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SIZE-DEPENDENT EFFECT OF ZINC OXIDE ON TOXICITY AND INFLAMMATORY POTENTIAL OF HUMAN MONOCYTES

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With the rapid development of nanomedicines, it is important to understand their potential immunotoxicity. Zinc oxide (ZnO) nanoparticles have several applications in the pharmaceutical and biomedicine industries. This study investigates the effect of particles size (nano and micro) of ZnO on viability, phagocytosis, and cytokine induction in human monocytes, THP-1 cells, a model of the innate immune system. Cells were incubated with nano (approximately 100 nm) and micro (approximately 5 μm) sized ZnO particles in a concentration range of 10–100 μg/ml. The parameters measured included the MTT assay, phagocytosis assay, enzyme-linked immunosorbent assay (ELISA), gene expression, and DNA analysis. ZnO particles significantly decreased cell viability in a size- and concentration-dependent manner associated with significant alterations in phagocytic capacity of monocytes. Exposure of THP-1 cells to both sizes of ZnO stimulated and increased release of proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, as well as chemokine IL-8, and upregulated the expression of monocyte chemoattractant protein-1 and cyclooxygenase-2 genes. However, ZnO particles did not markedly affect monocytes DNA. Collectively, these results suggest higher propensity of nano ZnO particles in inducing cytotoxicity and inflammation in human monocytes regardless of micro size, and caution needs to be taken concerning their biological application.

Particle size is an important factor in evolution of biological effects of respirable and insoluble particulate matter (Donaldson and Tran, 2002; Donaldson et al., 2002). Nanoparticles (NPs) have been widely studied in various medical fields, such as drug delivery, discovery of biomarkers, molecular diagnostics, and gene therapy (Zhao and Castranova, 2011). Despite the many proposed advantages of nanomaterials, increasing concerns have been expressed on their potential adverse human health and environmental effects (Gulumian and Vallyathan, 2010; Maynard et al., 2004; Price et al., 2010; Tabet et al., 2009; LeBlanc et al., 2009; Scuri et al., 2010; Kim et al., 2010). Prior to NP use for medical applications, their biological behavior and toxicological properties need to be assessed. Thus, it is necessary to understand the interactions of NP with biological systems.

As foreign particles, NP are able to affect host immune responses (Wan et al., 2008). Monocytes are among the first immune cells recruited to an invasion site in response to foreign materials. With the rapid and extensive research into the design of novel nanomedicines, it is critical that attention be directed to their potential immunotoxicity. Recently, studies focused on nano-immunotoxicity and found that some inorganic particles such as hydroxyapatite particles, nano-cobalt, and quantum dots activate

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monocytes to increase release of proinflammatory cytokines and reactive oxygen species (ROS) (Grandjean-Laquerriere et al., 2005; Lee et al., 2009). Zinc oxide nanoparticles (ZnO NP) have several applications in the pharmaceutical industry and biomedicine (Hong et al., 2011; Nag et al., 2011; Rasmussen et al., 2010; Yang and Xie, 2006) and are commonly used in sunscreens (Osmond and McCall, 2010; Zhao and Castranova, 2011) and as antibacterial agents (Applerot et al., 2009). Hanley et al. (2009) demonstrated that ZnO NP specifically target cancer cells and could possibly be developed as an alternative anticancer therapeutic agent. Hence, it is imperative to rigorously characterize the health and safety aspects of human exposure to ZnO NP.

Most human diseases are associated with local or systemic inflammatory responses. Epidemiological and experimental studies showed that individuals with preexisting inflammatory conditions are more prone to the adverse effects of environmental injury (Pope et al., 1995; Hollingsworth et al., 2007; Manzo et al., 2010). Indeed, aggravation of preexisting inflammation was noted after exposure to particulate matter (PM) air pollution and various types of NP (Hussain et al., 2011; Inoue et al., 2006; Kamata et al., 2011). Inflammatory cytokines play an important role in the intercellular signaling cascade leading to tissue recruitment of leukocytes challenged by a pathogen or by PM. Chemokines are secondary proinflammatory mediators that are typically induced by primary proinflammatory mediators such as interleukin-1 (IL-1) or tumor necrosis factor (TNF). Monocyte chemoattractant protein-1 (MCP-1) stimulates both chemotaxis of monocytes and several cellular events associated with chemotaxis. Monocytes represent an important source of IL-8 (Groves and Jiang, 1995), which acts as a potent chemoattractant for neutrophils (Huber et al., 1991).

Evidence indicates that risks associated with NP exposure require investigation due to evidence that these particles may be more inflammogenic and toxic than larger particles comprising of the same material. Investigators demonstrated that ultrafine particles (average diameter $\geq$ 100 nm) exert marked toxic effects and are more likely to induce inflammatory responses and lead to development of PM-mediated lung diseases compared to same mass of fine particles (Ferin et al., 1990, 1991, 1992; Li et al., 1996). Animal studies with carbon black and titanium dioxide particles noted that particles smaller than 30 nm have a greater ability to induce lung inflammation than larger particles with the same nominal composition (Churg et al., 1999; Li et al., 1999). Both Donaldson et al. (2002) and Oberdorster (2001) concluded that ultrafine particles of low-solubility, low-toxicity materials are more inflammogenic in rat lung than larger particles from the same material, and postulated that the effects are related to surface area and involve oxidative stress.

Vernath et al. (2007) found low potency of both nano- and micrometer metal oxide particles to induce proinflammatory cytokines. In studies conducted in immortalized rodent lung epithelial cells, alveolar macrophage cell lines, and primary alveolar macrophages, ZnO NP failed to induce TNF-α (Beyerle et al., 2009; Sayes et al., 2007). In addition, Prach et al. (2012) also noted that ZnO NP and bulk ZnO exerted little effect on immunological markers of inflammation such as HLA DR, CD14, and TNF-α. In contrast, Hanley et al. (2009) demonstrated that ZnO NP induced the expression of immunoregulatory cytokines. Gojava et al. (2007) observed that ZnO NPs in human aortic endothelial cells increased IL-8 and MCP-1 cytokine mRNA expression.

Therefore, the present study investigated the effects of particles size (nano and micro) of ZnO on viability, phagocytosis, and cytokine induction in human monocytes, THP-1 cells. These undifferentiated THP-1 cells express many of the properties of monocytes and represent a model of innate immune system (Tsuchiya et al., 1980). These cells are an essential link between the adaptive and innate immune responses because they (1) develop into various forms of antigen-presenting cells including macrophages and dendritic cells, and (2) are often used as a model to study human

inflammatory responses, which enables elucidation of interactions of NP with innate immune cells (Dobrovolskaia et al., 2008; Nicholas and Sumbayev, 2009).

MATERIALS AND METHODS

Particle Preparation and Characterization

ZnO nanopowder (<100 nm) and micro ZnO (<5 μm) were purchased from Sigma-Aldrich. Physicochemical properties of particles were analyzed using transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential analyzer. The morphology and size of NP in the stock dispersion were determined by TEM. Dry powder of particles was suspended in cell culture medium at a concentration of 1 mg/ml, and sonicated at room temperature for 10 min to form a homogeneous suspension. After sonication and stabilization, TEM samples were prepared by drop coating of the stock suspension on carbon-coated copper grids. The films on the grids were allowed to dry prior to measurement. TEM measurements were performed at an accelerating voltage of 120 kV (model 1200EX, JEOL Ltd., Tokyo, Japan). ZetaPALS (Brookhaven Instruments Corporation, Holtsville, NY) was used to determine the hydrodynamic size and zeta potential of particle suspension in cell culture medium.

