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 SiO₂, Fe₂O₃, ZnO, CeO₂, TiO₂, and an Ag/SiO₂ 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 CeO₂, 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 hours after exposures to Ag/SiO₂ 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.

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1. Introduction

A large and increasing variety of engineered nanomaterials (ENMs) are now common components of many industrial and consumer products [1–3], and more are being designed for novel diagnostic, antimicrobial, and therapeutic applications [4–11]. 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 [12–14]. The chemical compositions, small sizes and associated large surface-to-volume ratios of these materials suggest potential for harmful biological interactions [1,15–19]. 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 [2,20–22,14,23–29].

Most nanotoxicology studies have focused on direct toxicity, either physiological impairment or death *in vivo*, or various indicators of cytotoxicity *in vitro* [15,16,20–22,24–26,30]. 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 [31–33]. 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

76 and equipped to detect, engulf and destroy inhaled threats, the lung macrophage is also at high risk in
77 inhalation exposures to ENMs. ENMs are readily and rapidly taken up by macrophages, and insoluble
78 nano-sized particles can persist within macrophages for extended periods of time [34,35], raising the
79 likelihood that ENM exposure could lead to both immediate and long-term macrophage dysfunction.
80 Indeed, reported effects of ENM exposures on macrophages include changes in phagocytic function, as
81 well as altered gene expression, cytokine secretion, reactive oxygen and nitrogen species production,
82 and surface markers [36–40].

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84 The few studies of ENM effects on macrophage function published to date have focused on one or two
85 ENMs, and most have employed non-human macrophages [37–40], and effects on phagocytosis of
86 opsonized beads or killed bacterial particles [36,38–40]. Given the growing variety of ENMs that pose
87 potential exposure risks, and evidence from these early studies of ENM-induced macrophage
88 dysfunction, there is a need for broader systematic study and development of efficient methods for
89 screening ENM-induced macrophage dysfunction in relevant human cells. Moreover, since effective
90 macrophage defense requires both phagocytosis and subsequent killing of inhaled pathogens, it is
91 important to examine effects on both of these functions, using relevant live pathogenic bacteria. In
92 addition, since opsonizing serum proteins are not normally present in alveolar fluid, macrophage
93 function should be studied in the context of unopsonized as well as opsonized phagocytosis.

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95 In the present study we have begun to address the needs outlined above. We employed human THP-1
96 macrophages, differentiated to maximize resemblance to primary lung macrophages, to examine the
97 effects of a panel of industry-relevant metal oxide ENMs, including SiO₂, Fe₂O₃, ZnO, CeO₂, TiO₂, and
98 a composite of 10 percent Ag supported on SiO₂ particles (Ag/SiO₂) [41,42]. We evaluated effects of
99 test ENMs on key macrophage functions, including phagocytosis of both opsonized and unopsonized
100 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 [43,44]. These assessment were carried out using automated fluorescence scanning confocal microscopy and image analysis techniques, adapted from methods previously developed by the authors [45,46], as well as flow cytometry and lysis and CFU assays.

2. Materials and Methods

2.1. ENM powders and characterization

SiO₂, Fe₂O₃, CeO₂, and the composite Ag/SiO₂ ENM, consisting of 10 percent silver supported on SiO₂ particles, were produced by flame spray pyrolysis using the Harvard Versatile Engineered Nanomaterial Generation System (VENGES) as previously described [41,42,47]. TiO₂ 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

$$d_{\text{BET}} = \frac{6}{\text{SSA} \times \rho_{\text{p}}},$$

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 [48]. 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} .

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126 **2.2. ENM dispersal and characterization in suspension**

127 Dispersions were prepared based on a protocol recently developed by the authors [49] . Sonication was
128 performed in deionized water (DI H₂O) using the critical dispersion sonication energy (DSE_{cr}), which
129 was determined as previously described for each ENM [49]. ENMs were dispersed at 5 mg cm⁻³ in 3 ml
130 of solute in 15 ml conical polyethylene tubes using a Sonifer Cell Disruptor W200P probe sonicator
131 (Heat Systems-Ultrasonics, Inc., Plainview, L.I., NY), calibrated by the calorimetric calibration method
132 previously described [49,50]. Stock DI H₂O suspensions were then diluted to final concentrations in
133 either RPMI + 10% heat-inactivated FBS or PBS + 0.5% BSA and vortexed for 30 seconds. Dispersions
134 were analyzed for hydrodynamic diameter (d_H), polydispersity index (PdI), zeta potential (ζ), and
135 specific conductance (σ) by DLS using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK).
136 Effective density of particles and their formed agglomerates in suspension were determined using the
137 Volume centrifugation method (VCM) as previously described[51]. Fate and transport modeling to
138 determine mean delivered doses for each test ENM over the range of administered initial concentrations
139 was performed using the distorted grid (DG) computational model recently published by the authors[52].

