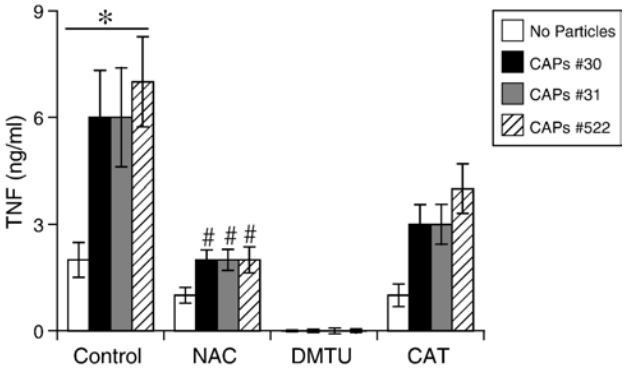

Fig. 1. Effect of antioxidants on particle-induced TNF release by primed AMs. Cells were primed for 3 h and treated with either with no particles (light grey) or with three CAPs samples (100 μg/ml, CAPs #30, black; CAPs #31, white; CAPs #522, dark grey) for 20 h. The effect of NAC (20 mM), DMTU (20 mM) and catalase (CAT, 5μM) on TNF release was measured, $n=6$. There were 21 comparisons made and significance was accepted when $p \leq 0.00238$. Antioxidant inhibition of TNF release within control and particle groups was noted (*). There was also significant difference noted in the ability of NAC to cause inhibition of particle-mediated TNF release when compared to NAC inhibition of control cell TNF release (#).

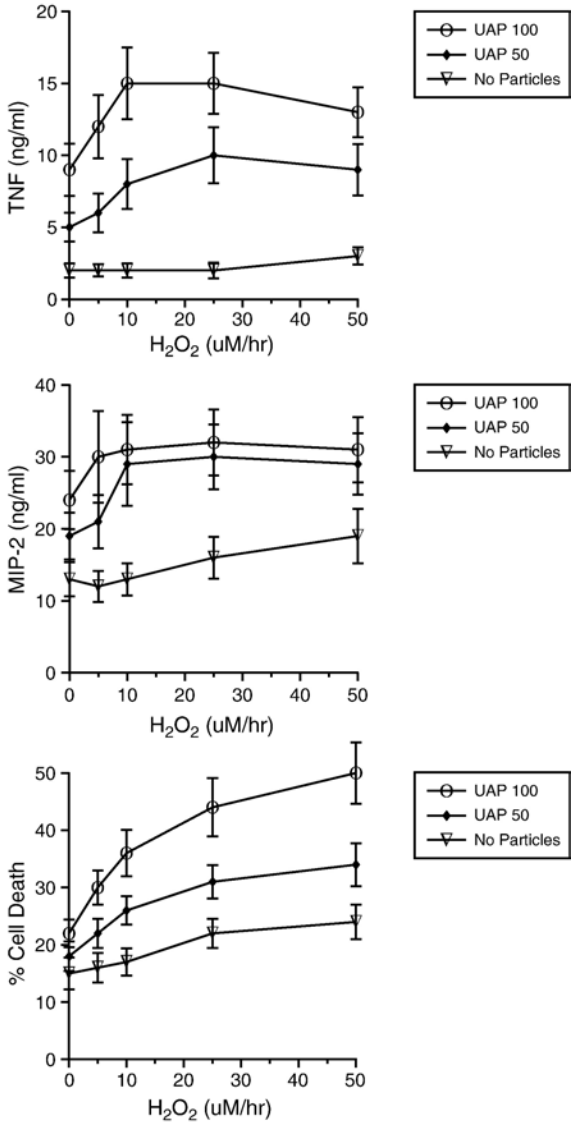

Fig. 2. Effect of exogenous oxidants on primed AM responses to UAP. Rat AMs were primed for 3 h then incubated with media only (open circles), UAP 50 μg/ml (open squares) or UAP 100 μg/ml (solid triangles) for 20 h, $n=8$. At the same time, cells were treated with H_2O_2 produced at several different rates (by adding glucose oxidase). After 20 h, TNF release (upper), MIP-2 release (middle) and cell death (lower) were quantified.

