INFORMATION OF INFLAMMASOME DEPENDENT PYROPTOSIS BY CARBON BLACK NANOPARTICLES*

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Running Title: Nanoparticles induce pyroptosis

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Inhalation of nanoparticles has been implicated in respiratory morbidity and mortality. In particular, carbon black nanoparticles are found in many different environmental exposures. Macrophages take up inhaled nanoparticles and respond via release of inflammatory mediators and in some cases, cell death. Based on new data, we propose that exposure of macrophages (both a macrophage cell line and primary human alveolar macrophages) to carbon black nanoparticles induces pyroptosis, an inflammasome-dependent form of cell death. Exposure of macrophages to carbon black nanoparticles resulted in inflammasome activation as defined by cleavage of caspase 1 to its active form and downstream IL-1β release. The cell death that occurred with carbon black nanoparticle exposure was identified as pyroptosis by the protective effect of a caspase 1 inhibitor and a pyroptosis inhibitor. These data demonstrate carbon black nanoparticle exposure was identified as pyroptosis by the protective effect of a caspase 1 inhibitor and a pyroptosis inhibitor. These data demonstrate carbon black nanoparticle exposure activates caspase 1, increases IL-1β release after LPS priming and induces the proinflammatory cell death, pyroptosis. The identification of pyroptosis as a cellular response to carbon nanoparticle exposure is novel, and relates to environmental and health impacts of carbon-based particulates.

Macrophages are critical regulators of local immune homeostasis. They are highly adaptive components of the innate immune system and respond in diverse ways to pathogens and other potential danger signals (1-3). In the lung, the alveolar macrophage is the first line of defense against environmental exposures. Alveolar macrophages phagocytose particulate matter, release inflammatory cytokines, and interact with other cells and molecules through the expression of surface receptors. One way in which an immune response is generated in alveolar macrophages is through the phagocytosis of deposited particles within the respiratory tract (4).

The nanoparticle industry has expanded substantially in recent years. A variety of engineered carbon nanoparticles are used in consumer products such as car tires, rubber, and printer toner cartridges (5). Nanoparticles are also being used as novel means of drug delivery. Additionally, carbonaceous nanoparticles are present as an environmental contaminant. Combustion processes are a significant source of carbon nanoparticles. Elemental carbon-based nanoparticles with a diameter of less than 100 nm are a major part of diesel exhaust and ambient pollution (6).

Particulate ambient pollution is known to cause adverse health effects in susceptible individuals, and aggravates existing respiratory conditions such as asthma and COPD (7). Even moderate levels of ambient air particulates are known to induce acute adverse health effects such as mortality in heart and lung diseases and chronic lung morbidity (8). Ultrafine particles are unique in their ability to bypass mucociliary clearance mechanisms and penetrate into deeper regions of the respiratory tract (9-12). Although bulk elemental carbon is considered chemically inert, (as in diamond and graphite), seemingly inert substances have been shown to elicit an inflammatory response when exposure occurs with nanoscale particles compared to an equivalent mass dose of larger particles (11-15). Carbon black (CB) nanoparticles can cause cytotoxic injury, increase levels of proinflammatory chemokines and inhibit cell growth (16). There are several explanations for this increased toxicity, including the increased surface...
area of nanoparticles (10,12,14,17-22). In a previous study, acute adverse effects of different types of carbonaceous nanoparticles instilled in mice strongly correlated with particle size and surface area (23). A surface area threshold of ~20 cm$^2$ was defined for acute lung inflammation in mice, below which no inflammatory responses were observed (23). CB nanoparticles showed higher surface reactivity compared with a similar dose of larger particles (24). CB nanoparticles have also been shown to induce oxidative stress in alveolar macrophages, and it is believed that this capacity for oxidation may be mediated by particle surface functionality (19,25-29). A recent study showed that the oxidative potency of CB nanoparticles correlates with their surface area and inflammatory responses (30). A possible mechanism for CB nanoparticles particle-related inflammation involves direct and indirect reactive oxygen species (ROS) generation by particle-cell interactions, which in turn activates redox-sensitive transcription of proinflammatory genes (30). ROS have been implicated as the cause of significant inflammation and, in some cases, cell death (31).

One possible outcome of macrophage exposure to nanoparticles is cell death. Cell death may be categorized according to several characteristics including non-inflammatory or pro-inflammatory, and accidental or programmed. Apoptosis, perhaps the best characterized of these mechanisms, is a programmed and non-inflammatory process. It is characterized by distinctive DNA cleavage, as well as activation of the executioner caspases, 3 and 9 (31,32). In contrast to apoptosis, necrosis is defined as an accidental and pro-inflammatory form of cell death, in which the plasma membrane loses its integrity, allowing rapid fluid influx, leading to cell swelling and lysis (32-34). Pyroptosis is a recently described mechanism of cell death, sharing unique characteristics with both necrosis and apoptosis (32-36). It is defined by its dependence on inflammasome activation and caspase 1 activity. Inflammasomes, which can differ in their subunit composition, have been shown to activate caspase 1, which, in the setting of a microbial stimulus, activates the proinflammatory cytokines IL-1β and IL-18 (37) (33,34). Like apoptosis, pyroptosis is a form of programmed cell death. But unlike apoptosis, pyroptosis is characterized by loss of membrane integrity. This is due to caspase 1-dependent insertion of a pore into the membrane, leading to fluid influx, cell swelling and lysis (38). Pyroptosis ultimately leads to release of cellular contents and inflammation (32-34,36,38,39).