Cell Culture

The human monocytic cell line THP-1 was obtained from the National Centre for Cell Sciences, Pune, India. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 m*M*), streptomycin (100 μg/ml), and penicillin (100 U/ml). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were exposed to different concentrations over a range (10–100 μg/ml) for 24 h, with nano and micro ZnO suspension. Cells free of ZnO particles were used as control cells throughout each assay.

Assessment of Cytotoxicity

THP-1 cells were incubated with nano and micro ZnO at a concentration of 10, 20, 40, 80, and 100 μg/ml for 24 h. Following incubation, cytotoxicity of particles was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Denizot and Lang, 1986). Briefly, MTT (20 μl/well of 5 mg/ml stock) was added and incubated for 4 h. Supernatants were removed by centrifugation and then 300 μl dimethyl sulfoxide (DMSO) was added. After thorough mixing, optical density at 570 nm was detected by microplate reader (Biotek, USA). Control values (without ZnO particles) were set at 100% viable and all values were expressed as percent of control, and respective TC-50 (particle concentration inducing 50% cell mortality) concentrations were calculated using GraphPad Prism software.

Lactate Dehydrogenase (LDH) Leakage Assay

Release of lactate dehydrogenase (LDH) to the cell culture medium indicates cell membrane damage (Wroblewski and LaDue, 1955). After exposure of ZnO particles to THP-1 cells, cell culture supernatant was collected by centrifugation for LDH activity analysis. LDH catalyzes the conversion of NAD^+ to NADH. A solution of lactate and NAD^+ was added to culture supernatant. Thus the rate of NAD^+ reduction is directly proportional to LDH activity. The decrease in absorbance every minute for 5 min was recorded at 340 nm (Bio-Tek, USA), and enzyme activity was expressed in units per liter.

Phagocytosis Assay

THP-1 cells were exposed to varying concentration of TC-50 (i.e., ½TC50, TC50, and 2TC50) of nano and micro ZnO particles for 4 h. After exposure, the phagocytic ability of

THP-1 cells was assessed by measuring their ability to phagocytose 1-μm latex beads (1-μm latex beads, carboxylate modified polystyrene, fluorescent yellow-green). The method used was modified from the method of Schroeder and Kinden (1983). After exposure, cells were washed twice with phosphate-buffered saline (PBS) to remove excess particles. Culture medium containing latex beads at a bead-to-cell ratio of 10:1 was transferred to the culture wells. Monocyte and bead suspensions were then incubated for 1 h to allow for phagocytosis. Beads not phagocytosed were removed by centrifugation at 225 × g for 5 min and cell pellet was then resuspended in PBS pH 7.2. The process was repeated 3 times, and finally cells were vortexed for 10 s and fluorescence of the cells was determined at excitation and emission wavelengths set at 440 nm and 485 nm, respectively. Cell viability during phagocytosis assay was monitored by trypan blue exclusion. Viability was 95 ± 5% throughout the assay. Representative microscopic images of phagocytosed latex beads at TC-50 concentration of test particles were also taken. After washing, the cells were seen under a microscope. Differential interference contrast (DIC) images or paired DIC and fluorescence images of phagocytosed beads by monocytes were captured using a Zeiss Axio Scope A1 microscope (Zeiss, Germany) with 40× dry objective.

Cytokine Analysis

To investigate the influence of size of ZnO particles on cytokine production, an enzyme-linked immunosorbent assay (ELISA) was performed. For determination of IL-1β, IL-6, TNF-α, and IL-8, monocytes were cultured at 1 × 10^6 cells/ml and exposed to a TC-50 concentration of nano and micro ZnO particles (17.69 and 38.35 μg/ml, respectively) for 6, 18, 24, and 48 h. After particle exposure, cell-free supernatants were harvested via successive 10-min centrifugations (1500 × g , 6500 × g, and 12,000 × g) and stored at –80°C until analysis. ELISA was performed according to the manufacturer's protocol (Abcam ELISA Kit), and absorbance values were measured using a microplate reader (Biotek Instruments, USA). At a minimum, three independent experiments were performed and concentrations were calculated from the linear regression equation derived from a set of standard absorbance values.

Gene Expression Analysis

Cells were exposed to a TC-50 concentration of ZnO particles (nano ZnO: 17.69 μg/ml, and micro ZnO: 38.35 μg/ml) for different time periods, namely, 6, 18, and 24 h. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, USA). The concentration and integrity of RNA were measured using a multimode microplate reader (Bio-Tek, USA) prior to the experiment. An Enhanced Avian HS reverse-transcription polymerase chain reaction (RT-PCR) kit (Sigma, USA) was used for the amplification of COX-2, MCP-1, and 18s-rRNA gene, according to the manufacturer's instructions. Amplified c-DNA products were separated on 1.2% agarose gel by electrophoresis. The primer sequences of amplified genes are shown in Table 1.

Detection of DNA Damage

THP-1 cells exposed to nano and micro ZnO particles (0, 10, 20, 40, 80, or 100 μg/ml) for 24 h were collected and washed with PBS. Cells were incubated for 3 h in lysis buffer (20 m*M* Tris-HCl, pH 8, 5 m*M* ethylenediamine tetraacetic acid [EDTA], 0.1 *M* NaCl, 0.5% sodium dodecyl sulfate [SDS], and 100 μg/ml RNase) at 37°C. After incubation, phenol:chloroform (1:1) mixture was used to extract DNA; then DNA was precipitated by adding an equal volume of ice-cold absolute isopropanol. The precipitated DNA was then dissolved in 50 μl of 1× TE (10 m*M* Tris, 1 m*M* EDTA, pH 8) buffer. Then 20 μg DNA was loaded onto 1.2% agarose gel; electrophoresis was carried out at 60 V for 2 h with TBE as the running buffer. DNA on agarose gel was visualized under ultraviolet (UV) light (Allen et al., 1997).

TABLE 1. Primer Sequence of Cyclooxygenase-2 (COX-2), Monocyte Chemoatractant Protein-1 (MCP-1), and 18s rRNA Gene Used in This Study

Primer name	Primer sequences	T_m	Number of cycles performed
COX-2	F: TTCAAATGAGATTGTGGGAAAATT R: AGATCATCTCTGCCTGAGTATCTT	59.1	35
MCP-1	F: AATCAATGCCCCAGTCACCTGC R: CGCAGTTTGGGTTTGCTTGTCC	62.1	35
18s rRNA	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	58.3	35

Statistical Analysis

Statistical analysis was carried out with GraphPad Prism 4 statistical software (GraphPad Software, Inc., San Diego, CA). Values are presented as mean ± SEM of three independent replicate experiments, unless otherwise stated. One-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons was used to evaluate the various responses induced by different concentration of particles, and statistical comparisons between particle sizes were performed with two-way ANOVA, followed by a Bonferroni post hoc test. Differences were considered statistically significant when the p was $<.05$.