Enhanced cytokine release (both TNF and MIP-2) was also observed (Fig. 3) when three CAPs samples were incubated with primed AMs in the presence or absence of H_2O_2 (similar to results with UAP). The inert particle TiO_2 was tested and showed no ability to increase cytokine release in the presence of exogenous H_2O_2. In addition, each of the CAPs samples significantly increased AM cell death when combined with H_2O_2 while TiO_2 did not. No significant differences in nitrite release were measured, with one exception: CAPs #816 caused slightly increased nitrite production by AMs when combined with H_2O_2 in comparison to H_2O_2 alone (Fig. 3).

Suspensions of CAPs contain both water-soluble and -insoluble components. We previously observed that the insoluble fraction of CAPs suspensions contains the majority

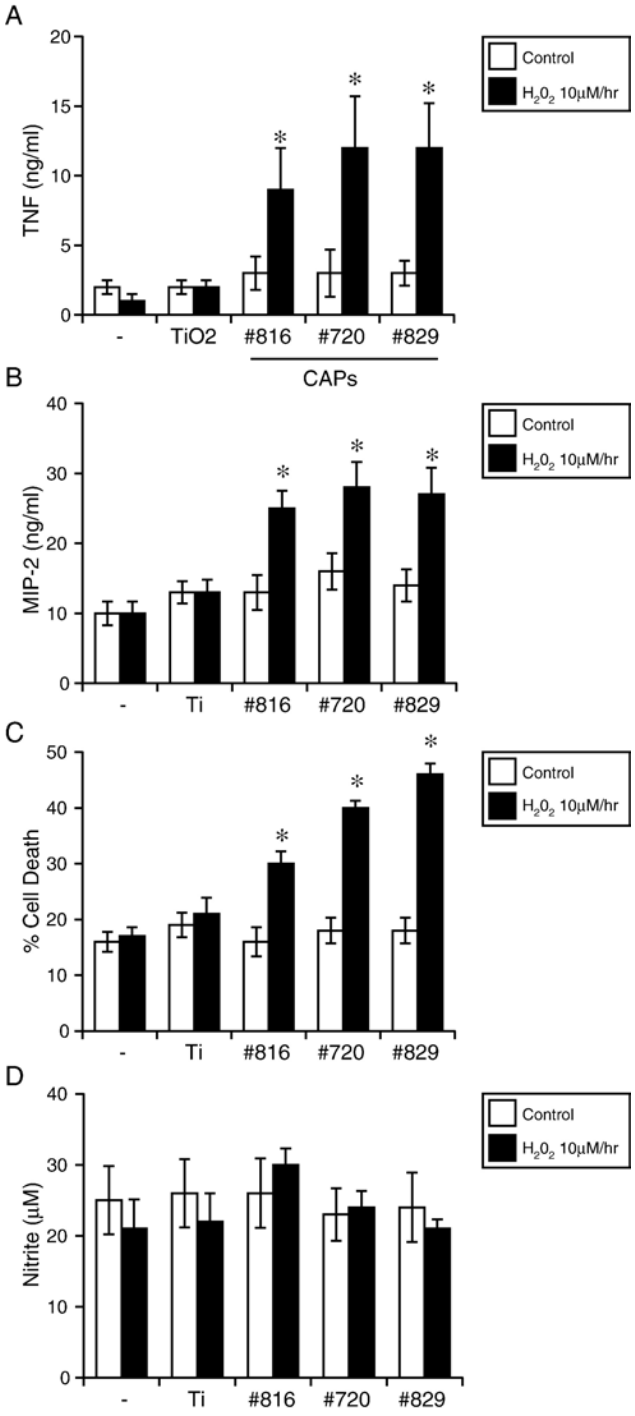

Fig. 3. Effect of exogenous oxidants on primed AM responses to CAPs. Primary AMs were treated with LPS for 3 h and then cultured with 4 different particles at 100 μg/ml (TiO_2, CAPs #816, CAPs #720, CAPs #829) for 20 h, $n=3$. Cells were also treated with nothing (light grey bars) or with H_2O_2 at 10 μM/h (dark grey bars). There were four parameters measured including AM release of TNF (A), MIP-2 (B), AM cell death (C) and release of nitrite (D). Comparisons were made to determine if changes seen in particle plus H_2O_2 groups are significantly different from no particle plus H_2O_2 effects (*). Significance was accepted when $p \leq 0.0125$.

of bioactivity for AMs (Imrich et al., 2000). To determine which CAPs components are active in the presence of exogenous H_2O_2, CAPs were separated into soluble (CAPS-s) and insoluble (CAPs-i) fractions. These fractions were tested along with the whole CAPs suspension in the presence or absence of H_2O_2. Results summarized in Fig. 4 show that without additional H_2O_2, the insoluble fraction (CAPs-i) contains most of the bioactivity responsible for increased TNF release. In contrast, in the presence of exogenous H_2O_2, the soluble component(s) (CAPs-s) contribute a substantial portion of bioactivity. A similar result was seen for both MIP-2 release and cell death assays: most of the additional bioactivity in the presence of exogenous H_2O_2 derives from the soluble fraction. In contrast, there were no significant differences in nitrite released during particle or H_2O_2 treatment (Fig. 4).