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141 **2.3. Biotinylation of latex beads**

142 1 μ m diameter green fluorescent latex beads were purchased from Life Technologies, and biotinylated
143 as described previously [46]. Briefly, 100 mg of tissue culture grade BSA (Sigma Aldrich) dissolved in
144 15 ml of warm (37° C) PBS, and 50 mg of biotin-X-NHS (EMD Millipore, Billerica, MA) dissolved in 5
145 ml warm PBS, were vortexed together and incubated at room temperature for 1 hour, and twice dialyzed
146 overnight at 4° C against 3 L of fresh PBS in two 10 ml aliquots in 12 ml capacity 7K MWCO Slide-A-
147 Lyzer dialysis cassettes (ThermoFisher Scientific, Waltham, MA). Volume of recovered samples was
148 adjusted to 10 ml by concentrating in 30K MWCO 15 ml capacity Amicon Ultra centrifugal filters units
149 (EMD Millipore). Two samples of 20×10^{10} carboxylated 1 μ m green fluorescent latex beads (Life

Technologies) suspended in deionized water were centrifuged at $2,000 \times g$ for 10 minutes, 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 hour at room temperature. Samples were then centrifuged at $4,200 \times g$ for 20 minutes, pellets resuspended 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 $4,200 \times g$ for 20 minutes and pellets re-suspended and incubated at room temperature for 1 hour 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 twice in 10 ml and finally resuspended in 5 ml of PBS containing 0.2% BSA and 0.01% sodium azide (final 4×10^{10} beads ml^{-1}), divided into 1 ml aliquots and stored at 4°C .

2.4. Preparation of bead suspensions

Beads were suspended at 2×10^9 beads/ml in 1.5 ml PBS, sonicated with a probe sonicator for 15 seconds, and diluted to 2×10^8 beads/ml in either RPMI + 0.3% BSA (unopsonized) or 10% Human AB serum (opsonized), and incubated at room temperature for 30 min prior to use to allow opsonization to occur.

2.5. *Francisella tularensis* suspensions

Francisella tularensis, subsp. holoarctica live vaccine strain (LVS) expressing green fluorescent protein (GFP) was generously provided by Dr. Larry S. Schlesinger (Ohio State University, Columbus, Ohio). Bacteria were grown to logarithmic phase in Modified Mueller-Hinton broth with tetracycline (10 μg

175 ml⁻¹, selection agent) and aliquots stored at -80° C. Six days prior to experiments bacteria were thawed
176 and streaked on Cystine Heart agar plates enriched with 1% chocolized hemoglobin and containing 10
177 µg ml⁻¹ tetracycline. A ~1 cm³ sample of bacteria was collected from streak plates with an inoculating
178 loop, suspended in 8 ml of PBS, and the suspension was filtered through a 5 µm syringe filter. A₆₀₀ of a
179 1:5 dilution was measured and CFU ml⁻¹ estimated from a standard A₆₀₀/CFU curve. The suspension
180 was diluted to 5 × 10⁸ CFU ml⁻¹ stock for opsonized (RPMI + 10% human serum) or 1 × 10⁹ ml⁻¹ for
181 unopsonized (RPMI + 0.3% BSA) uptake. Suspensions were incubated at room temperature for 30 min
182 prior to use to allow opsonization to occur.

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184 **2.6. Macrophages**

185 THP-1 monocytes were obtained from ATCC (Manassas, VA), and were cultured in RPMI
186 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U ml⁻¹ penicillin, 100 µg ml⁻¹
187 streptomycin, and 10 mM HEPES. THP-1 monocytes were differentiated using the modified PMA
188 protocol proposed by Diagneault et al. [53], which was shown to produces macrophages that closely
189 resemble primary human macrophages in terms of morphology, phenotypic markers and function.
190 Briefly, cells were resuspended at a 3.2 × 10⁵ ml⁻¹ in RPMI + 10% FBS containing 200 nM PMA,
191 dispensed into 96-well black-walled imaging plates (BD, Franklin Lakes, NJ) at 8.0 × 10⁵ cells per well
192 or into 6-well cell culture plates (BD) at 1 × 10⁶ cells per well, incubated for 3 days, washed with PBS,
193 and incubated for an additional 4 days in RPMI + 10% FBS without PMA.

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195 **2.7. Cytotoxicity assay**

196 Adherent THP-1 macrophages in 96-well microplate format were incubated at 37° C and 5% CO₂ for
197 four hours with 100 µl (96-well plates) of ENM suspensions. For immediate cytotoxicity evaluation
198 cells were then immediately incubated with 50 µl per well PBS + 2 µM Calcein AM (Life Technologies,
199 Carlsbad, CA) + 4 µM Ethidium Homodimer 1 (Life Technologies) + 2 µg ml⁻¹ Hoechst 33342 (Life

Technologies) + 2.5 $\mu\text{g ml}^{-1}$ HCS CellMask™ Blue stain (Life Technologies) at 37° C and 5% CO₂ for 40 minutes. For delayed (24 hour) toxicity evaluation, following the four hour ENM incubation, cells were washed twice with PBS, incubated overnight in RPMI + 10% FBS, and then stained as described above. At the end of the stain incubation, the stain mixture was replaced with PBS, and scanning confocal images were acquired immediately as described below.