RESULTS

Particle Characterization

TEM analysis was performed to determine the morphology and size of the particles. The particles were nearly spherical and cubical in shape (Figure 1). The primary sizes of the particles estimated from TEM images are presented in Table 2. Since NP often form agglomerates in a solution, the hydrodynamic sizes of the dispersed particles and their agglomerates in cell culture medium were estimated using ZetaPALS. These values were larger than the particle size measured by TEM (Table 2). Data indicate a strong agglomeration of particles in aqueous solutions. This was further corroborated by zeta potential measurements of 17.50 mV for nano ZnO and –8.60 mV for micro ZnO, illustrating that the charge of the hydrated particles was not sufficient to repel each other by electrostatic forces.

Effect of ZnO Particle on Cell Viability

Both sizes of ZnO particles induced a concentration-dependant decrease in cell viability. Significant size selective differences were seen at concentration of 20–100 μg/ml (Figure 2). The calculated TC-50 values of ZnO particles presented in Table 3 demonstrated lower toxicity of micro ZnO than nano ZnO.

Influence of ZnO Particle on LDH Leakage

According to the LDH activity assay, both size of ZnO produced a concentration-dependent rise in LDH activity (Figure 3). Nano ZnO showed greater release of LDH at all concentrations compared to micro ZnO. Significant

TABLE 2. Particle Characterization

Particles	Description	Average size[a]	Size using TEM[b] (nm)	Size in media[c] (nm)	PDI[c]	Zeta potential[c] (mV)
ZnO nano	Zinc oxide nanopowder	<100 nm	94.5 ± 7.7	253.6 ± 2.5	0.311 ± 0.003	17.50 ± 1.17
ZnO micro	Zinc oxide	<5 μm	158.3 ± 9.8	391.2 ± 0.8	0.266 ± 0.006	−8.60 ± 3.29

Note. PDI: polydispersity index.
[a]According to the manufacturer Sigma–Aldrich.
[b]Using transmission electron microscopy.
[c]Using Zeta PALS.

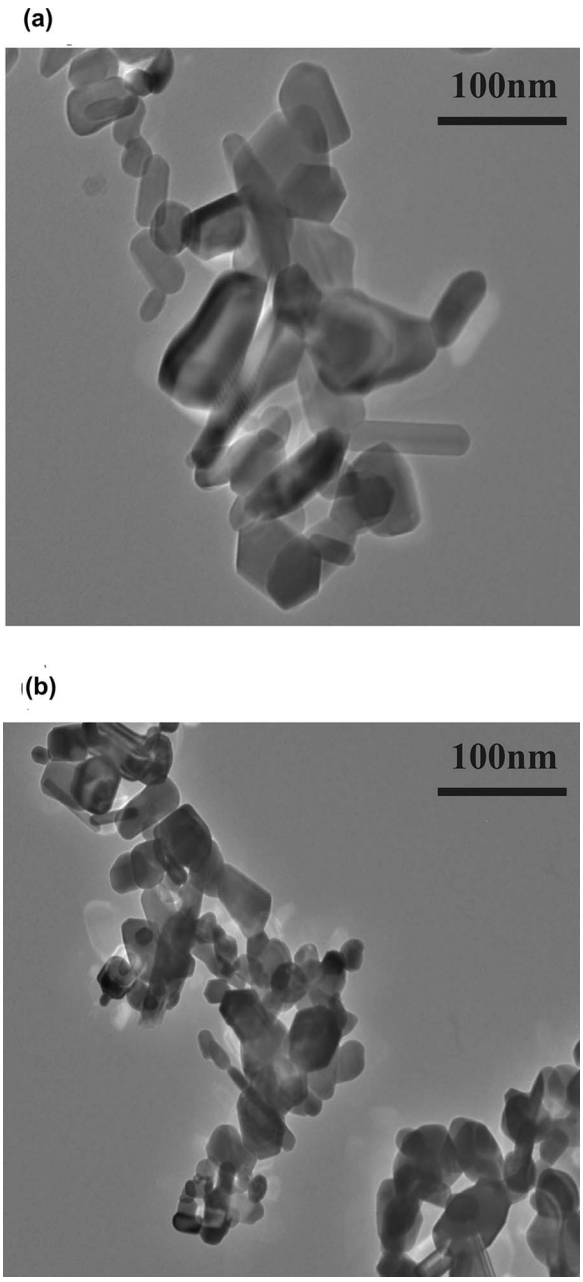

FIGURE 1. Transmission electron micrograph of Zinc Oxide particles (a) Nano ZnO and (b) Micro ZnO. Scale bar is 100 nm.

size selective differences between nano and micro ZnO was seen at 40 μg/ml and higher concentrations (Figure 3).

Effect of ZnO Particle on Phagocytic Capacity of THP-1 Cells

This parameter measured the phagocytic ability of the monocytes after the uptake of test particles. This assay was carried out by taking three different concentrations of test particles, namely, ½TC-50, TC-50, and 2TC-50 for each particle type. A significant reduction in the phagocytosis of indicator latex beads occurred after exposure to TC-50 and 2TC-50 concentrations of nano ZnO particles and micro ZnO particles (Figure 4). In addition, phagocytosis was significantly impaired following exposure to nano ZnO than at micron size. Fluorescent imaging (Figure 5) represents monocytes phagocytosing latex beads. Qualitatively control cells were found to phagocytose more beads compared to particle-exposed monocytes. Nano ZnO reduced phagocytic capacity to a greater extent than micro ZnO.

Influence of ZnO Particle on Induction of Proinflammatory Cytokines

Monocytes are able to secrete inflammatory mediators such as cytokines upon stimulation by various agents. In this study cellular release of the proinflammatory cytokines (IL-1β, TNF-α, IL-6) and chemokine (IL-8) into the culture medium was measured when monocytes were exposed to ZnO (nano and micron) particles at their respective TC-50 concentrations (nano ZnO: 17.69 μg/ml, and micro ZnO: 38.35 μg/ml) for different durations (6, 18, 24, and 48 h) (Figure 6). It was interesting to note that ZnO particles did not release of IL-1β up to 24 h and induced release only after 48 h of exposure (Figure 6a). Significantly more IL-1β was released by nano size ZnO than micro ZnO. The release of IL-6 by both sizes of ZnO was significant after 18 h (Figure 6b). Both particles showed time dependent increase in secretion of IL-6, with nano-sized particles consistently more effective than larger particles at all time points. Similarly, ZnO particles also induced release of TNF-α in to culture medium (Figure 6c). The nano-size particles were found to be more effective in inducing TNF-α secretion at all time points. THP-1 cells exhibited a time-dependent elevation in IL-8 production after exposure to nano ZnO particles at all time points (Figure 6d), whereas micro ZnO showed time-dependent