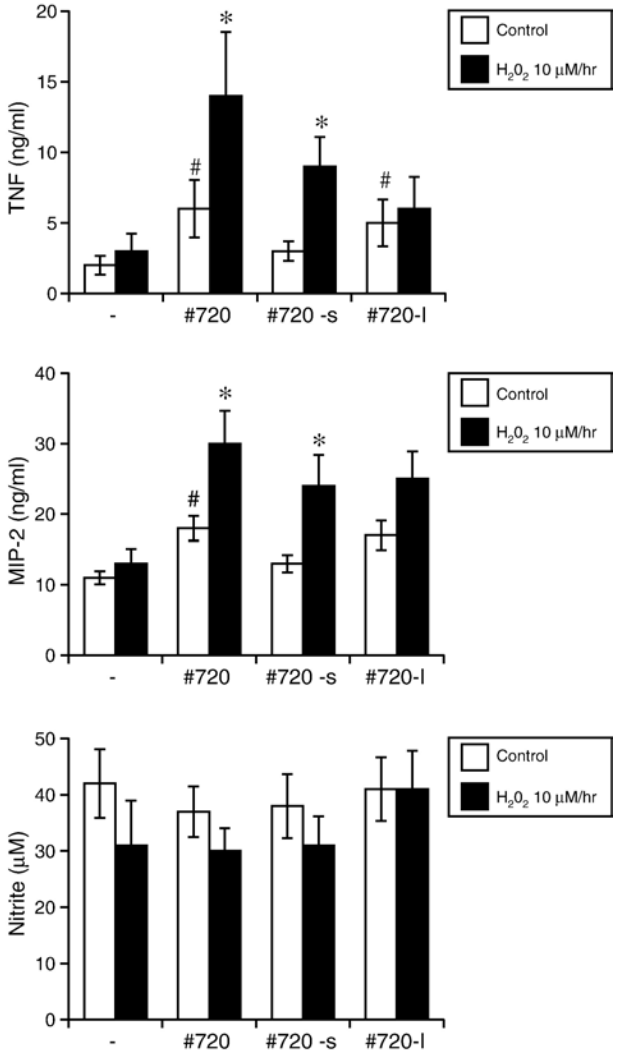

Fig. 4. Effect of exogenous oxidants on primed AM responses to CAPs fractions. Rat AMs were primed for 3 h and treated with no particles or with 100 μg/ml of CAPs # 720 and either soluble (720-s) or insoluble (720-i) fractions of CAPs #720 for 20 h, $n=6$. To determine which fraction contains bioactivity in the presence or absence of H_2O_2 cells were also treated with H_2O_2 produced at 10 μM/h (dark grey bars) or with media only (light grey bars). Three parameters were measured including TNF release (upper), MIP-2 release (middle) and cell death (lower). There were 6 comparisons made and significance was accepted when $p \leq 0.0071$. Significant increases were noted (*) when comparing the bioactivity of particles or particle fractions with cells only. There were also significant changes caused by particles plus H_2O_2 when comparing to changes seen in cells plus H_2O_2 without particles (#).

Desferoxamine (DFO) differentially inhibits CAPs-mediated effects

CAPs contain oxidant-reactive metals such as iron that could mediate effects through direct interaction with H_2O_2 to generate secondary, reactive oxidants. We sought to test the hypothesis that oxidant-dependent bioactivity of CAPs is dependent on iron. Our strategy was to use two concentrations of DFO, a highly effective iron chelator that is relatively cell-impermeant (Halliwell and Gutteridge, 1999). The first, lower DFO concentration (0.05 mM) does not affect intracellular iron status of AMs but is an effective extracellular chelator of iron and can block iron-mediated events initiated outside the cell. The second, higher DFO concentration (2 mM) is one that does affect intracellular iron status as well as extracellular iron-dependent events. By comparing the effectiveness of high and low DFO concentrations we sought to determine whether the bioactivity of CAPs components derives from extracellular or intracellular, iron-dependent events.