The recent expansion of the nanotechnology industry, as well as the continually growing sources of combustion derived pollution, warrants investigation into the potential health effects of these nanoparticles. In this study, we examined the effect of CB nanoparticles on the inflammasome and pyroptosis. The data show that macrophage exposure to 20 ± 6 nm CB nanoparticles induces caspase 1 activation and IL-1β release and the pro-inflammatory form of cell death, pyroptosis.

**Experimental Procedures**

*Source of manufactured nanomaterial.* TiO$_2$ and carbon black (CB) nanoparticles were purchased from Degussa, GmbH (Düsseldorf, Germany). Manufacturer’s stated average diameters of titanium dioxide (TiO$_2$) nanoparticles (Degussa P25) and CB nanoparticles (Degussa Printex 90) are 21 nm and 14 nm, respectively. The nanoparticles were used as received from the manufacturer without modification.

*Bulk characterization of nanoparticles.* Powder X-ray diffraction (XRD) was used to identify crystalline phases of the sample. XRD was performed using a Bruker D-5000 q–q diffractometer with Kevex-sensitive detector (Madison, WI). High resolution transmission electron microscopy (HRTEM) (JEOL JEM-2100F, Japan) operating at 200 kV was used to image the nanoparticles and measure their diameters to compare the average diameter to the manufacturer’s specifications. Samples for TEM analysis were deposited from methanol suspensions onto Cu grids. Dynamic light scattering (DLS) (Beckman Coulter Delsa Nano C, Brea, CA) was used to measure hydrodynamic diameter of the nanoparticle aggregates in reduced serum media (OptiMEM, Invitrogen) which was used as a cell culture media in the cytotoxicity experiments. Inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis was performed to check for metal impurities in CB nanoparticles. The nanoparticles were digested in
concentrated nitric acid at 90ºC prior to the ICP analysis. The digested solutions were filtered and centrifuged for 30 minutes at 14,000 rpm in order to remove nanoparticles and aggregates that were not dissolved. The final solutions were analyzed by ICP-OES (Varian 720 ES, Walnut Creek, CA).

**Surface characterization of nanoparticles.** Surface area and surface composition of the TiO₂ and CB nanoparticles were examined. Surface area measurements of powdered samples were performed on an automated multipoint BET surface area apparatus (Quantachrome Nova 4200e, Boynton Beach, FL) using nitrogen gas as the adsorbent. Samples were degassed at 100ºC for 24 hours under vacuum before the analysis. Surface area of TiO₂ and CB nanoparticles was calculated using 7-point BET method. X-ray photoelectron spectroscopy (XPS) was used to probe the surface chemical composition characteristics of the powdered samples (Ultra-Axis DLD, Kratos, Manchester, UK). The system has been described before (40).

**Demonstration of intracellular CB nanoparticles (TEM).** Samples were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Post fixation was carried out for 1 hour at room temperature with a buffered 1% osmium tetroxide solution reduced with 1.5% potassium ferrocyanide. Samples were en bloc stained using 2.5% uranyl acetate. Cells were then rinsed and dehydrated. Infiltration of Spurr’s epoxy resin and acetone were carried out over several days to 100% resin and cured overnight in a 70ºC oven. Sections of 100 nm thickness were cut using a Leica EM UC6 ultramicrotome. Grids were then counterstained with 5% uranyl acetate for 12 minutes and Reynold’s lead citrate for 5 minutes. Samples were imaged using a JEOL 1230 transmission electron microscope.

**Cell culture.** RAW264.7 cells were obtained from ATCC (#TIB-71) and maintained in D-MEM with 10 % fetal bovine serum and gentamycin, 40 μg /ml. Cells were sub-cultured every two to three days. Experiments were run in 6 well Costar tissue culture plates, 96 well assay plates or coverslip chamber slides.

**Human alveolar macrophages.** To obtain normal human alveolar macrophages, subjects were recruited who were nonsmokers with no underlying medical conditions and on no medications other than possible birth control. After informed consent was obtained, subjects underwent standard flexible bronchoscopy. Bronchoalveolar lavage was performed by instilling 20 ml of normal saline into a tertiary bronchus up to five times in three different lung segments. The first collection out of five was discarded for possible contamination from upper airway secretions or by lidocaine, which is used to locally anesthetize the subject during the procedure. The remaining lavage was transported to the laboratory where fluid was filtered through sterile gauze and centrifuged at 200 x g for 5 min to pellet cellular material. Cells were washed twice in PBS and finally re-suspended in RPMI plus Glutamax for cell culture. Cyto-prep slides were also made with the cells, and were stained with Wright stain. Slides were microscopically examined to ensure that greater than 95% of the cells were macrophages (41-43).