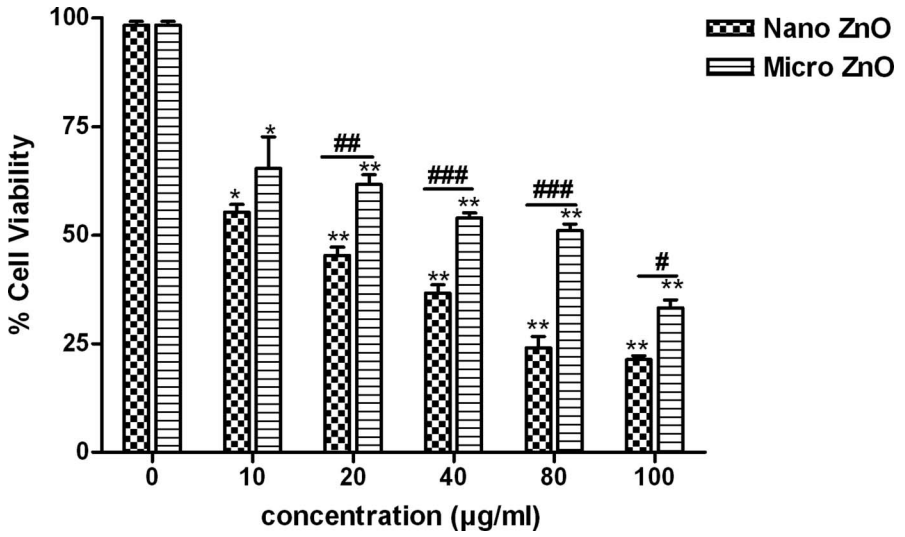

FIGURE 2. Percentage metabolic activity of THP-1 cells upon exposure to varying concentrations of ZnO particles, as determined by the MTT-assay. Cells were exposed with 10, 20, 40, 80 and 100 µg/ml of nano and micro ZnO particles for 24 h. The results are expressed as a percentage relative to controls and are presented as mean ± SEM of three independent replicate experiments. Significance indicated by: *$p < 0.05$ and **$p < 0.01$ versus control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicates significant difference between the particle size.

production of IL-8 up to 24 h; this later decreased after 48 h, demonstrating a significant difference between the particle sizes in terms of induced effects.

Influence of ZnO Particle on COX-2 and MCP-1 mRNA Expression

A significant upregulation of COX-2 and MCP-1 gene expression was observed with both sizes of ZnO particles (Figure 7). The expression was dependent on the size of particle and duration of exposure. The rise in exposure time showed gradual increase in COX-2 mRNA expression. Nano ZnO was found to induce COX-2 gene expression at an early time point (6 h), and this later decreased at 24 h, while micro ZnO induced COX-2 gene expression at 24 h. Similarly MCP-1 gene expression was also upregulated by ZnO particles; however, a time-dependent effect was not seen with both sizes of ZnO.

Effect of ZnO Particle on DNA

THP-1 cells exposed to both sizes of ZnO particles at a concentration range of 10–100 µg/ml for 24 h did not show any marked effect on DNA (Figure 8).

TABLE 3. Calculated TC-50 Concentrations

	ZnO particles	
Cell type	Nano ZnO	Micro ZnO
Human monocytes (THP-1)	17.69 µg/ml (15.75 to 19.87)	38.35 µg/ml (24.52 to 58.13)

DISCUSSION

Nanoparticles interact with various components of the immune system and either enhance or inhibit functions (Dobrovolskaia et al., 2009; Dobrovolskaia and McNeil, 2007; Hussain et al., 2011; Zolnik et al., 2010). Modulation of the immune function by NP may be useful or detrimental, depending on the intended use (Zolnik et al., 2010). Concerns are raised, however, when an engineered nanomaterial not intended for interaction with the immune system alters its function. High levels of cytokines upon treatment with NP are usually associated with toxicity, adverse reactions, and low therapeutic efficacy. Evaluation

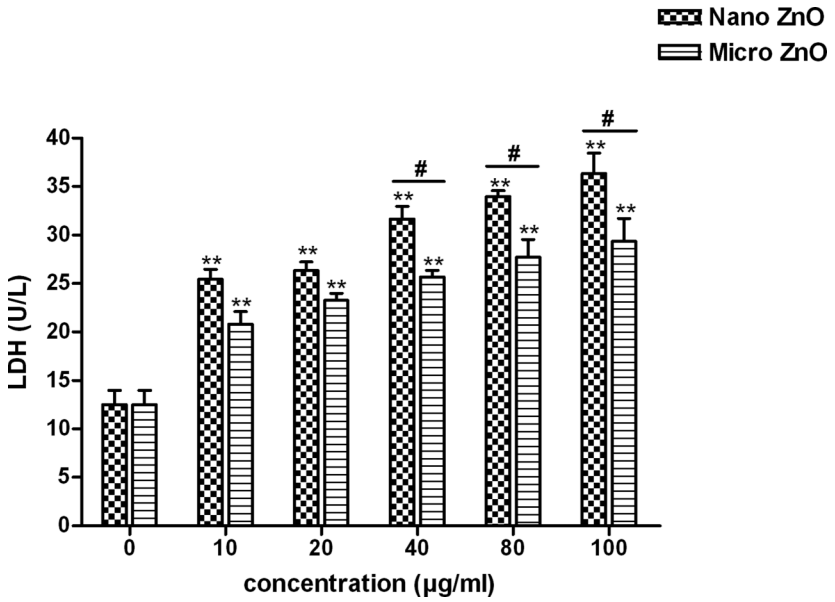

FIGURE 3. Lactate dehydrogenage leakage from THP-1 cells after 24 h exposure to ZnO particles. Cells were exposed with 10, 20, 40, 80 and 100 μg/ml of nano and micro ZnO particles for 24 h. The results are expressed in activity unit per liter and are presented as mean ± SEM of three independent replicate experiments. Significance indicated by: $^{**}p < 0.01$ versus control. $^{\#}p < 0.05$, indicates significant difference between the particle size.

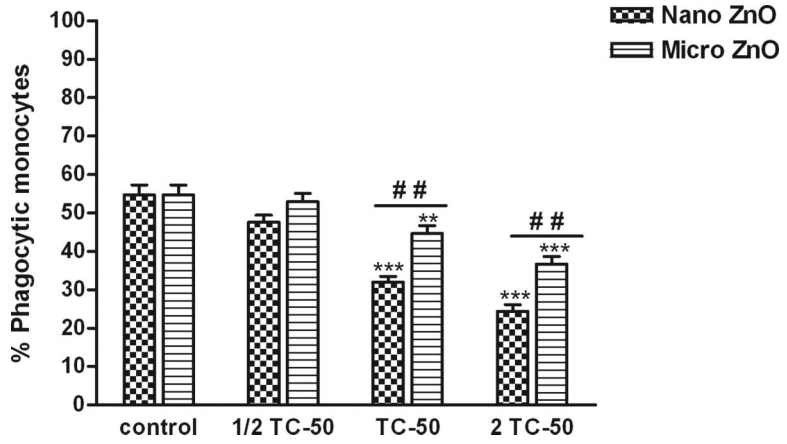

FIGURE 4. Percentage of cells capable of phagocytosing indicator latex beads after the uptake of test particles (i.e., phagocytic monocytes). Cells were exposed to $^{1}/_{2}$ TC-50, TC-50 and 2TC-50 concentrations of nano ZnO (8.85 μg/ml, 17.69 μg/ml, and 35.38 μg/ml resp.) and micro ZnO (19.18 μg/ml, 38.35 μg/ml and 76.70 μg/ml resp.) for 4 h. The results are expressed as a percentage relative to controls and are presented as mean ± SEM of three independent replicate experiments. Significance indicated by: $^{**}p < 0.01$ and $^{***}p < 0.05$ versus control. $^{\#\#}p < 0.01$ indicates significant difference between the particle size.

of immunotoxicity of NP by measuring levels of cytokines or other immune indicators is of particular importance for their clinical safety and for maximizing therapeutic benefits (Elsabahy and Wooley, 2013).