To first determine if CAPs can indeed react with H_2O_2 and generate secondary oxidants, the oxidant generating capacity of CAPs fractions were measured in the presence or absence of H_2O_2 using the fluorescent reporter DCFH (OxyBurst-BSA). Results seen in Fig. 5 indicate that the soluble fraction (CAPs-s) is highly reactive with H_2O_2 (large increase in fluorescence) and this reactivity is completely inhibited by the lower concentration of DFO (0.05 mM). The insoluble fraction showed comparatively moderate reactivity that was also DFO-sensitive. The oxidant-reactive probe showed no increased fluorescence when incubated with H_2O_2 alone (Fig. 5).

To determine cellular iron status after treatment with both low and high DFO concentrations, transferrin receptor (TfR) mRNA levels were measured. Cells acquire iron primarily via the TfR and induction of TfR mRNA is a well-documented and highly sensitive indicator of cellular iron depletion (Knutson et al., 2003). Fig. 6 shows Northern blot analysis of TfR mRNA

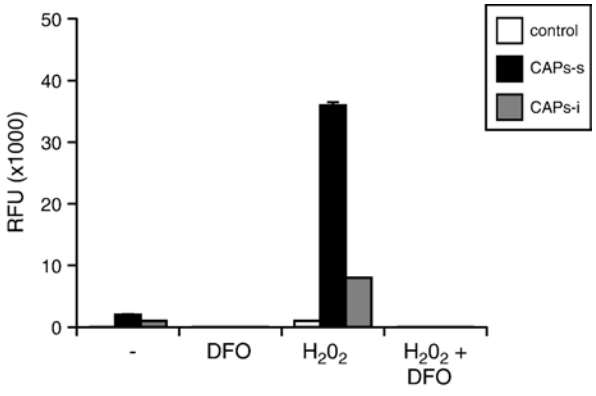

Fig. 5. Oxidant capacity of CAPs fractions. Fractions of CAPs were generated from a whole suspension at 50 μg/ml. The soluble fraction (CAPS-s, light grey) and insoluble fraction (CAPs-i, dark grey) and water vehicle control (black) were incubated in the presence or absence of 10 μM H_2O_2 with the oxidant reactive probe OxyBurst-BSA. Also included was the metal chelator desferoxamine (DFO) at 0.05 mM. The relative fluorescence units (RFU) were measured after 1-h incubation. Data are shown as means ± SD, but due to minimal variability error bars are difficult to see (e.g. for CAPs + H_2O_2: 36079 ± 518).

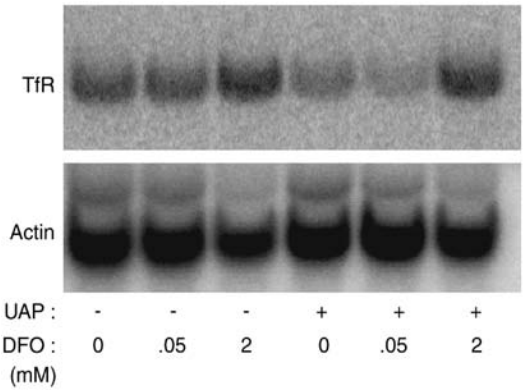

Fig. 6. Northern blot analysis of DFO effects on transferrin receptor mRNA. To assess the effectiveness of DFO in rat AMs, Northern blot analysis of transferrin receptor mRNA was performed. Exposure of rat AMs to 2 mM but not 0.05 mM DFO causes increased expression of Tfr mRNA, both in the absence and in the presence of CAPs. No changes were observed in expression of the beta-actin mRNA.

expression in primed AMs treated with or without UAP particles and DFO. Treatment with 2 mM DFO increased TfR mRNA levels 2- and 3-fold in control and UAP groups, respectively, indicating cellular iron depletion at this DFO concentration. No changes in TfR mRNA levels were observed in cells treated with 0.05 mM DFO, or in the expression of β-actin mRNA in any of the groups.