The cells were then placed in culture and exposed to CB nanoparticles. All procedures and protocols described in this communication were approved by the University of Iowa Institutional Review Board. Written informed consent was obtained and all clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki.

**Whole cell protein isolation.** Whole cell protein was obtained by lysing the cells on ice for 20 minutes in 200 μl of lysis buffer (0.05 M Tris pH 7.4, 0.15 M NaCl, 1% NP-40, with added protease and phosphatase inhibitors: 1 protease minitab (Roche Biochemicals)/10 ml and 100 ul 100X phosphatase inhibitor cocktail (Calbiochem)/10 ml. The lysates were sonicated for 20 seconds, kept at 4º for 30 minutes, spun at 15,000 g for 10 minutes and the supernatant saved. Protein determinations were made using the Bradford Protein assay from Bio-Rad. Cell lysates were stored at −70º until use.

**Cell supernatant protein isolation.** To isolate proteins from cell supernatants, macrophages were cultured in Opti-MEM® from Invitrogen to allow for reduced serum culture. Cell supernatant protein was obtained by concentrating the supernatants in Amicon p10 filter tubes, spun at 3,000 g for 30 minutes. Protein determinations were made using the Bradford Protein assay from
Bio-Rad. Concentrated supernatants were stored at −70° until use.

**Western analysis.** Western analysis for the presence of active caspase 1 was performed on whole cell proteins and concentrated supernatants from RAW cell experiments. 30 μg of protein was mixed 1:1 with 2x sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue and 1.25 M Tris pH 6.8, all chemicals from Sigma Chemical Co.) heated to 95° for 5 minutes and loaded onto a 10% SDS-PAGE gel and run at 100 V for 90 minutes. Cell proteins were transferred to PVDF (Bio-Rad Hercules, CA) by semi-dry transfer (BioRad). Equal loading of the protein groups on the blots was evaluated using Ponceaus S, a staining solution designed for staining proteins on PVDF membranes or by stripping and reprobing with antibodies to beta actin or GAPDH. The PVDF was dried and then incubated with the primary antibody overnight in 5% milk. The blots were washed x4 with TTBS and incubated for 1 hour with horseradish-peroxidase conjugated anti-rabbit or mouse IgG antibody. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus, Amersham, Arlington Heights, IL). An autoradiograph was obtained, with exposure times of 10 seconds to 2 minutes.

**IL-1 β release.** For these studies, RAW cells were cultured in standard medium for 24 hours with and without LPS (10 ng/ml). After the culture period, the supernatants were harvested and stored at −70° until assayed. The amount of IL-1 β in the supernatant was measured by ELISA (R & D Systems, Minneapolis, MN).

**Cell survival analysis.** For analysis of cell survival, macrophages were cultured in 96 well tissue culture plates. Following incubations with nanoparticles, plasma membrane integrity was assayed by two methods (LDH release and PrestoBlue assay). Triplicate cultures were performed on all experiments. LDH released into the supernatant was monitored using CytoTox-ONE™ Homogeneous Membrane Integrity Assay which measures LDH release via a coupled fluorescent assay (Promega). PrestoBlue Cell Viability Assay was done following the manufacturer’s protocol.

**Whole cell DNA Isolation/DNA analysis.** For DNA analysis, cells were cultured in 100 mm tissue culture plates. Following incubations with particles, whole cells were harvested in 500 μl PBS and DNA was isolated (DNAeasy Blood and Tissue Kit, Qiagen, Valencia, CA). DNA concentration was measured and equal concentrations were loaded and run on a SYBR Green gel (E-Gel 2% with SYBR Safe, Invitrogen). The gel was visualized with ultraviolet light, and samples were examined for laddering.

**Quantitative RT-PCR.** Total RNA was isolated using the RNAqueous – 4PCR kit (Ambion Inc., Austin, TX) following the manufacturer’s instructions. RNA quality and quantity were assessed with Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA) using the Experion RNA StdSens Analysis Kit according to the manufacturer’s protocol. RNA quality was considered adequate for use if the 28S/18S ratio was >1.2 and the RNA Quality Indicator (RQI) was >7. Total RNA (300 ng) was reverse-transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad) following the manufacturer’s instructions. PCR reactions were performed as previously described (44). Specificity of the amplification was confirmed using melting curve analysis. Data were collected and recorded by CFX Manager Software and expressed as a function of threshold cycle (C_T).