The physicochemical characteristics of nano and micro ZnO particles were extensively characterized. The primary size of micro ZnO particles provided by the supplier was not compatible with the measurements made in the lab. The supplier reported that the mean size of the micro ZnO particles was <5 μm; however, as demonstrated in Table 2, the measured particle sizes by TEM were not identical for micro ZnO particles. Indeed, the average size of nano ZnO was in agreement with the size provided

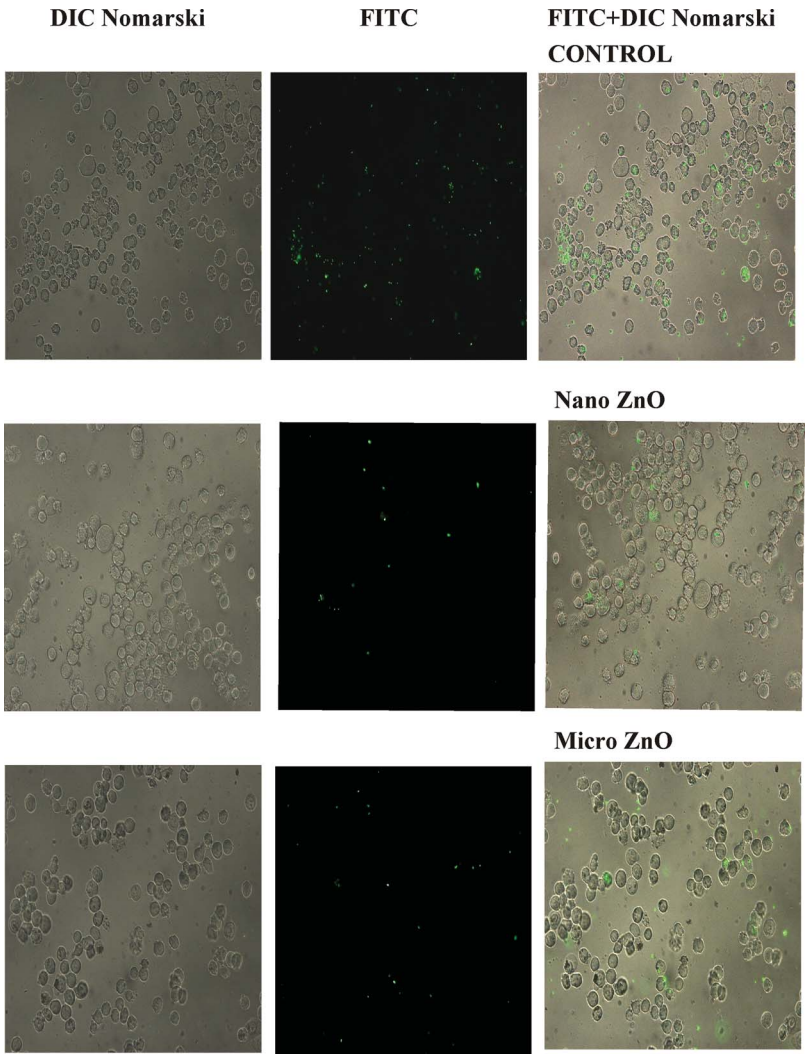

FIGURE 5. Photomicroghraphs of THP-1 cells phagocytosing latex beads (magnification 400X).

by the supplier. Not surprisingly, as compared to measurements in the dry phase (measured by TEM), the mean particle sizes and size distributions of particles (measured by DLS) were enhanced when measured in aqueous media. Due to high specific surface area and high surface energy level, NP have the propensity to aggregate together to form micro-size particles that are more stable in the environment (He et al., 2006; Petosa et al., 2010). To minimize the effects of particle aggregation and sedimentation, colloidal suspensions of ZnO particles were always freshly prepared and sonicated before each experiment.

In this study, cytotoxicity of nano-sized ZnO was compared to its micro size in human monocytes (THP-1 cells). Our results revealed that nano ZnO particles were more potent than the micro size in inducing cytotoxicity in THP-1 cells. In addition, significant differences detected with TC-50 values of ZnO particle (Table 3), suggested greater toxicity of nano ZnO than micron size toward monocytes. These results are in agreement with the findings of Prach et al. (2012). Both sizes of particle followed concentration-dependent cytotoxicity in monocytes. It was further demonstrated that the phagocytic ability of monocytes

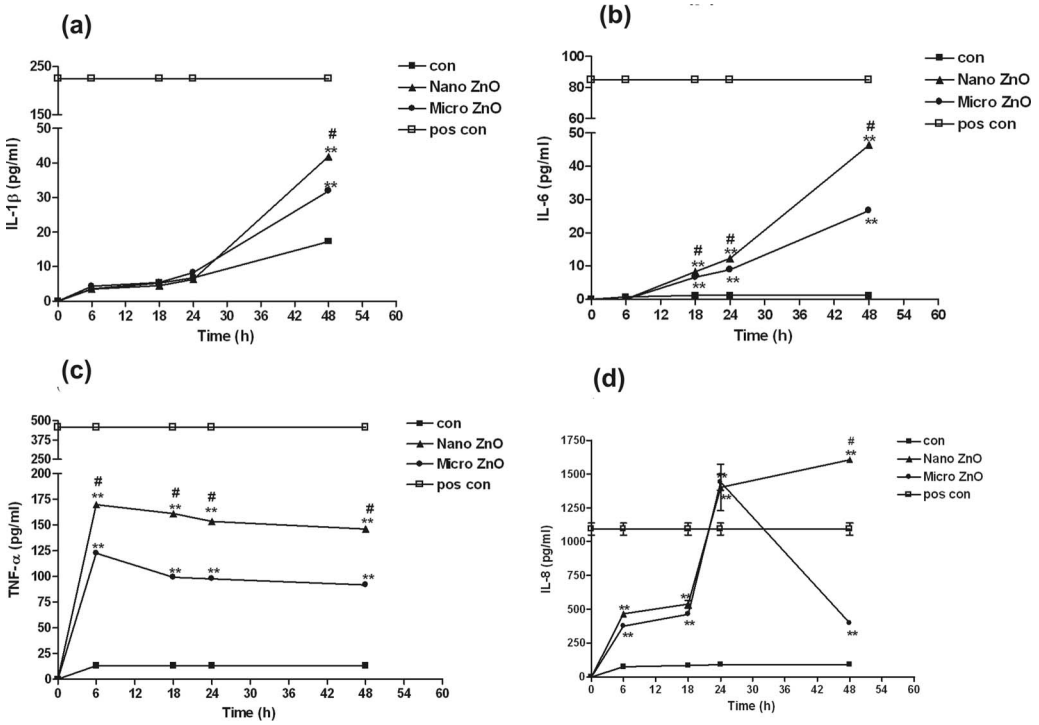

FIGURE 6. Proinflammatory cytokine and chemokine level in THP-1 cells were exposed to ZnO particles at their TC-50 concentration (nano ZnO: 17.69 μg/ml and micro ZnO: 38.35 μg/ml) for different time (0, 6, 18, 24 and 48 h) as determined by ELISA. A positive control for each assay was run in parallel as per manufacturer's instruction. (a) IL-1β level (b) IL-6 level (c) TNF-α level and (d) IL-8 level. Results are presented as mean ± SEM of three independent replicate experiments. Significance indicated by: $^{**}p < 0.01$ versus control. $^{\#}p < 0.01$ indicates significant difference between the particle size.