Primed AMs were treated with CAPs fractions (100 μg/ml), exogenous H_2O_2 (10 μM/h) and the chelator DFO (0.05 and 2 mM). The data are reported in two sets. The first examines the

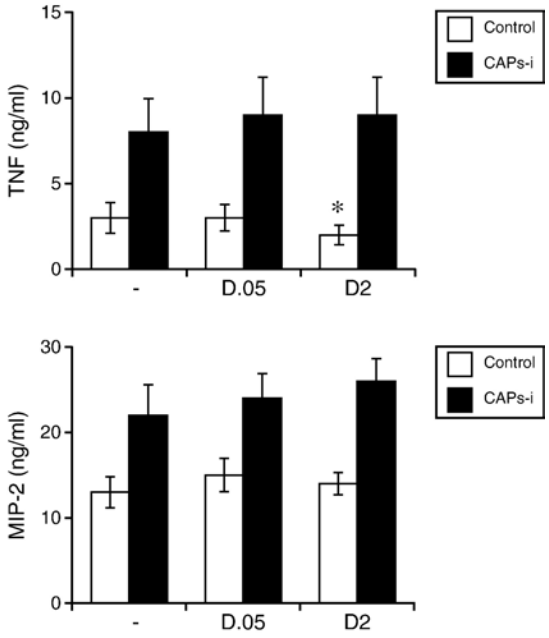

Fig. 7. Effect of DFO on response to CAPs insoluble fraction. Rat AMs were primed and then incubated for 20 h with nothing (light grey bars) or the insoluble fraction of CAPs (CAPs-i, black bars), $n = 4$. The CAPs-i was separated from a complete CAPs suspension at 100 μg/ml. Both control and CAPs-i-treated cells were also incubated with medium only or DFO at 0.05 mM (D.05) or DFO at 2 mM (D2). Both TNF (upper) and MIP-2 (lower) release were measured. DFO had minimal effects on the increased cytokine release induced by CAPs-i.

effects of DFO on cytokine release mediated by the insoluble fraction (CAPs-i) (Fig. 7). The data show that elevated production of TNF in the presence of CAPs-i was not inhibited by DFO at either concentration. Production of MIP-2 by AMs treated with CAPs-i was also insensitive to both high and low DFO concentrations (Fig. 7).

The second data set examines whether DFO inhibits the reactivity of CAPs-s in the presence of exogenous H_2O_2 (Fig. 8). In contrast to results with CAPs-i (Fig. 6), TNF production by AMs treated with H_2O_2 plus CAPs-s was significantly inhibited by DFO at 0.05 mM and completely inhibited by 2 mM DFO. Measurement of iron content in these soluble fraction samples confirmed the presence of iron (for #720, 816 and 829 for 7.4, 9.6 and 7.2 μM soluble iron, respectively). A similar result was seen with MIP-2 production where all CAPs-s bioactivity was removed in the presence of 2 mM DFO and 0.05 mM DFO caused partial inhibition (Fig. 8). Finally, the toxic effects on AM viability of CAPs-s plus H_2O_2 showed a trend towards inhibition that was not statistically significant at the lower 0.05 mM concentration and complete inhibition by 2 mM DFO.