**RESULTS**

**Nanoparticle characterization.** The results of particle characterization are summarized in Table 1. Primary particle diameters obtained from the TEM images (Figure 1A) indicate that TiO2 nanoparticles have diameter of 23 ± 3 nm and CB nanoparticles have diameter of 20 ± 6 nm (Table 1). For the CB nanoparticles, this result is close to
the manufacturer’s specified diameter of 14 nm. The aggregate size distributions of TiO$_2$ and CB nanoparticles were measured using DLS in OptiMEM media. Both types of nanoparticles form aggregates in the cell culture media. TiO$_2$ nanoparticles have bimodal size distribution with peaks at 146 ± 60 nm and 985 ± 450 nm with a larger size distribution dominating over a smaller size distribution. CB nanoparticles have aggregate sizes of 175 ± 80 nm (Table 1). The XRD analysis of TiO$_2$ nanoparticles indicates that there are anatase and rutile crystalline phases present in the sample (45). A diffraction pattern for CB nanoparticles is similar to the literature reports for CB (46) and indicates crystalline phases of graphitic domains. XPS analysis of TiO$_2$ nanoparticles indicates 33, 61 and 6% of Ti, O and C, respectively, showing close to stoichiometric TiO$_2$ ratio and residual hydrocarbon and hydroxyl groups present on the surface of the sample (45). A diffraction pattern for CB nanoparticles is similar to the literature reports for CB (46) and indicates crystalline phases of graphitic domains. XPS analysis of TiO$_2$ nanoparticles indicates 33, 61 and 6% of Ti, O and C, respectively, showing close to stoichiometric TiO$_2$ ratio and residual hydrocarbon and hydroxyl groups present on the surface of the sample (Figure 1B). High resolution XPS analysis of CB nanoparticles shows the presence of carbon at 285.0 eV with traces of oxygen at 530.0 eV (Figure 1B). ICP-OES analysis of CB nanoparticles digested in concentrated nitric acid revealed small amounts of metal impurities such as Ca (0.02%) and K (0.04%) present in the sample. The BET specific surface areas of TiO$_2$ and CB nanoparticles calculated using the 7-point BET method are 41 ± 1 m$^2$/g and 279 ± 6 m$^2$/g, respectively (Table 1). CB is considered to be a microporous material. The external surface area of CB nanoparticles calculated using the Halsey method (47) is 176 ± 9 m$^2$/g.

**CB nanoparticle exposure induces macrophage cell death (RAW264.7 cells).** To evaluate the effect of nanoparticles on macrophage viability, RAW264.7 cells were seeded into 96 well tissue culture plates and cultured for 24 hours with TiO$_2$ (30 μg/cm$^2$) or CB nanoparticles (30 μg/cm$^2$). At the end of the culture period, one group was treated with ATP as a positive control for cell death (31,38,45-47). Samples were assayed for LDH release. Figure 2A shows that CB nanoparticles, and not TiO$_2$ nanoparticles, induce LDH release from macrophages. To confirm cell death with an alternative assay, a PrestoBlue cell viability reagent was used. PrestoBlue analyzes cell death by determining the level of reducing activity associated with living cells. RAW264.7 cells were seeded into 96 well tissue culture plates and cultured for 24 hours in the same conditions. As with the LDH release assay, the PrestoBlue analysis showed cell killing with exposure to CB nanoparticles and not TiO$_2$ nanoparticles.

To further characterize the cell death caused by CB nanoparticle exposure, both time and dose response experiments were performed. Figure 2B demonstrates an increase in LDH release by 12 hours that continues increasing through 24 hours of particle exposure. Optimal effects were seen at a dose of 30 μg/cm$^2$. As a composite, these data show that CB nanoparticles, and not TiO$_2$ nanoparticles, decrease plasma membrane integrity leading to cell death.

**Nanoparticle-induced plasma membrane disruption is characterized by an increase in cell size.** The LDH and PrestoBlue assays suggest that exposure to CB nanoparticles disrupts the plasma membrane. To determine whether this reduced plasma membrane integrity affected cell size, cell size was quantified after exposure to CB nanoparticles. In the first experiment (Figure 3A), cells were exposed to CB nanoparticles and images were obtained immediately (15 minutes) and again after 24 hours. Cell size was determined by drawing circles around representative cells from 10 fields and calculating area using Image J software. Figure 3A shows an increase in cell size after exposure to CB nanoparticles (mean of 162.7 ± 17.57 mm$^2$ for control cells compared to 276.9 ± 39.99 mm$^2$ for CB nanoparticle exposed macrophages). To analyze volume changes, macrophages were loaded with a fluorescent tracer, cell tracker green CMFDA (Invitrogen). Figure 3B shows an increase in volume with CB nanoparticle exposure. To confirm that the macrophages were internalizing the particles, transmission electron microscopy was used to analyze particle exposed macrophages. Figure 3C demonstrates that CB nanoparticles are taken up by macrophages and appear to localize both in the cytosol and in membrane bound vesicles.