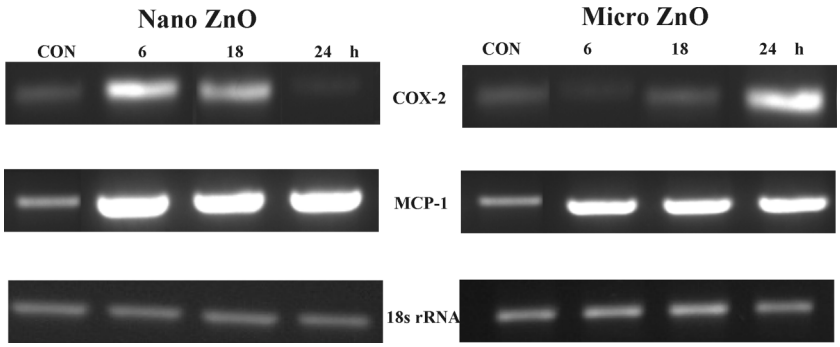

FIGURE 7. Effects of ZnO particles (nano and micro) on mRNA levels of COX-2 and MCP-1 gene. THP-1 cells were exposed to ZnO particles at their TC-50 concentration (nano ZnO: 17.69 μg/ml and micro ZnO: 38.35 μg/ml) for different time (0, 6, 18, and 24 h).

was significantly impaired following exposure to ZnO particles. Nano ZnO particles significantly impaired phagocytic capacity of monocytes more compared to the micro size. One of the plausible mechanisms for this might be the greater surface area and smaller diameter of NP, occupying more space in monocytes. Thus, the capacity of monocytes to further phagocytose latex beads was decreased. Increase in concentration of NP may lead to inhibition of phagocytosis of latex beads. In addition to this, microparticles with larger size are phagocytosed to a lesser extent compared to nano-size particles, allowing easy access to latex beads to get phagocytosed by monocytes. To the best of our knowledge, no apparent studies demonstrated the effect of ZnO particles on phagocytic capacity of monocytes. Our

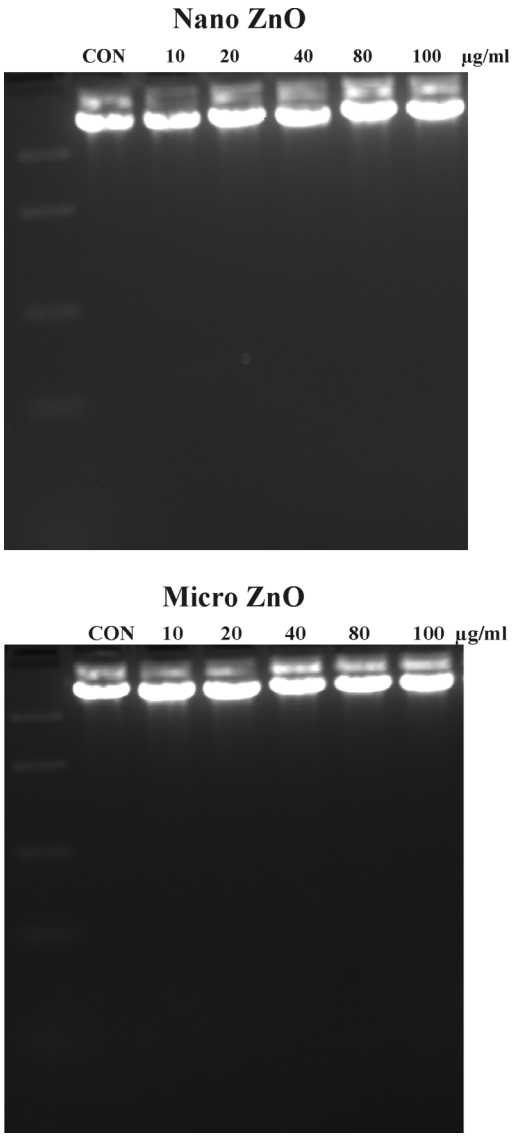

FIGURE 8. Effect of nano and micro ZnO on DNA. THP-1 cell were exposed with 10, 20, 40, 80 and 100 µg/ml of ZnO particles for 24 h. Equal amount of DNA (20 µg) was loaded on agarose gel and visualized under UV light.

study provides preliminary data on alterations in phagocytic function of monocyte by ZnO particles; further uptake and molecular studies need to be carried out to explore alterations in phagocytosis.

Our results showed induction of proinflammatory cytokines IL-1β, IL-6, and TNF-α by ZnO particles. Many cytokines including IL-1, IL-6, and TNF-α activate functions of inflammatory cells during acute inflammatory responses. These cytokines increase the vascular permeability and thus produce swelling and redness associated with inflammation. IL-1 and IL-6 are responsible for fever reactions; TNF-α stimulates endothelial cells and is responsible for hypotension (Elsabahy and Wooley, 2013). In the present study, release of IL-1β, TNF-α, and IL-6 rose gradually with increasing exposure duration and decrease in particle size. It was observed that release of IL-1β and TNF-α cytokines was more profound with nano ZnO than micro ZnO. Our results are consistent with the study of Heng et al. (2011), who showed elevation in TNF-α and IL-6 secretion upon exposure to ZnO NP of different shapes. Thus, the response of THP-1 cells to proinflamatory cytokine release was dependent on size of ZnO particles. The inflammatory marker COX-2 gene expression was upregulated upon exposure to ZnO particles. The increase in expression was seen earlier in cells exposed to nano ZnO, compared to micro ZnO. COX-2, an inducible isoform of cyclooxygenase, is induced by several mitogenic and proinflammatory stimuli including LPS, interleukin-1 (IL-1α and IL-1β), and TNF-α (Diaz et al., 1998; Reddy et al., 2003). Results of the present investigation indicated induction of COX-2, IL-1β, and TNF-α was size dependent. In the present study, gene expression of MCP-1, a CC chemokine, and release of IL-8, a member of the CXC chemokine subfamily, were studied. MCP-1 stimulates both chemotaxis of monocytes and several cellular events associated with chemotaxis. IL-8 is a chemokine that plays a key role in the activation of neutrophils and their recruitment to the site of inflammation (Huber et al., 1991). The higher expression of MCP-1 and time-dependent increase in IL-8 release by nano ZnO indicated more potency for chemotaxis than for its micro counterpart. Gojava et al. (2007) also reported a rise in IL-8 and MCP-1 mRNA expression in human aortic endothelial cells following exposure to ZnO NP.

In assessing toxicity, DNA damage to macrophages is an important outcome since (i) these cells remove inhaled NP (Bunn et al., 2001), and (ii) DNA damage is considered to be

an important initial event in disease including carcinogenesis (Evans et al., 2004). Our results did not show any DNA damage by ZnO particles, suggesting no genotoxic or carcinogenic potential of ZnO particle.

In conclusion, ZnO particles of both sizes induced cytotoxicity and altered the phagocytic capacity with no marked effect on DNA in human monocytes. Further, both sizes of ZnO particles were found to induce various cytokines release and upregulated the expression of chemokine and other inflammatory mediators, thus indicating the potential of this material in induce inflammation. The induction of inflammatory markers rose with decrease in particle size, and increased with duration of exposure. The nano ZnO particles demonstrated more potential to induce toxicity and inflammation than micro-size ZnO in THP-1 cells. The magnitude of induction of proinflammatory cytokines and chemokine, and their delivery to desired areas, may be an important parameter when considering ZnO NP for biomedical purposes to achieve the desired therapeutic response and clinical safety. The variation in release of cytokines from THP-1 cells by both sizes of ZnO described in this study emphasize the need for careful in vitro cytokine profiling with its mechanism across a spectrum of relevant cell types, followed by appropriate in vivo studies to provide useful and potentially predictive screening data regarding the relative adverse effects of metal oxide NP.