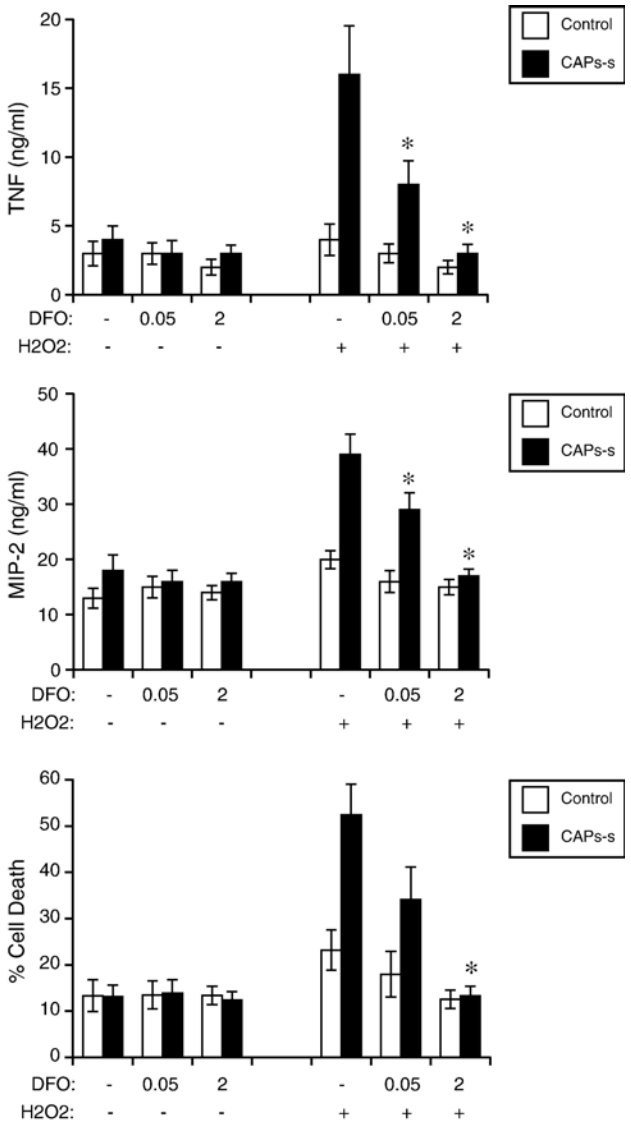

Fig. 8. Effect of DFO in response to the soluble fraction of CAPs. Rat AMs were primed and then incubated for 20 h with medium only (light grey bars) or the soluble fraction of CAPs (CAPs-s, black bars), $n=4$. The CAPs-s was separated from a complete CAPs suspension at 100 μg/ml. Both control and CAPs-s-treated cells were also incubated with medium only or DFO at 0.05 mM (D.05) or DFO at 2 mM (D2). Finally, cells with or without DFO were also treated with H_2O_2 at 10 μM/h. Three parameters were measured including TNF release (upper), MIP-2 release (middle) and cell death (lower). There were three comparisons made and significance was accepted when $p \leq 0.0167$. There was significant inhibition by DFO (*) of CAPs-s bioactivity in the presence of H_2O_2.

Discussion

The data presented address the role of oxidants in particle-mediated cytokine release by primed AMs. Our strategy was to use the positive control particle UAP (SRM 1649) to determine optimal treatment conditions, which were then used for study of the more relevant particles of interest, concentrated ambient particles (CAPs). The results show that, in primed AMs both UAP-mediated and CAPs-mediated TNF release are oxidant-dependent events and that H_2O_2 is involved. The antioxidant DMTU, a scavenger of H_2O_2, OH and hypochlorous acid, was dramatically effective at reducing TNF levels and catalase, a specific H_2O_2 scavenger, showed partial effects (Tables 1 and 2; Fig. 1). Since baseline TNF release (due to priming alone) is similarly inhibited by DMTU and catalase, we postulate that particles are triggering an oxidant-dependent mechanism for TNF release that overlaps with the cellular machinery induced by LPS priming. When comparing CAPs and UAP, there were differences noted in the effectiveness of NAC, supporting the value of studies on 'real-world' ambient particles. While NAC was not significantly effective at blocking UAP mediated TNF release, this antioxidant did significantly inhibit CAPs-mediated TNF production. Contrary to our postulate based on catalase and DMTU results, NAC caused significantly greater inhibition of CAPs-mediated TNF release when compared to inhibition of TNF released by 'primed-only' cells. However, this finding is nonetheless consistent with the oxidant dependence for CAPs effects indicated by use of DMTU and catalase and may reflect the ability of NAC to act as a direct free radical scavenger in addition to augmenting cellular glutathione levels. Finally, the inclusion of nitrite measurements provided additional useful information. While NAC had minimal effects on UAP-mediated TNF release, it dramatically reduced nitrite release by LPS-primed AMs, indicating an adequate level of NAC was used. In addition, in the absence of antioxidants, nitrite release remained unchanged while TNF production was increased (Table 1). Together these results support the conclusion that nitric oxide is not required for PM-mediated TNF release by primed AMs.

One goal of these studies was to learn more about how particles modulate oxidant-dependent cytokine release in primed AMs. Our previous work established that the bioactive components of CAPs suspensions (TNF and MIP-2 release) reside in the insoluble fraction (Imrich et al., 2000). Several studies have treated AMs in vitro with particle suspensions in the presence of oxidant reactive probes and found increased oxidant production. In one study both soluble and insoluble

fractions of CAPs caused increased oxidant stress in hamster AMs in vitro (Goldsmith et al., 1998). This increased oxidant production was significantly inhibited by the iron chelator desferoxamine (DFO), suggesting a portion of oxidant activity caused by CAPs (present as both soluble and insoluble fractions) was due to secondary oxidant generation mediated by iron (Goldsmith et al., 1998). Our working hypothesis was that in primed AMs, CAPs-i particles are bound and ingested by the AM. The particle dissolves in the phagolysosome and iron interacts with intracellular H_2O_2 to produce secondary oxidants, causing enhanced release of TNF. In support of this postulate, when H_2O_2 was added to the culture system both TNF and MIP-2 levels were increased by UAP and CAPs (Figs. 3 and 4) while treatment with H_2O_2 alone was insufficient. Such particle plus H_2O_2 effects were component-specific since the inert particle control TiO_2 did not stimulate AMs to produce more TNF when combined with H_2O_2 (Fig. 4).