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206 **2.8. ROS production and mitochondrial integrity assay**

Adherent THP-1 macrophages in 96-well microplate format were pre-loaded with ROS probe by incubating in 50 μl per well of PBS + 10 μM CM-H₂DCFDA (Life Technologies) at 37° C and 5% CO₂ for 30 minutes. Stain solution was then replaced with 100 μl of ENM suspensions and cells were incubated for four hours at 37° C and 5% CO₂. For evaluation of immediate effects, cells were then washed with PBS and incubated with 50 μl per well PBS + 100 nM MitoTrackerRed-CMXRos (Life Technologies) + 2 $\mu\text{g ml}^{-1}$ Hoechst 33342 (Life Technologies) + 2.5 $\mu\text{g ml}^{-1}$ HCS CellMask™ Blue stain (Life Technologies) at 37° C and 5% CO₂ for 30 minutes. For 24 hour evaluation, following the four hour ENM incubation, cells were washed twice with PBS, incubated overnight in RPMI + 10% FBS, and then stained as described above. At the end of the stain incubation, the stain mixture was replaced with PBS, and scanning confocal images were acquired immediately as described below.

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218 **2.9. Latex bead phagocytosis assay**

Cells were incubated for 4 hours at 37° C and 5% CO₂ with 100 μl (96-well plates) or 1.5 ml (6-well plates) of ENM suspensions, washed twice with PBS, incubated for 30 minutes with RPMI + 10% FBS +/- cytochalasin D (15 μM), incubated for 20 minutes with 50 μl (96-well) or 750 μl (6-well) of biotinylated bead suspensions to allow binding, washed twice with PBS, and incubated with RPMI + 10% FBS +/- cytochalasin D (15 μM) for an additional 30 minutes for internalization. Cells in 6-well plates were washed twice with cold PBS and processed for either flow cytometry or lysis and CFU counting as

described below. Cells in 96-well plates were washed once with cold PBS, incubated at 4° C for 1 hour with 4 µg ml⁻¹ AlexaFluor594-streptavidin (Life Technologies, Carlsbad, CA) in PBS + 1% BSA, washed twice with cold PBS, fixed with 4% formaldehyde at room temperature for 10 minutes, washed twice with PBS, incubated with 50 µl per well PBS + 2 µg ml⁻¹ Hoechst 33342 (Life Technologies) + 2.5 µg ml⁻¹ HCS CellMask™ Blue stain (Life Technologies) at room temperature for 1 hour, washed with PBS and stored in 200 µl per well PBS at 4°C prior to scanning.

2.10. Bacterial phagocytosis assay

Cells were incubated for 4 hours at 37° C and 5% CO₂ with 100 µl (96-well plates) or 1.5 ml (6-well plates) of ENM suspensions, washed twice with PBS, incubated for 30 minutes with RPMI + 10% FBS +/- cytochalasin D (15 µM), followed by incubation for 2 hours with 50 µl (96-well) or 750 µl (6-well) of FT suspension. Following phagocytosis, cells were washed twice with PBS and processed for scanning cytometry, flow cytometry or lysis and CFU counting. Cells for scanning cytometry were fixed with 4% formaldehyde at room temperature for 10 minutes, washed twice with PBS, incubated with 50 µl per well PBS + 2 µg ml⁻¹ Hoechst 33342 (Life Technologies) + 2.5 µg ml⁻¹ HCS CellMask™ Blue stain (Life Technologies) at room temperature for 1 hour, and washed twice with PBS before storage in 200 µl per well PBS at 4°C prior to scanning. Cells were processed for flow cytometry or lysis and CFU counting as described below.

2.11. Bacterial killing assay

Cells were incubated for 2 hours at 37° C and 5% CO₂ with 50 µl (96-well) or 750 µl (6-well) of FT suspension for phagocytosis, washed twice with PBS, incubated for 30 minutes with RPMI/10% FBS + 100 µg/ml gentamicin to kill external bacteria, washed twice with PBS, incubated for 4 hours at 37° C and 5% CO₂ with 100 µl (96-well plates) or 1.5 ml (6-well plates) of ENM suspensions, washed twice with PBS, incubated for an additional 18 hours in RPMI + 10% FBS, washed twice with PBS and

250 processed for scanning cytometry, as described above, or for flow cytometry or lysis and CFU counting,
251 as described below.

252

253 **2.12. Scanning cytometry and analysis**

254 Confocal fluorescence microscopy was performed using a BD Pathway 855 High-Content Bioimager
255 with a 20X NA075 objective (Olympus), with laser auto-focus, 2x2 binning and flat field correction.
256 Collapsed confocal stack (from Z-stack images at 1.5 μm intervals) were acquired using standard
257 excitation, emission, and dichroic filters for green (Calcein AM, CM-H2DCFDA, GFP-expressing *F.*
258 *tularensis*, green fluorescent beads), blue (Hoechst/CellMask Blue) and red (Ethidium Homodimer 1,
259 MitoTrackerRed-CMXRos, AlexaFluor594) channels. Sufficient image fields were collected from
260 triplicate wells to provide a minimum of 500 cells for quantitative analysis of each sample condition.
261 Image analysis and quantification was performed using our custom MATLAB [®] (The Mathworks,
262 Natick, MA) software as previously described [45,46].