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REFERENCES

Allen, R. T., Hunter, W. J. 3rd, and Agrawal, D. K. 1997. Morphological and biochemical characterization and analysis of apoptosis. *J. Pharmacol. Toxicol. Methods* 37: 215–228.

Applerot, G., Lipovsky, A., Dror, R., Perkas, N., Nitzan, Y., Lubart, R., and Gedanken, A. 2009. Enhanced antibacterial activity of nanocrystalline ZnO due to increased ROS-mediated cell injury. *Adv. Funct. Mater*. 19: 842–852.

Beyerle, A., Schulz, H., Kissel, T., and Stoeger, T. 2009. Screening strategy to avoid toxicological hazards of inhaled nanoparticles for drug delivery: The use of a-quartz and nano zinc oxide particles as benchmark. *J. Phys. Conf. Ser*. 151: 012034.

Bunn, H.J., Dinsdale, D., Smith, T., and Grigg, J. 2001. Ultrafine particles in alveolar macrophages from normal children. *Thorax* 56: 932–934.

Collins, A. R. 2004. The comet assay for DNA damage and repair: Principles, applications, and limitations. *Mol. Biotechnol*. 26: 249–261.

Churg, A., Gilks, B., and Dai, J. 1999. Induction of fibrogenic mediators by fine and ultrafine titanium dioxide in rat tracheal explants. *Am. J. Physiol*. 277: L975–L982.

Denizot, F., and Lang, R. 1986. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89: 271–277.

Diaz, A., Chepenik, K. P., Korn, J. H., Reginato, A. M., and Jimenez, S. A. 1998. Differential regulation of cyclooxygenases 1 and 2 by interleukin 1 beta, tumor necrosis factor-alpha, and transforming growth factor-beta 1 in human lung fibroblasts. *Exp. Cell. Res*. 241: 222–229.

Dobrovolskaia, M. A., and McNeil, S. E. 2007. Immunological properties of engineered nanomaterials. *Nat. Nanotechnol*. 2: 469–478.

Dobrovolskaia, M. A., Germolec, D. R., and Weaver, J. L. 2009. Evaluation of nanoparticle immunotoxicity. *Nat. Nanotechnol*. 4: 411–414.

Dobrovolskaia, M. A., Aggarwal, P., Hall, J. B., and McNeil, S. E. 2008. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. *Mol. Pharmacol*. 5: 487–495.

Donaldson, K., and Tran, C. L. 2002. Inflammation caused by particles and fibers. *Inhal. Toxicol*. 14:5–27.

Donaldson, K., Brown, D., Coulter, A., Duffin, R., MacNee, W., Renwick, L., Tran, L., and Stone, V. 2002. The pulmonary toxicology of ultrafine particles. *J. Aerosol. Med*. 15: 213–220.

Elsabahy, M., and Wooley K. L. 2013. Cytokines as biomarkers of nanoparticle immunotoxicity. *Chem. Soc. Rev*. 42: 5555–5576.

Evans, J. R., Helmy, A. E., Cluroe, A., and Purushotham, A. D. 2004. Lymphoma of the breast- case report and review of the literature. *J. BUON* 9: 307–311.

Ferin, J., Oberdorster, G., and Penney, D. P. 1992. Pulmonary retention of ultrafine and fine particles in rats. *Am. J. Respir. Cell Mol. Biol*. 6: 535–542.

Ferin, J., Oberdorster, G., Penney, D. P., Soderholm, S. C., Gelein, R., and Piper, H. C. 1990. Increased pulmonary toxicity of ultrafines? I. Particle clearance, translocation, morphology. *J. Aerosol. Sci*. 21: 381–384.

Ferin, J., Oberdorster, G., Soderholm, S. C., and Gelein, R. 1991. Pulmonary tissue access of ultrafine particles. *J. Aerosol. Med*. 4:57–68.

Gojova, A., Guo, B., Kota, R. S., Rutledge, J. C., Kennedy, I. M., and Barakat, A. I. 2007. Induction of Inflammation in vascular endothelial cells by metal oxide nanoparticles: Effect of particle composition. *Environ. Health. Perspect*. 115: 403–409.

Grandjean-Laquerriere, A., Laquerriere, P., Guenounou, M., Laurent-Maquin, D., and Phillips, T.M. 2005. Importance of the surface area ratio on cytokines production by human monocytes in vitro induced by various hydroxyapatite particles. *Biomaterials* 26: 2361–2369.

Groves, D. T., and Jiang, Y. 1995. Chemokine, a family of chemotactic cytokines. *Crit. Rev. Oral Biol. Med*. 6: 109–118.

Gulumian, M., and Vallyathan, V. 2010. Nanoparticles and potential human health implications: Past and future directions. Preface. *J. Toxicol. Environ. Health A* 73: 339–340.

Hanley, C., Hurber, A., Hanna, C., Punnoose, A., Zhang, J., and Wingett, D. G. 2009. The influences of cell type and ZnO nanoparticle size on immune cell cytotoxicity and cytokine induction. *Nanoscale Res. Lett*. 4: 1409–1420.

He, Y. T., Wan, J., and Tokunaga, T. 2008. Kinetic stability of hematite nanoparticles: the effect of particle sizes. *J. Nanopart. Res*. 10: 321–332.

Heng, B. C., Zhao, X., Tan, E.C., Khamis, N., Assodani, A., Xiong, S., Ruedl, C., Ng, K. W., and Loo, J. S. 2011. Evaluation of the cytotoxic and inflammatory potential of differentially shaped zinc oxide nanoparticles. *Arch. Toxicol*. 85: 1517–1528.

Hollingsworth, J. W., Maruoka, S., Li, Z., Potts, E. N., Brass, D. M., Garantziotis, S., Fong A., Foster, W. M., and Schwartz, D. A. 2007. Ambient ozone primes pulmonary innate immunity in mice. *J. Immunol*. 179: 4367–4375.

Hong, H., Shi, J., Yang, Y., Zhang, Y., Engle, J. W., Nickles, R. J., Wang, X., and Cai, W. 2011. Cancer-targeted optical imaging with fluorescent zinc oxide nanowires. *Nano Lett*. 11: 3744–3750.

Huber, A., Kunkel, S., Todd, R. 3rd, and Weiss, S., 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* 254: 99–102.

Hussain, S., Vanoirbeek, J. A., and Hoet, P. H. 2012. Interactions of nanomaterials with the immune system. *Nanomed. Nanobiotechnol*. 4: 169–183.

Hussain, S., Vanoirbeek, J. A., Luyts, K., De Vooght, V., Verbeken, E., Thomassen, L. C., Martens, J. A., Dinsdale, D., Boland, S., Marano, F., Nemery, B., and Hoet, P. H.

2011. Lung exposure to nanoparticles modulates an asthmatic response in a mouse model. *Eur. Respir. J*. 37: 299–309.

Inoue, K., Takano, H., Yanagisawa, R., Hirano, S., Sakurai, M., Shimada, A., and Yoshikawa, T. 2006. Effects of airway exposure to nanoparticles on lung inflammation induced by bacterial endotoxin in mice. *Environ. Health Perspect*. 114: 1325–1330.