Experiments using CAPs fractions led to some intriguing results. The ability of both CAPs-s and CAPs-i to react directly with H_2O_2 was confirmed when each were incubated with H_2O_2 and a DCFH-based oxidant reactive probe (OxyBurst-BSA, Fig. 6). Both soluble and insoluble fractions of CAPs were reactive with H_2O_2 and all reactivity was inhibited with DFO, supporting our hypothesis that iron from PM could mediate the H_2O_2-dependent cytokine release observed in primed AMs. However, when H_2O_2 was added to the cell culture system, CAPs-i showed no additional bioactivity (Fig. 4) while the soluble fraction became highly bioactive. It is possible that adding exogenous H_2O_2 does not necessarily mimic intracellular H_2O_2 production. We therefore sought to more directly test whether iron from CAPs-i is generating secondary oxidants from intracellular H_2O_2 (and thereby causing enhanced cytokine release). Rat AMs were treated with a high concentration of DFO to chelate intracellular iron and reduce any iron-dependent reactions (Figs. 6 and 7). Particle-mediated release of both TNF and MIP-2 were not affected by 2 mM DFO while this higher concentration depleted the cell of iron, supporting the conclusion that enhanced cytokine release caused by CAPs-i is independent of iron. Although contrary to our original hypothesis, this conclusion is consistent with results of elemental analysis applied to a panel of insoluble CAPs fractions reported previously (Imrich et al., 2000). In these analyses, the CAPs-i samples had similar bioactivity in primed AMs, including some samples which had no measurable iron content. In the present study of CAPs fractions, the high bioactivity of CAPs-s in the presence of exogenous H_2O_2 was inhibited by a low concentration of DFO (Fig. 8), one that had no effect on intracellular iron status (Fig. 6). Also, this low DFO concentration was effective at inhibiting all CAPs reactivity with H_2O_2 as reported by increased DCFH fluorescence (Fig. 5). This suggests soluble iron from CAPs-s plus H_2O_2 are in part, acting outside the cell at or near the plasma membrane surface to enhance cytokine release. The marked decreased viability caused by CAPs plus H_2O_2 is consistent with an environment where reactive oxidants are being generated outside the cell. Whether mediators released by cell death contribute to signaling for TNF release remains to be investigated. The bioactivity of CAPs-s plus H_2O_2 was completely inhibited using 2 mM DFO, indicating that all increases in cytokine release and cell death were caused by soluble iron.

It is worth noting that we intentionally chose CAPs samples with different bioactivities for different parts of our study. Specifically, for the anti-oxidant studies we chose CAPs samples at the higher end of bioactivity by pre-screening a panel of samples, as previously reported (Imrich et al., 2000). In contrast, for studies investigating possible enhancement of bioactivity by exogenous oxidants, we chose CAPs samples with relatively lower bioactivity to better allow detection of increases (samples #720, 816, 829, see Fig. 3).

These data support the conclusion that CAPs can mediate their effects in primed AMs by acting on oxidant-sensitive cytokine release in at least two distinct ways. In the primed cell, insoluble components of PM mediate enhanced TNF production that is H_2O_2 dependent (catalase-sensitive) yet independent of iron. In the presence of exogenous H_2O_2, soluble iron present in CAPs mediates cytokine release as well as increased cell death. The data suggest that within an inflamed alveolar milieu of a susceptible individual with preexisting lung disease, soluble iron released from inhaled air pollution particles may interact with oxidants released by PMNs or other lung cells. This interaction of soluble components, H_2O_2 and AMs may constitute a second pathway to enhanced inflammation. Diminished lung antioxidant status in certain disease states, e.g. asthma, may also contribute (Kelly et al., 1999). We have used LPS-primed AMs to model the activated macrophages present in chronic inflammatory disease, but studies using primary macrophages from patients or from disease models are needed to further test these findings. The data also support the potential utility of in vitro co-culture models to allow more detailed analysis of the complex mechanisms of particle effects.

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