**CB nanoparticles activate the inflammasome (caspase 1 and IL-1β release).** Both necrosis and pyroptosis are characterized by LDH release and loss of plasma membrane integrity. We asked if CB nanoparticle exposure was activating the inflammasome (central to the pyroptosis form of cell death). The inflammasome’s two primary activation markers, caspase 1 activity and IL-1β release, were measured. Cells were primed for 3
hours with LPS (10 ng/ml). After priming, media was replaced, and fresh LPS was added, along with CB and TiO$_2$ nanoparticles for an additional 6 hours as previously described (48). Western analysis was performed using whole cell lysates, as well as concentrated supernatants. Figure 4A shows that in RAW264.7 cells, significantly more of the 20 kD cleaved caspase 1 was present in both the lysates and supernatants of cells exposed to CB nanoparticles as opposed to those exposed to TiO$_2$ nanoparticles. Recent studies have defined the release of active caspase 1 as a valid measure of inflammasome activity (49-53).

To determine if caspase 1 activation also occurred in a relevant human primary cell, human alveolar macrophages were cultured in the same conditions. Cell lysates and supernatants were harvested and Western analysis performed for active caspase 1. Figure 4B shows that in human alveolar macrophages, CB nanoparticle exposure caused activation of caspase 1 as demonstrated by the presence of the 20 kD cleaved caspase 1 in both cell lysates and supernatants. To confirm the caspase 1 activation, IL-1$\beta$ release was measured using an ELISA. Figure 4C demonstrates that with LPS priming both RAW264.7 cells and human alveolar macrophages increase IL-1$\beta$ release with CB nanoparticle exposure (p<0.01).

Figure 4C shows that CB alone does not induce IL-1$\beta$ protein release from macrophages. To further examine the role of LPS priming in the CB augmentation of IL-1$\beta$ release, we examined LPS or CB exposed cells for IL-1$\beta$, IL-18 and TNF$\alpha$ mRNAs. We found that LPS, but not CB, induced transcript up regulation (Figure 4D). This supports our conclusion that CB alone activates the inflammasome leading to cell death (pyroptosis), while CB plus a microbial stimulus leads to both pyroptosis and IL-1$\beta$ release.

To confirm that the increase in IL-1$\beta$ release with CB nanoparticles was due to caspase 1 activation, an experiment was performed using the caspase 1 inhibitor, YVAD. Figure 4E shows that the increase in IL-1$\beta$ release with CB nanoparticles is blocked in the YVAD exposed cells. As a composite, these data suggest that CB nanoparticle exposure in macrophages activates the inflammasome as shown by caspase 1 activation and IL-1$\beta$ release.

**CB nanoparticle-induced cell death is not due to apoptosis.** Because the CB nanoparticle-induced cell death is characterized by loss of plasma membrane integrity, it is unlikely that it is an apoptotic process. However, to confirm that apoptosis was not involved we examined the effect of CB nanoparticles on apoptosis. Macrophages were exposed to CB or TiO$_2$ nanoparticles for 16-24 hours. Stauroporine was used as a positive control for apoptosis. Following the incubation period, DNA was isolated from whole cell lysates. The DNA concentration was measured, and equal amounts were run on a SYBR green gel and visualized using ultraviolet light. Figure 5A shows that, while the cells exposed to staurosporine show distinctive DNA laddering characteristic of apoptosis, none of the CB nanoparticle exposed cells show DNA laddering. In a second set of experiments, macrophages from CB nanoparticle exposed cells were lysed, proteins isolated and Western analysis performed for activation of two caspases linked to apoptosis, caspase 3 and caspase 9 (31). Figure 5B shows that caspases 3 and 9 were activated in cells exposed to staurosporine but not in those exposed to CB nanoparticles. Taken in combination with the LDH and PrestoBlue assays, this demonstrates that CB nanoparticles induce non-apoptotic cell death.

**Inhibition of caspase 1 blocks CB nanoparticle-induced cell death in macrophages.** In light of the observation that CB nanoparticles activate the inflammasome and induce cell death in macrophages, we next sought to characterize the specific mechanism of cell death. The cell death modality pyroptosis is characterized by caspase 1 activation and the subsequent opening of cell membrane pores, resulting in an influx of extracellular fluid and eventual cell lysis (38). To confirm that the observed non-apoptotic cell death was pyroptosis, we examined the effects of a caspase 1 inhibitor (YVAD) and a pyroptosis inhibitor (glycine) on RAW264.7 cells exposed to CB nanoparticles. RAW264.7 cells were seeded into 96 well tissue culture plates and cultured for 24 hours with CB nanoparticles (30 $\mu$g/cm$^2$), CB nanoparticles in the presence of YVAD, or CB nanoparticles in the presence of glycine. At the end of the culture period, all samples were assayed for LDH release. Figure 6A shows that, as previously demonstrated, CB nanoparticle exposed
cells showed decreased membrane integrity as evidenced by LDH release. Both YVAD and glycine attenuated the effects of CB nanoparticle-induced LDH release. Cells exposed to CB nanoparticles in the presence of either of these inhibitors released near control levels of LDH.

