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Effects of engineered nanomaterial exposure on macrophage innate immune function

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ABSTRACT

Increasing use of engineered nanomaterials (ENMs) means increased human exposures. Potential adverse effects include those on the immune system, ranging from direct toxicity to impairment of defenses against environmental pathogens and toxins. Effects on lung macrophages may be especially prominent, because they serve to clear foreign materials like ENMs and bacterial pathogens. We investigated the effects of 4 hour exposures over a range of concentrations, of a panel of industry-relevant ENMs, including SiO_2, Fe_2O_3, ZnO, CeO_2, TiO_2, and an Ag/SiO_2 composite, on human THP-1 macrophages. Effects on phagocytosis of latex beads, and phagocytosis and killing of *Francisella tularensis* (FT), as well as viability, oxidative stress and mitochondrial integrity, were measured by automated scanning confocal microscopy and image analysis. Results revealed some notable patterns: 1) Phagocytosis of unopsonized beads was increased, whereas that of opsonized beads was decreased, by all ENMs, with the exception of ZnO, which reduced both opsonized and unopsonized uptake; 2) Uptake of opsonized and unopsonized FT was either impaired or unaffected by all ENMs, with the exception of CeO_2, which increased phagocytosis of unopsonized FT; 3) Macrophage killing of FT tended to improve with all ENMs; and 4) Viability was unaffected immediately following exposures with all ENMs tested, but was significantly decreased 24 h after exposures to Ag/SiO_2 and ZnO ENMs. The results reveal a complex landscape of ENM effects on macrophage host defenses, including both enhanced and reduced capacities, and underscore the importance of robust hazard assessment, including immunotoxicity assessment, of ENMs.

1. Introduction

A large and increasing variety of engineered nanomaterials (ENMs) are now common components of many industrial and consumer products (Pirela et al., 2015a, 2013; Xia et al., 2009), and more are being designed for novel diagnostic, antimicrobial, and therapeutic applications (Pyrgiotakis et al., 2015, 2014a; Jain et al., 2014; Key and Leary, 2014; Tong et al., 2014; Pyrgiotakis et al., 2016; Ma et al., 2016a; Servin and White, 2016). As such, the potential for workplace or environmental exposure to ENMs during their production and the life-cycle of products containing them, and for intentional exposure to therapeutic ENMs, is great (Pal et al., 2015; Sotiriou et al., 2016; Pirela et al., 2016). The chemical compositions, small sizes and associated large surface-to-volume ratios of these materials suggest potential for harmful biological interactions (Pirela et al., 2015a; Krewski et al., 2010; Oberdörster, 2012; Grassian et al., 2016; Konduru et al., 2015a; Lu et al., 2016). Over the last decade the toxicity of ENMs has been studied extensively in a variety of *in vivo* and *in vitro* systems, and toxicity of metal oxide ENMs in particular has been often reported (Pirela et al., 2013, 2016; Bakand et al., 2012; Demokritou et al., 2013; Lu et al., 2015; Pirela et al., 2015b; Watson et al., 2014; Yokel et al., 2014; Zhou et al., 2014; Konduru et al., 2014, 2015b; Cohen et al., 2014a).

Most nanotoxicology studies have focused on direct toxicity, either physiological impairment or death *in vivo*, or various indicators of cytotoxicity *in vitro* (Krewski et al., 2010; Oberdörster, 2012; Bakand et al., 2012; Demokritou et al., 2013; Lu et al., 2015; Watson et al., 2014; Yokel et al., 2014; Zhou et al., 2014; Setyawati et al., 2013). Recent studies suggest the potential for indirect hazardous effects of ENMs through their interaction with the immune system. ENMs can alter innate and adaptive immune cells and processes, possibly causing impaired protection or disrupting immune homeostasis, leading to or exacerbating existing allergic and inflammatory conditions (Wang et al., 2013; Smith et al., 2014; Roy et al., 2014a). In this study we have focused on a key component of the innate immune system, the macrophage.