Kamata, H., Tasaka, S., Inoue, K., Miyamoto, K., Nakano, Y., Shinoda, H., Kimizuka, Y., Fujiwara, H., Ishii, M., Hasegawa, N., Takamiya, R., Fujishima, S., Takano, H., and Ishizaka, A. 2011. Carbon black nanoparticles enhance bleomycin-induced lung inflammatory and fibrotic changes in mice. *Exp. Biol. Med. (Maywood)* 236: 315–324.

Kim, J. E., Lim, H. T., Minai Tehrani, A., Kwon, J. T., Shin, J. Y., Woo, C. G., Choi, M., Back, J., Jeong, D. J., Ha, Y. C., Chae, C. H., Song, K. S., Ahn, K. H., Lee, J., H., Sung, H. J., Yu, I. J., Beck, G. R, and Cho, M. H. 2010. Toxicity and clearance of intratracheally administered multiwalled carbon nanotubes from muine lung. *J. Toxicol Environ. Health A* 73: 1530–1543.

LeBlanc, A. J., Crumpston, J. L., Chen, B. T., Frazer, D., Castranova, V., and Nurkiewicz, T. R. 2009. Nanoparticle inhalation impairs endothelium-dependent vasodilation in subepicardial arterioles. *J. Toxicol. Environ. Health A* 72: 1576–1584.

Lee, H. M., Shin, D. M., Song, H. M., Yuk, J. M., Lee, Z. W., Lee, S. H., Hwang, S. M., Kim, J. M., Lee, C. S., and Jo, E. K. 2009. Nanoparticles up-regulate tumor necrosis factor-alpha and CXCL8 via reactive oxygen species and mitogen-activated protein kinase activation. *Toxicol. Appl. Pharmacol*. 238: 160–169.

Li, X. Y., Brown, D., Smith, S., MacNee, W., and Donaldson, K. 1999. Short-term inflammatory responses following intratracheal instillation of fine and ultrafine carbon black in rats. *Inhal. Toxicol*. 11: 709–731.

Li, X. Y., Gilmour, P. S., Donaldson, K., and MacNee, W. 1996. Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) *in vivo* and *in vitro*. *Thorax* 51: 1216–1222.

Manzo, N. D., Slade, R., Richards, J. H., McKee, J. K., Martin, L. D., and Dye, J. A. 2010. Susceptibility of inflamed alveolar and airway epithelial cells to injury induced by diesel exhaust of particles of varying organic carbon content. *J. Toxicol. Environ. Health A* 73: 565–580.

Maynard, A. D., Baron, P. A., Foley, M., Shvedova, A. A., Kisin, E. R., and Castranova, V. 2004. Exposure to carbon nanotube material: Aerosol release during the handling of unrefined single-walled carbon nanotube material. *J. Toxicol. Environ. Health A* 67: 87–107.

Nag, N., Bhattacharya, A., Gupta, R.K., Manivannan, K., Kahol, P.K., Wanekaya, A. K., Delong, R. K., and Ghosh, K. 2011. Glucose stabilized ZnO nanoparticles for biomedical applications. *J. Nanosci. Lett*. 1: 172–178.

Nicholas, S. A., and Sumbayev, W. 2009. The involvement of hypoxia-inducible factor 1 alpha in Toll-like receptor 7/8-mediated inflammatory response. *Cell Res*. 19: 973–983.

Oberdorster, G. 2001. Pulmonary effects of inhaled ultrafine particles. *Int. Arch. Occup. Environ. Health* 74: 1–8.

Osmond, M. J., and McCall, M. J. 2010. Zinc oxide nanoparticles in modern sunscreens: An analysis of potential exposure and hazard. *Nanotoxicology* 4: 15–41.

Petosa, A. R., Jaisi, D. P., Quevedo, I. R., Elimelech, M., and Tufenkji, N. 2010. Aggregation and deposition of engineered nanomaterials in aquatic environments: Role of physicochemical interactions. *Environ. Sci. Technol*. 44: 6532–6549.

Pope, C. A. 3rd, Thun, M. J., Namboodiri, M. M., Dockery, D. W., Evans, J. S., Speizer, F. E., and Heath, C. W., Jr. 1995. Particulate air pollution as a predictor of mortality in a prospective study of US adults. *Am. J. Respir. Crit. Care Med*. 151: 669–674.

Prach, M., Stone, V., and Proudfoot, L. 2012. Zinc oxide nanoparticles and monocytes: Impact of size, charge and solubility on

activation status. *Toxicol. Appl. Pharmacol*. 266: 19–26.

Price, H., Arthur, R., Sexton, K., Gregory, C., Hoogendoorn, B., Matthews, I., Jones, T., and BeruBe, K. 2010. Airborne particles in Swansea, UK: Their collection and characterization. *J. Toxicol. Environ. Health A* 73: 355–367.

Rasmussen, J. W., Martinez, E., Louka, P., and Wingett, D. G. 2010. Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. *Expert Opin. Drug. Deliv*. 7: 1063–1077.

Reddy, M. C., Subhashini, J., Mahipal, S. V. K., Bhat, V. B., Reddy, P. S., Kiranmai, G., Madyasatha, K. M., and Reddanna, P. 2003. C-phycocyanin, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochem. Biophys. Res. Commun*. 304: 385–392.

Sayes, C. M., Reed, K. L., and Warhiet, D. B. 2007. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. *Toxicol. Sci*. 97: 163–180.

Scuri, M., Chen, B. T., Castranova, V., Reynolds, J. S., Johnson, V. J., Samsell, L., Watson, C., and Piedimonte, G. 2010. Effects of titanium dioxide nanoparticle exposure on neuroimmune responses in rat airways. *J. Toxicol. Environ. Health A* 73: 1353–1369.

Schroeder, F., and Kinden, D. A. 1983. Measurement of phagocytosis using fluorescent latex beads. *J. Biochem. Biophys. Methods* 8: 15–27.

Tabet, L., Bussy, C., Amara, N., Setyan, A., Grodet, A., Rossi, M. J., Pairon, J. C., Boczkowski, J., and Lanone, S. 2009. Adverse effects of industrial multiwalled carbon nanotubes on human pulmonary cells. *J. Toxicol. Environ. Health A* 72: 60–73.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* 26: 171–176.

Veranth, J. M., Kaser, E. G., Veranth, M. M., Koch, M., and Yost, G. S. 2007. Cytokine responses of human lung cells (BEAS-2B) treated with micron-sized and nanoparticles of metal oxides compared to soil dusts. *Part. Fibre Toxicol*. 4: 2.

Wan, R., Mo, Y., Zhang, X., Chien, S., Tollerud, D. J., and Zhang, Q. 2008. Matrixmetalloproteinase-2 and -9 are induced differently by metal nanoparticles inhuman monocytes: The role of oxidative stress and protein tyrosine kinase activation. *Toxicol. Appl. Pharmcol*. 233: 276–285.

Wroblewski, F., and LaDue, J. S. 1955. Lactic dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med*. 90: 210–213.

Yang, Z., and Xie, C. 2006. Zn^{2+} release from zinc and zinc oxide particles in simulated uterine solution. *Colloids Surfact. B Biointerfaces* 47: 140–145.

Zhao, J., and Castranova, V. 2011. Toxicology of nanomaterials used in nanomedicine. *J. Toxicol. Environ. Health B* 14: 593–632.

Zolnik, B. S., Gonzalez-Fernandez, A., Sadrieh, N., and Dobrovolskaia, M. A. 2010. Nanoparticles and the immune system. *Endocrinology* 151: 458–465.