263

264 **2.13. Flow cytometry**

265 Cells were incubated for 20 minutes at 4° C in 2 ml per well cold PBS without Ca/Mg + 10 mM EDTA,
266 gently lifted by cell scraper, and aspirates and two subsequent washes collected in individual 15 ml
267 conical tubes. Cells were fixed by addition of an equal volume of 4% formaldehyde and incubation at
268 room temperature for 10 minutes, washed twice by centrifugation with PBS, resuspended in 0.5 ml per
269 sample of PBS and transferred to flow tubes. Cells were analyzed using a BD Canto II flow cytometer
270 (BD Biosciences, Sparks, MD). 10,000 cells were analyzed for each sample.

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272 **2.14. Lysis and CFU**

273 Cells were lysed by incubation for 5 minutes at room temperature in 2 ml per well 0.1% SDS in PBS
274 without Ca/Mg, followed by vigorous pipetting. Aspirates were diluted to 1:500,00 and 1:500,000 and

275 100 µl of each dilution was spread onto triplicate Cystine Heart agar + 1% chocolatzed hemoglobin
276 plates. Plates were incubated at 37° C for 48 hours, and colonies were counted manually.

277

278 **3. Results**

279 **3.1. ENM characterization and dosimetry**

280 Characterizations of ENM powders are shown in Table 1, and characterization of the corresponding
281 suspensions in culture media (RPMI + 10% heat-inactivated FBS) are shown in Table 2. All ENMs
282 formed agglomerates averaging between roughly 120 and 240 nm in diameter, with considerable
283 polydispersity in most cases, particularly for the ZnO material. Zeta potential was negative for all
284 suspended materials, consistent with corona formation by albumin and other serum proteins with
285 predominantly anionic surfaces. Effective densities ranged between roughly 1.1 (slightly greater than
286 media density) for Ag/SiO₂ and SiO₂, to approximately 2.1 for CeO₂. Effective mean delivered
287 concentrations (over the 4 hour exposures) determined for each initial concentration of each ENM using
288 the DG model, are presented in Table 3. At the highest initial concentration used (50 µg ml⁻¹) the
289 effective delivered concentrations among the test ENMs ranged from nearly identical to the initial
290 concentration (e.g. for Ag/SiO₂ and SiO₂ ENMs) to more than three times the initial concentration (for
291 TiO₂). Analysis of ENM suspensions (10 µg ml⁻¹) for endotoxin contamination by chromogenic LAL
292 assay showed no endotoxin present (< 0.1 EU ml⁻¹) in any of the ENMs used.

293

294 **3.2. Cytotoxicity of ENMs**

295 To assess ENM toxicity we examined cell viability by fluorescent live/dead staining with calcein AM
296 and ethidium homodimer-1. We also used fluorescent probes to assess oxidative stress (ROS production)
297 and mitochondrial function, both immediately and 24 hours after four hour ENM exposures (Figure 1).
298 No loss of viability was observed immediately after treatment (four hours) with any of the ENMs in our
299 panel. At 24 hours after exposure, however, significant dose-dependent decreases in viability were

300 observed for Ag/SiO₂ and ZnO ENM-treated cells. With the exception of Fe₂O₃, which caused modest
301 cell death 24 hours after exposure at an intermediate concentration (administered concentration of 12.5
302 µg ml⁻¹, delivered concentration of 16.38 µg ml⁻¹), none of the other ENMs affected viability at 24 hours.
303 Most ENMs caused minimal changes in ROS (as indicated by reduction of non-fluorescent CM-
304 H₂DCFDA to its fluorescent form) immediately or 24 hours after exposure, except at the lowest
305 concentration, where a 25 – 50 percent increase was observed at 24 hours. One exception to this pattern
306 was a significant decrease in ROS relative to control 24 hours after exposure with intermediate
307 concentrations of Fe₂O₃ ENM. Changes in mitochondrial integrity, as measured by mitochondrial
308 membrane potential-dependent uptake of fluorescent dye (MitoTrackerRed-CMXRos), followed
309 patterns that nearly mirrored those observed for changes in ROS. Specifically, increased dye uptake
310 (membrane potential) was observed in most cases 24 hours after exposure at the lowest concentration.
311 However, significant decreases in mitochondrial potential were observed at higher concentrations of
312 Fe₂O₃ and CeO₂ at both four and 24 hours, and of ZnO at four (but not 24) hours.

313

314 **3.3. Effect of ENMs on phagocytosis of latex beads**

315 To investigate the effect of ENM exposure on phagocytosis, THP-1 macrophages were treated with
316 ENM suspensions for four hours prior to assessment of uptake of opsonized and unopsonized 1 µm
317 fluorescent latex beads. Differential staining and quantification of external bound and internalized beads
318 by fluorescence scanning cytometry and automated image analysis was performed, as described
319 previously [45,46], and detailed in the methods section. The results of these studies are summarized in
320 Figure 2. Because changes in uptake can result either from altered binding or internalization of particles,
321 it is useful to differentiate between these two possible contributions. Since external bound and
322 internalized beads were differentially labeled and enumerated in our assay, we were able to calculate
323 total beads per cell (bound external + internal) as well as internalized beads per cell, and the percentage
324 of total beads internalized. In general, exposure to ENMs resulted in a decrease in both total and