**DISCUSSION**

In this study, we found that CB nanoparticles induced cell death in macrophages, and that this occurred in the absence of any detectable transition metals. These data show that following phagocytosis of the CB nanoparticles, macrophages increased in size - the opposite of the cellular condensation associated with apoptosis. Macrophage exposure to CB nanoparticles led to inflammasome activation, as characterized by caspase 1 activation and IL-1β release. The identification of the cell death undergone by exposed cells as pyroptosis was confirmed by the inhibiting effects of both a caspase 1 inhibitor and a pyroptosis inhibitor on CB nanoparticle-induced cell death (Figure 6B).

A daily dose of CB in the working environment can be as large as 120 µg/kg person, assuming a threshold limiting value for respirable carbon black of 2.5 mg/m³ (54). Additionally, an eight-hour average amount of elemental carbon detected on a heavily traveled roadway in Harlem was 6.2 µg/m³ (55), which corresponds to the alveoli burden of 7 µg, assuming a respiratory volume of 0.7 m³ per hour and 0.2 alveolar deposition fraction for 20 nm particles based on human deposition model (56). In the current study, 30 µg/cm² of CB nanoparticles were applied to cell culture wells. Although it is difficult to compare these doses with the mass per mass and mass per volume concentrations discussed above, it is within the mass range that is observed in occupational and environmental settings but does represent a substantial dose for respirable carbon black.

Pyroptosis, a pro-inflammatory form of cell death, proceeds through the activation of the inflammasome, leading to cleavage of caspase 1 into its active form. Once activated, caspase 1 cleaves the pro-inflammatory cytokines IL-1β and IL-18 into their active forms, allowing for their release into the extra-cellular environment. Although indicative of caspase 1 activation, the release of these inflammatory cytokines is not required for caspase 1 activation or pyroptosis. The production of pro-IL-1β and pro-IL-18 is mediated by toll like receptors (TLRs). The release of active IL-1β and IL-18 associated with pyroptosis in previous reports (36,57-65) has all included TLR stimulatory effects of microbial antigens. Thus, while priming cells with LPS prior to nanoparticle exposure allows for an additional confirmation of caspase 1 activation, inflammasome activation can occur separately from LPS priming. This is supported by previous reports, showing that caspase 1 induced cell death may proceed independently of IL-1β and IL-18 secretion when a microbial stimulus is not present (38,66).

Pyroptosis is characterized by a loss of membrane integrity (32-34,38). While the exact mechanism of this remains unknown, it has been demonstrated that membrane pore formation occurs, leading to cell swelling, and necrosis-like lysis. Our data supports pyroptosis after CB nanoparticle exposure with evidence of cell swelling, caspase 1 activation and cell death.

There have been a number of inflammasomes characterized with different subunits. Although this study does not specify which inflammasome CB nanoparticles activate, it has been shown that particulate matter including silica, asbestos, MSU, cholesterol crystals (37,67,68), and aluminum adjuvants (69) induce a caspase 1 dependent inflammatory response mediated by the NALP3 inflammasome (31,37,70-72). As such, these data suggest that CB nanoparticles activate the NALP3 inflammasome as well, though ongoing studies will further characterize the CB nanoparticle inflammasome. To the best of our knowledge, this is the first instance in which nanoparticles have been implicated in inducing pyroptosis (31).

Several mechanisms of inflammasome activation have been proposed, including the generation of ROS (13,18,73-78), potassium efflux (50), cathepsin B (79), and phagosomal destabilization(28,48,72,80). Disintegration of the cellular membrane by CB nanoparticles can cause the production of ROS. Aam and Fonnum showed that low doses of CB nanoparticles activate rat alveolar macrophages to produce ROS (28). They suggested that the ERK MAPK pathway participates in intracellular signaling leading to the ROS generation. Hornung et al demonstrated that
the NALP3 inflammasome activation induced by silica crystals and aluminum salts could be replicated via sterile lysosomal damage, implicating intracellular pH or cathepsin B activity in inflammasome activation (48). Even nanoparticles made from materials that are considered inert in bulk form have been found to induce pulmonary inflammation when exposure occurs with nanoscale particles. Although this study did not find TiO$_2$ nanoparticles to be inflammasome activating in macrophages, Yazdi et al showed that in human keratinocytes, nano-TiO$_2$ activates the NALP3 inflammasome and induces IL-1$\beta$ (51). Any or all of the potential mechanisms discussed may apply to CB nanoparticles’ mechanism of inflammasome activation. Further investigation into the ROS generated by alveolar macrophages in response to CB nanoparticle exposure is warranted.

The present study shows that macrophage exposure to CB nanoparticles activates the inflammasome leading to pyroptosis. CB merits further investigation into its mechanisms of inflammation modulation (increased IL-1$\beta$ release) and pyroptosis. As a primary component in ambient pollution and diesel exhaust, and a component of toners in printers used in office buildings worldwide, CB nanoparticles are a critical target for study. A better understanding of their mechanism of inflammasome activation may allow us to appropriately regulate potential health hazards.