In the lung, the macrophage is the first line of defense against inhaled pathogens and particles, and also coordinates early inflammatory and immune responses as needed. Because it is strategically positioned

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and equipped to detect, engulf and destroy inhaled threats, the lung macrophage is also at high risk in inhalation exposures to ENMs. ENMs are readily and rapidly taken up by macrophages, and insoluble nano-sized particles can persist within macrophages for extended periods of time (Lehnert, 1992; Lay et al., 1998), raising the likelihood that ENM exposure could lead to both immediate and long-term macrophage dysfunction. Indeed, reported effects of ENM exposures on macrophages include changes in phagocytic function, as well as altered gene expression, cytokine secretion, reactive oxygen and nitrogen species production, and surface markers (Sahu et al., 2014; Lin et al., 2014; Liu et al., 2010; Bancos et al., 2015; Kodali et al., 2013).

The few studies of ENM effects on macrophage function published to date have focused on one or two ENMs, and most have employed non-human macrophages (Lin et al., 2014; Liu et al., 2010; Bancos et al., 2015; Kodali et al., 2013), and effects on phagocytosis of opsonized beads or killed bacterial particles (Sahu et al., 2014; Liu et al., 2010; Bancos et al., 2015; Kodali et al., 2013). Given the growing variety of ENMs that pose potential exposure risks, and evidence from these early studies of ENM-induced macrophage dysfunction, there is a need for broader systematic study and development of efficient methods for screening ENM-induced macrophage dysfunction in relevant human cells. Moreover, since effective macrophage defense requires both phagocytosis and subsequent killing of inhaled pathogens, it is important to examine effects on both of these functions, using relevant live pathogenic bacteria. In addition, since opsonizing serum proteins are not normally present in alveolar fluid, macrophage function should be studied in the context of unopsonized as well as opsonized phagocytosis.

In the present study we have begun to address the needs outlined above. We employed human THP-1 macrophages, differentiated to maximize resemblance to primary lung macrophages, to examine the effects of a panel of industry-relevant metal oxide ENMs, including SiO_2, Fe_2O_3, ZnO, CeO_2, TiO_2, and a composite of 10% Ag supported on SiO_2 particles (Ag/SiO_2) (Demokritou et al., 2010; Sotiriou et al., 2012). We evaluated effects of test ENMs on key macrophage functions, including phagocytosis of both opsonized and unopsonized latex beads, and phagocytosis and killing of live *Francisella tularensis* (FT) using the live vaccine strain (LVS) of that pathogen. *Francisella tularensis* is a highly infectious Gram-negative intracellular pathogen, which causes the potentially fatal disease known as tularemia in humans and animals, and has been categorized as a category A select agent because of its potential for rapid dissemination (Santic et al., 2010; Darling et al., 2002). These assessment were carried out using automated fluorescence scanning confocal microscopy and image analysis techniques, adapted from methods previously developed by the authors (DeLoid et al., 2009; Sulahian et al., 2008), as well as flow cytometry and lysis and CFU assays.

2. Materials and methods

2.1. ENM powders and characterization

SiO_2, Fe_2O_3, CeO_2, and the composite Ag/SiO_2 ENM, consisting of 10% silver supported on SiO_2 particles, were produced by flame spray pyrolysis using the Harvard Versatile Engineered Nanomaterial Generation System (VENGES) as previously described (Demokritou et al., 2010; Sotiriou et al., 2012, 2014). TiO_2 and ZnO ENM powders were purchased from EVONIK (Essen, Germany), and Alfa Aesar (Ward Hill, MA, USA), respectively. ENMs were tested for endotoxin with a Limulus Amebocyte Lysate (LAL) chromogenic quantitation kit from ThermoFisher (Waltham, MA, USA) using 10 μg ml^{-1} suspensions of ENMs in water and following manufacturers' instructions. Specific surface area, *SSA*, was determined by the nitrogen adsorption/Brunauer-Emmett-Teller (BET) method using a Micrometrics Tristar 3000 (Micrometrics, Inc., Norcross, GA, USA). Equivalent primary particle diameter, d_{BET}, was calculated, assuming spherical particles, as