internalized opsonized beads, and a decrease in both total and internalized unopsonized beads. However, in most cases, the differences between individual treatment measurements and controls did not reach statistical significance (by one-way ANOVA with Dunnett's test for multiple comparisons, Figure 2). Example fluorescence confocal images illustrating differential staining of external bound beads and reduced uptake after exposure to Ag/SiO₂ ENM are shown in Figure 3 a. With most ENMs, these changes were most pronounced at the lowest concentrations tested (3.125 – 6.25 µg ml⁻¹). An exception to this pattern was observed with ZnO ENMs, which caused increased internalization of unopsonized beads at low concentrations, but decreased internalization at high concentrations. Although none of the differences in unopsonized beads internalized between mean control and individual concentration exposures with ZnO reached significance, correlation analysis revealed a significant dose-dependent decrease in internalization ($r = -.873$, $p = 0.023$). Otherwise the pattern observed with ZnO exposure was identical to that observed with the other ENMs, with a particularly sharp and statistically significant decrease in total and internalized opsonized beads. The percent of total beads internalized, also shown in Figure 2, provides further insight into the functional cause of changes in numbers of beads internalized per cell. For all ENMs but ZnO, exposure caused little change in percent internalization, suggesting that regardless of the number of particles bound, the fraction of those bound particles that were subsequently taken up was unchanged, and that observed changes in internalized beads per cell was primarily a result of altered recognition and binding of particles. In this respect, ZnO was a notable exception, causing a dramatic and statistically significant concentration-dependent reduction in percent internalization for both opsonized and unopsonized beads, suggesting an impairment by ZnO of the internalization machinery as the primary cause of reduced uptake. As discussed below in greater detail, the effects of ZnO and Ag/SiO₂ on phagocytosis may be related to the delayed cytotoxicity observed 24 hours after treatment with these ENMs, rather than a direct effect on phagocytic processes.

349 To explore whether similar results would be obtained using a different methodology, we used flow
350 cytometry to evaluate the effects of Ag/SiO₂ ENM on macrophage function. The data show a
351 statistically significant and marked reduction in phagocytosis of opsonized beads (as well as a trend to
352 increase phagocytosis of unopsonized beads, though not statistically significant). The flow cytometry
353 results, shown in figure 3 b-c, are consistent with those observed using the scanning cytometry approach.
354 In cells exposed to Ag/SiO₂ ENM, the area under the higher intensity, right-shifted set of peaks,
355 corresponding to cells containing one or more fluorescent beads, was substantially diminished, relative
356 to untreated controls, although not as much as seen in cells treated with the positive control, the actin
357 polymerization inhibitor cytochalasin-D (Figure 3 b). This corresponded to a 50% decrease relative to
358 untreated controls in mean fluorescence for cells exposed at 50 µg ml⁻¹ Ag/SiO₂ (Figure 3 c), identical to
359 the decrease observed by scanning cytometry for the same treatment (Figure 2).

360

361 **3.4. Effects of ENMs on phagocytosis and killing of *Francisella tularensis***

362 *Francisella tularensis* (FT) is gram negative intracellular pathogen that causes the potentially fatal
363 disease tularemia in humans and animals [43], and has been categorized as a category A select agent
364 because of its potential for weaponization and dissemination [44]. Using the differential staining and
365 scanning cytometry approach we examined the effect of ENM exposure on phagocytosis and killing of
366 the live vaccine strain (LVS) of FT. For these experiments we employed a GFP-expressing clone of the
367 bacterium, with indirect immunofluorescence staining to selectively label external bacteria. To assess
368 effects on phagocytosis, THP-1 macrophages were exposed to ENMs prior to phagocytosis of opsonized
369 or unopsonized bacteria. For assessment of bacterial killing, macrophages were treated with ENM
370 suspensions after phagocytosis to ensure equal uptake in all samples. The results of these studies are
371 summarized in Figure 4. Sample confocal images illustrating differential staining of external bound FT
372 are shown in Figure 5 a. Treatment with ZnO ENMs caused a striking dose-dependent reduction in total
373 and internalized unopsonized FT, but only a modest reduction of the same for opsonized FT. Treatment

374 with Ag/SiO₂ also impaired both opsonized and unopsonized phagocytosis of FT. All other ENMs (SiO₂,
375 Fe₂O₃, CeO₂ and TiO₂) caused slight impairment of opsonized, but not of unopsonized FT phagocytosis.
376 A notable exception was observed with CeO₂ ENM, which produced a significant dose-dependent
377 increase in the number of internalized unopsonized FT per cell. Percent internalization was also
378 significantly diminished after treatment with both Ag/SiO₂ and ZnO ENMs, suggesting that these ENMs
379 impaired both binding and internalization. As discussed below, effects of ZnO and Ag/SiO₂ on FT
380 phagocytosis may be related to the delayed cytotoxicity caused by these ENMs.

381

382 All ENMs appeared to produce modest improvement in killing of both opsonized and unopsonized FT,
383 as indicated by decreased bacteria per cell at 24 hours (Figure 4). Although the trend was consistent
384 among ENMs, statistically significant differences were observed only at the highest administered
385 concentration (50 µg ml⁻¹) of Ag/SiO₂ and TiO₂. It should be noted that the effective delivered
386 concentration of TiO₂ was three times greater than that of Ag/SiO₂, and that therefore Ag/SiO₂ was
387 more potent in eliciting this response than TiO₂.