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**FOOTNOTES**

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The abbreviations used are: BET, Brunauer-Emmett-Teller; CB, carbon black; CMFDA, 5-chloromethylfluorescein diacetate; COPD, chronic obstructive pulmonary disease; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbant assay; ICP-OES, inductively coupled plasma optical emission spectroscopy; IL-1 β, interleukin 1 β; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NALP3, NACHT domain, leucine-rich-repeat (LRR) domain, and pyrin domain (PYD)-containing protein 3; ROS, reactive oxygen species; TEM, transmission electron microscopy; TTBS, tris buffered saline; XPS, x-ray photoelectron spectroscopy; XRD, x-ray diffraction; YVAD, benzylxycarbonyl-Tyr-Val-Ala-Asp(OMe)-fluoromethylketone.
FIGURE LEGENDS

Figure 1. Nanoparticle characterization. A. High resolution TEM images of CB and TiO$_2$ nanoparticles. The scale bar in both images is 20 nm. Both nanoparticles form aggregates when in solution. Particles were sonicated for 1 hour and vortexed vigorously prior to cell exposure. Primary particle diameters were obtained from the images. B. Surface composition of TiO$_2$ and CB nanoparticles was measured using X-ray photoelectron spectroscopy.

Figure 2. CB nanoparticles induce macrophage cell death. A. RAW264.7 cells were exposed to CB (30 μg/cm$^2$) or TiO$_2$ (30 μg/cm$^2$) nanoparticles for 24 hours. One group was treated with ATP as a positive control for LDH release. At the end of the culture period, LDH analysis was performed to determine cell viability. The experiment was repeated using identical conditions. A PrestoBlue cell viability assay was performed as indicated by the manufacturer. Significance was determined using nonpaired Student’s $t$ test. B. RAW264.7 cells were exposed to CB nanoparticles (30 μg/cm$^2$) for 4, 12, and 24 hours. ATP was used as a positive control for LDH release. At the end of the culture period, an LDH assay was performed to determine cell viability. Additionally, RAW264.7 cells were exposed to CB nanoparticles at 3 μg/cm$^2$, 15 μg/cm$^2$, or 30 μg/cm$^2$ for 24 hours. An LDH assay was performed. Significance was determined using nonpaired Student’s $t$ test.

Figure 3. CB nanoparticles cause an increase in macrophage cell size. A. Bright field images of CB nanoparticle exposed cells were obtained 15 minutes after exposure and again after 24 hours. Circles were drawn around representative cells from 10 fields and then used to calculate cellular area. B. Fluorescent images of cells were obtained before CB nanoparticle exposure and again, 24 hours after exposure. Cells were loaded with cell tracker green CMFDA. Average cellular volume was calculated from the level of fluorescence per cell. C. Transmission electron microscopy images of control and CB nanoparticle exposed cells.

Figure 4. CB nanoparticles activate the inflammasome. A. Activation of caspase 1 in RAW264.7 cells. 3x10$^6$ RAW264.7 cells per well were seeded into culture plates. Cells were primed for 3 hours with LPS (10 ng/ml). After 3 hours, media was aspirated off of cells and replaced with fresh. LPS was re-added, and CB (30 μg/cm$^2$) or TiO$_2$ (30 μg/cm$^2$) nanoparticles were added for an additional 6 hours. After the culture period, whole cell lysates were harvested in Western Lysis Buffer. Supernatants were concentrated and proteins analyzed. Western analysis for caspase 1 was performed on cellular lysates and concentrated supernatants. B. 3x10$^6$ human alveolar macrophages per well were seeded into culture plates. Cells were cultured as above, except for an extended second incubation time (16-24 hours). Supernatants were harvested and IL-1β levels measured by ELISA. Significance was determined using nonpaired Student’s $t$ test. C. 1x10$^5$ RAW264.7 cells per well were seeded into culture plates. Cells were cultured as above, except for an extended second incubation time (16-24 hours). Supernatants were harvested and IL-1β levels measured by ELISA. Significance was determined using nonpaired Student’s $t$ test. D. Expression of IL-1β, IL-18, and TNFα mRNA in human alveolar macrophages exposed to LPS or CB nanoparticles. 3x10$^6$ human alveolar macrophages per well were seeded into culture plates. Cells were incubated with CB nanoparticles (30 μg/cm$^2$) or LPS (10 ng/ml) for 4 hours. RNA was isolated and qRT-PCR performed. * p< 0.01 by Student’s t-test (compared to control). E. 1x10$^6$ RAW264.7 cells per well were seeded into culture plates. Cells were primed for 3 hours with LPS (10 ng/ml). For the second incubation period, LPS was re-added and CB nanoparticles (30 μg/cm$^2$), TiO$_2$ nanoparticles (30 μg/cm$^2$), YVAD (100 μM), and ATP were added for an additional 24 hours. Supernatants were harvested and IL-1β levels measured by ELISA.