$$d_{BET} = \frac{6}{SSA \times \rho_p},$$

where ρ_p is the particle density, which was obtained for each particle from the densities of component materials, at 20 °C, reported in the CRC handbook of Chemistry and Physics (Haynes, 2011). Particle crystal size and diameter was also determined by X-ray diffraction using a Scintag XDS2000 powder diffractometer (Scintag Inc., Cupertino, CA, USA), reported here as d_{XRD}.

2.2. ENM dispersal and characterization in suspension

Dispersions were prepared based on a protocol recently developed by the authors (Cohen et al., 2013). Sonication was performed in deionized water (DI H_2O) using the critical dispersion sonication energy (DSE_{cr}), which was determined as previously described for each ENM (Cohen et al., 2013). ENMs were dispersed at 5 mg cm^{-3} in 3 ml of solute in 15 ml conical polyethylene tubes using a Sonifer Cell Disruptor W200P probe sonicator (Heat Systems-Ultrasonics, Inc., Plainview, L.I., NY), calibrated by the calorimetric calibration method previously described (Cohen et al., 2013; Taurozzi et al., 2011). Stock DI H_2O suspensions were then diluted to final concentrations in either RPMI + 10% heat-inactivated FBS or PBS + 0.5% BSA and vortexed for 30 s. Dispersions were analyzed for hydrodynamic diameter (d_H), polydispersity index (PdI), zeta potential (ζ), and specific conductance (σ) by DLS using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Effective density of particles and their formed agglomerates in suspension were determined using the Volume centrifugation method (VCM) as previously described (DeLoid et al., 2014). Fate and transport modeling to determine mean delivered doses for each test ENM over the range of administered initial concentrations was performed using the distorted grid (DG) computational model recently published by the authors (DeLoid et al., 2015).

2.3. Biotinylation of latex beads

1 μm diameter green fluorescent latex beads were purchased from Life Technologies, and biotinylated as described previously (Sulahian et al., 2008). Briefly, 100 mg of tissue culture grade BSA (Sigma Aldrich) dissolved in 15 ml of warm (37 °C) PBS, and 50 mg of biotin-X-NHS (EMD Millipore, Billerica, MA) dissolved in 5 ml warm PBS, were vortexed together and incubated at room temperature for 1 h, and twice dialyzed overnight at 4 °C against 3 l of fresh PBS in two 10 ml aliquots in 12 ml capacity 7 K MWCO Slide-A-Lyzer dialysis cassettes (ThermoFisher Scientific, Waltham, MA). Volume of recovered samples was adjusted to 10 ml by concentrating in 30 K MWCO 15 ml capacity Amicon Ultra centrifugal filters units (EMD Millipore). Two samples of 20×10^{10} carboxylated 1 μm green fluorescent latex beads (Life Technologies) suspended in deionized water were centrifuged at 2000 ×*g* for 10 min, pellets washed and then resuspended, both times in 40 ml 0.1 M 2-(N morpholino) ethanesulfonic acid (MES) buffer (pH 6.0) (SantaCruz Biotechnology, Dallas, TX) containing 10 mg/ml of the water soluble carbodiimide (WSC) N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (SantaCruz), and incubated for 1 h at room temperature. Samples were then centrifuged at 4200 ×*g* for 20 min, pellets re-suspended and washed in 20 ml 0.5 X PBS (diluted in deionized water), and resuspended in 10 ml deionized water (activated bead suspension).

Each 10 ml sample of activated bead suspension was combined with 10 ml of biotin-BSA solution and incubated while rocking overnight at room temperature. Samples were centrifuged at 4200 ×g for 20 min and pellets re-suspended and incubated at room temperature for 1 h in 20 ml 0.5 X PBS with 40 mM glycine (to displace unreacted WSC/carboxyls through glycine's primary amine). Beads were then washed