388

389 The effects of Ag/SiO₂ and CeO₂ ENMs on FT phagocytosis and killing were also examined by flow
390 cytometry and by lysis and CFU assay. The flow cytometry results were generally consistent with those
391 from scanning cytometry (Figure 5 b-d). CeO₂ ENM treatment sharply increased phagocytosis of
392 unopsonized FT, especially at the lowest concentration (Figure 5 b), and Ag/SiO₂ ENM significantly
393 decreased phagocytosis of both opsonized and unopsonized FT at the highest concentration tested
394 (Figure 5 c), and produced a modest reduction in bacterial load at 24 hours. (Figure 5 d). Lysis and
395 CFU results (Figure 5 e-g) were also generally consistent, but less pronounced, with CeO₂ ENM
396 increasing phagocytosis (Figure 5 e) and improving killing of unopsonized FT (Figure 5 g), and Ag/SiO₂
397 ENM reducing phagocytosis of both opsonized and unopsonized FT at the highest concentration (Figure
398 5 e).

399

400 **4. Discussion**

401 The biological effects of ENMs are highly dependent upon intrinsic particle properties such as
402 composition, size, shape, surface chemistry and extrinsic culture media properties such as ionic strength,
403 which define agglomeration state [49,54–56]. Surprisingly, we observed broad similarities in the
404 response patterns in this study among a compositionally diverse set of ENMs. Exposure to most ENMs
405 tested impaired THP-1 phagocytosis of human serum-opsonized latex beads, but improved phagocytosis
406 of unopsonized beads. In most cases the percentage of total beads associated with cells that were
407 phagocytosed was unaffected, suggesting that the observed changes in phagocytosis were at least in part
408 due to changes in binding of beads to cell surface receptors. Likewise, in the FT phagocytosis and
409 killing assay, exposure to most ENMs resulted in reduced phagocytosis of opsonized FT and either
410 reduced or unchanged levels of phagocytosis of unopsonized FT. There was, again, no change in percent
411 internalization. Finally, all ENMs in our panel, to at least some degree, improved killing of internalized
412 FT at 24 hours.

413

414 The general decrease in phagocytosis of opsonized beads and FT observed here is also consistent with several
415 previous studies described above [36–40]. It might be argued that macrophage over-filling by ENM particles
416 was the cause. However, this would be inconsistent with the increased phagocytosis we observed for
417 unopsonized beads, as well as the fact that the effect was typically observed at the lowest ENM concentration
418 tested. Another possible mechanism for decreased phagocytosis of opsonized particles is decreased surface
419 receptor availability resulting from internalization during receptor-bound ENM particles and agglomerates.
420 Such “ligand-hijacking” is consistent with our finding that percent internalization was usually unchanged,
421 suggesting a receptor defect or deficiency. Moreover the timing of our phagocytosis challenges immediately
422 after ENM exposure would have limited the opportunity for cells to recycle or replenish endocytosed receptors.
423 Receptor internalization has also been proposed by Kodali et. al as a possible mechanism for reduced

424 phagocytosis following ENM exposure [40].

425

426 In this study we did not attempt to identify or quantify expression of the specific receptors involved in
427 phagocytosis of beads or FT, or in ENM uptake. Additional studies along these lines may help us to better
428 understand the difference between effects observed on opsonized and unopsonized uptake. It is likely, however,
429 since we used pooled human serum as the opsonizing agent, that uptake of our opsonized beads and FT was
430 mediated at least in part by the complement receptor (Cr) and the Fcγ receptor, whereas unopsonized beads and
431 FT were more likely recognized and bound by scavenger receptors (SR) such as SR-A and MARCO [57], or by
432 other non-specific receptors. It should also be noted that serum proteins, including complement and IgG, do not
433 normally occur in alveolar fluid, and that true opsonization, in the sense of marking particles or pathogens for
434 recognition by specific receptors, would therefore not occur. Thus, phagocytosis by alveolar macrophages *in*
435 *vivo* probably occurs primarily through SR and other non-specific receptors.

436

437 It is also worth noting that in biological fluids, proteins are typically adsorbed onto the surface of ENMs to form
438 protein coronas, which in turn play an important role in determining receptor type and mechanism of uptake, as
439 well as subsequent intracellular transport and toxicity [58–63]. Since ENMs in our studies were suspended in
440 media containing heat-inactivated FBS, it is likely that binding and uptake of ENMs was mediated by SR or
441 non-specific receptors. Indeed, several recent studies have demonstrated a prominent role for scavenger
442 receptors in uptake of ENMs by macrophages and epithelial cells [60–63]. Whereas depletion of SR during
443 ENM uptake is consistent with the observed reduction in uptake of unopsonized FT, it would leave unexplained
444 our findings of diminished binding and uptake of opsonized beads and FT, and of enhanced binding and uptake
445 of unopsonized beads. Moreover, SRs comprise a diverse “superfamily” of receptors [64], and it is not unlikely
446 that the specific SRs mediating ENM uptake differ from those mediating unopsonized bead and microbe uptake.
447 Given this, ligand-hijacking would seem to be an implausible explanation for the general pattern of effects of
448 ENM exposure on phagocytosis observed in the present study.