Figure 5. Nanoparticle-induced cell death is not apoptosis. A. 5x10$^6$ RAW264.7 cells were seeded into 100 mm culture dishes and incubated with LPS (10 ng/ml), CB nanoparticles (30 μg/cm$^2$), or both
for 24 hours. One group was exposed to staurosporine (1 μM) as a positive control for apoptosis. Following the culture period, DNA was isolated. DNA concentration was measured and equal concentrations were loaded and run on a SYBR Green gel. The gel was visualized with ultraviolet light, and samples were examined for laddering. B. 3x10^6 RAW264.7 cells per well were seeded into culture plates. Cells were exposed to CB nanoparticles (30 μg/cm²), TiO₂ nanoparticles (30 μg/cm²), or staurosporine (1 μM) for 24 hours. Proteins were isolated and Western analysis for the apoptosis-associated caspases 3 and 9 was performed.

**Figure 6.** Blocking caspase 1 activation protects macrophages from CB nanoparticles toxicity. A. RAW264.7 cells were exposed to CB nanoparticles (30 μg/cm²), CB nanoparticles in combination with the pyroptosis inhibitor glycine (5 mM), CB nanoparticles in combination with the caspase 1 inhibitor YVAD (100 μM), or TiO₂ nanoparticles (30 μg/cm²) for 24 hours. At the end of the culture period, LDH analysis was performed to determine cell viability. B. The diagram summarizes the CB nanoparticle induced pathway to inflammasome activation and pyroptosis, as supported by this study.
Table 1. Summary of physicochemical characterization data of the nanoparticles.

<table>
<thead>
<tr>
<th></th>
<th>Detection method</th>
<th>TiO$_2$</th>
<th>Carbon Black</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary particle size</td>
<td>TEM</td>
<td>23 ± 3 nm</td>
<td>20 ± 6 nm</td>
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<tr>
<td>Phase</td>
<td>XRD</td>
<td>Anatase/rutile</td>
<td>Graphitic domains</td>
</tr>
<tr>
<td>BET surface area</td>
<td>BET</td>
<td>41 ± 1 m$^2$/g</td>
<td>279 ± 6 m$^2$/g</td>
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<td>Aggregate size in OptiMEM media</td>
<td>DLS</td>
<td>146 ± 60 nm</td>
<td>175 ± 80 nm</td>
</tr>
</tbody>
</table>
Reisetter et al, 2011

Figure 1

A. HRTEM images of carbon black and TiO$_2$ nanoparticles.

![HRTEM images](image)

B. XPS spectra of carbon black and TiO$_2$ nanoparticles

![XPS spectra](image)
Reisetter et al, 2011

Figure 2

A.
Lactate Dehydrogenase Release Assay

Presto Blue Viability Assay

B.
Carbon Black: Time Course

Carbon Black: Dose Response

* p < 0.01
Reisetter et al, 2011

Figure 3

A.

Control

Carbon Black

B.

Control

Carbon Black

1272 +/- 41.5 mm$^3$

2324 +/- 194.2 mm$^3$

$p < 0.01$

C.

Control

Carbon Black

Cell size (mm$^2$)

Time of CB exposure

0 200 400 600

15 min 24 hr

$p < 0.05$
Figure 4.

A. Control
LPS
LPS+CB
LPS+TiO2

Caspase 1 precursor, 45 kD
Cleaved caspase 1, 20 kD
β Actin, 42 kD

Raw 264.7 cell lysates

B. Control
LPS
LPS+CB

Caspase 1 precursor, 45 kD
Cleaved caspase 1, 20 kD
Unknown band

Alv. macrophage cell lysates

C. RAW 264.7 Cells

IL-1β, pg/ml

Control
LPS
LPS+CB

p<0.01

D. RAW 264.7 Cells

IL-1β mRNA (fold increase)

Control
CB
LPS

IL-1β, pg/ml

Control
LPS
LPS+CB

E. Alveolar Macrophages

IL-18 mRNA (fold increase)

Control
CB
LPS

TNF-α mRNA (fold increase)

Control
CB
LPS

p<0.01

ns

*
Reisetter et al, 2011

Figure 5

A.

DNA Ladder

Control CB LPS LPS + CB Staurosporine

B.

Pro-caspase 3 35kD
Cleaved Caspase 3 12,17 kD
GAPDH, 36 kD

Caspase 3

Pro-caspase 9 51kD
Cleaved Caspase 9 35,37 kD
GAPDH, 36 kD

Caspase 9
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Figure 6

A.

B.

Nanoparticle exposure
(carbon black)

LPS

Inflammasome

Pro-IL1β

Cleaved and released
IL1β

Caspase 1 → YVAD

Pore formation → Glycine

Cell swelling and Death

** p< 0.01
Induction of inflammasome dependent pyroptosis by carbon black nanoparticles
Anna C. Reisetter, Larissa V. Stebounova, Jonas Baltrusaitis, Linda Powers, Amit Gupta, Vicki H. Grassian and Martha M. Monick

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