449

450 Although outcomes for most ENMs examined conformed to the pattern described above, there were
451 notable exceptions. In particular, ZnO ENM exposure led to a dramatic dose-dependent reduction in
452 phagocytosis of both opsonized and unopsonized beads, and near complete abrogation of phagocytosis
453 of unopsonized FT, but only a slight reduction in uptake of opsonized FT. In addition, whereas most
454 ENMs had no effect on percent internalization of either beads or FT, diminished uptake resulting from
455 ZnO ENM exposure was accompanied by a significant and sharp reduction in percent internalization,
456 suggesting an impairment of the associated cytoskeletal machinery. Part or all of these effects may be a
457 consequence of cytotoxicity caused by ZnO ENM exposure. Although no loss of viability was observed
458 immediately after exposure, severe and dose-dependent loss of viability was observed 24 hours after
459 exposure, suggesting that although not detectable by our methods, cell death was underway, which one
460 might easily imagine impacting other cell functions such as phagocytosis. Several studies have found
461 that ENM exposure can lead to activation of inflammasomes, with activation of caspase-1, which in turn
462 activates and leads to release of IL-1 β , and which through action on other targets also initiates a type of
463 programmed cell death known as pyroptosis [65–67]. It is possible then, that the severe dysfunction
464 observed after ZnO ENM exposure was associated with this process, rather than any direct effects on
465 phagocytic pathways. However, this still leaves unexplained the observation that opsonized
466 phagocytosis of FT was not substantially impacted by ZnO exposure. It should also be noted that
467 Ag/SiO₂ ENM, which caused delayed toxicity similar to that seen with ZnO, did not significantly impair
468 uptake of unopsonized beads or FT, although its negative effects on opsonized bead and FT uptake may,
469 as in the case of ZnO, may be a result of cellular derangements underway, but not manifested as frank
470 cell death, until 24 hours after exposure.

471

472 Another notable exception to the pattern observed for most ENMs is that of CeO₂ ENM, which was the
473 only ENM tested that caused a statistically significant dose-dependent increase in phagocytosis (of

unopsonized FT by scanning cytometry assays ($r = 0.897$, $p = .0292$, Figure 4). Like most other ENMs, CeO₂ also appeared to improve killing of both opsonized and unopsonized FT by scanning cytometry, although this only reached statistical significance at the highest concentration tested for opsonized FT. Improved phagocytosis and killing of FT after exposure to CeO₂ ENM were also observed by both flow cytometry and cell lysis and CFU (Figure 5). The combination of increased uptake and killing of FT by CeO₂ ENM at concentrations that did not affect viability either immediately or 24 hours after exposure (Figure 1), suggests a possible novel therapeutic application for this ENM. Because of its unique autoregenerative free-radical scavenging properties, CeO₂ has been proposed as a nanotherapeutic agent for disorders associated with oxidative stress [68–71]. It is not clear from our data whether these reported antioxidant properties of CeO₂ ENMs contributed to the facilitation of FT uptake or killing observed in this study. Although ROS generation following CeO₂ ENM exposure was not pronounced, it did not differ substantially from that observed with other ENMs. Further work is required to explore the efficacy and mechanism of this potential therapeutic use for CeO₂ ENMs.

While oxidative stress (ROS production) is well known to be a precursor to cytotoxicity and genotoxicity generally, which could in turn affect innate immune functions, ROS production also plays an important role in killing of intracellular bacteria, including FT [72–75]. Indeed, FT has been shown to downregulate host cellular ROS responses and to produce superoxide dismutases in order to provide protection against ROS within macrophages [73–75]. It is thus possible that changes in ROS production caused by ENM exposure could alter FT killing by macrophages. However, in the case of the Fe₂O₃ ENM tested here, which caused a significant decrease in ROS production, there was no associated statistically significant change in killing function. Additional studies are needed to determine whether the improvements in bacterial killing observed with some ENMs (Ag/SiO₂ and TiO₂) can be attributed to changes in ROS production in infected cells that were pre-treated with ENMs.

499

500 Finally, mass transport (sedimentation and diffusion) over time plays an important role in determining
501 effective dose delivered to cells, and since toxicology is by nature comparative, it is imperative that
502 colloidal characterization include relevant transport properties (agglomerate/particle size and effective
503 density), and that analysis of particle kinetics by computational fate and transport modeling be
504 performed to provide dose metrics needed to properly interpret dose responses [49,51,76]. Accordingly,
505 dose responses have been presented here in terms of delivered dose (mean concentration at the bottom of
506 the cell culture well over the duration of the exposure. It is worth noting that as a result of differences in
507 transport properties among the suspended ENMs, the effective dose ranges tested also differed among
508 the test ENMs in these studies.

509

510 **5. Conclusion**

511

512 In this study we have demonstrated that ENM exposure can significantly alter key macrophage functions
513 in either a detrimental or potentially advantageous way. It is important that hazard evaluation of
514 potentially airborne ENMs include assessment of macrophage function. Inhalation is the most likely
515 route of accidental ENM exposure, and lung macrophages, which provide the first line of defense
516 against inhaled pathogens and particles, not only lie in direct line of fire, but are disposed to take up and
517 concentrate ENMs that reach the alveolar space. Moreover, it is important to note that phagocytosis and
518 killing by macrophages can proceed by a variety of mechanisms and signaling pathways, depending on
519 the nature of the pathogen or particle, as well as the specific proteins or opsonins present. This point is
520 underscored by the differences between effects of ENMs on serum-opsonized and unopsonized
521 phagocytosis of beads and FT observed in the present study. Furthermore, we have previously shown
522 that even among different strains of *Staphylococcus aureus*, macrophage uptake appears to proceed via

different signaling pathways [45]. Focus in future work should therefore be given to developing a robust, but practical panel of harmonized macrophage functional screening assays.

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FIGURE LEGENDS

Figure 1. NP panel cytotoxicity, mitochondrial function, and oxidative stress - scanning cytometry.

Adherent PMA-matured THP-1 cells were incubated for 4 hours with indicated concentrations of nanoparticles, and stained either immediately or after wash and replacement with fresh media and overnight incubation. Cytotoxicity (% viability), ROS production (% control ROS) and mitochondrial function (% control fluorescence) are indicated for 4 h (immediately after exposure, solid lines) and 24 h (dashed lines) time points. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; Statistically significant dose/response correlation coefficients (r) and P values are indicated in inset boxes.

Figure 2. NP panel polystyrene bead phagocytosis screen, scanning cytometry, dose response.

Adherent PMA-matured THP-1 cells were incubated for 4 hours with indicated concentrations of nanoparticles prior to incubation with green fluorescent polystyrene beads. Total beads per cell, internal beads per cell, and percent internalized ($100 \times$ internal beads per cell/total beads per cell) are indicated for unopsonized (solid lines) and opsonized (dashed lines) beads. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; Statistically significant dose/response correlation coefficients (r) and P values are indicated in inset boxes.

Figure 3. Ag/SiO₂ polystyrene bead phagocytosis, images and flow cytometry. Adherent PMA-matured THP-1 cells were incubated for 4 hours with indicated concentrations of 10% Ag/SiO₂ prior to incubation with opsonized or unopsonized 1 μ m biotinylated green fluorescent polystyrene beads. (a) representative confocal fluorescence images from latex bead phagocytosis experiments. All beads are seen in the green channel, and external beads, labeled with streptavidin-AlexaFluor 594, are seen in the red channel. (b) Representative cell fluorescence histograms from bead phagocytosis flow cytometry

786 experiments. (c) quantification of flow cytometry (mean percent control fluorescence) for bead
787 phagocytosis by cells treated with cytochalasin D control or indicated concentrations of Ag/SiO₂ ENMs.
788
789 **Figure 4.** NP panel *F. tularensis* phagocytosis and killing screen, scanning cytometry, dose response.
790 For phagocytosis experiments, adherent PMA-matured THP-1 cells were incubated for 4 hours with
791 indicated concentrations of ENMs prior to incubation for 2 hours with *F. tularensis*. For bacterial killing
792 experiments cells were incubated for 2 hours with *F. tularensis*, then treated for 4 hours with ENM
793 suspensions, washed, and incubated overnight (total 24 hours). Phagocytosis (total bacteria per cell,
794 internal bacteria per cell, and percent internalized) and bacterial killing (bacteria per cell at 24 hours) are
795 indicated for unopsonized (solid lines) and opsonized (dashed lines) beads. * = p<0.05; ** = p<0.01;
796 ***=p<0.001; Statistically significant dose/response correlation coefficients (r) and P values are
797 indicated in inset boxes.

798
799 **Figure 5.** *F. tularensis* phagocytosis and killing, scanning cytometry, images, flow cytometry and
800 lysis/cfu. Adherent PMA-matured THP-1 cells were incubated for 4 hours with indicated concentrations
801 of 10% Ag/SiO₂ or CeO₂ prior to or following incubation for 2 hours with *F. tularensis*. (a)
802 representative confocal fluorescence images from FT phagocytosis experiments. All bacteria (GFP-
803 expressing) are seen in the green channel, and external bacteria, labeled by indirect immunofluorescence
804 staining with AlexaFluor 594, are seen in the red channel. (b, c) quantification of flow cytometry (mean
805 percent control fluorescence) for FT phagocytosis by cells treated with cytochalasin D control or
806 indicated concentrations of either CeO₂ or Ag/SiO₂ ENMs. (d) quantification of flow cytometry for FT
807 killing by cells treated with indicated concentrations of Ag/SiO₂ ENM. (e, f) quantification of cell lysis
808 and CFU count experiments (percent control CFU) for FT phagocytosis by cells treated with indicated
809 concentrations of CeO₂ or Ag/SiO₂ ENMs. (g) quantification of cell lysis and CFU count experiments

810 for FT phagocytosis by cells treated with indicated concentrations of CeO₂ ENM. Results represent
811 means ± standard deviation, N=3; * = p<0.05; ** = p<0.01; ***=p<0.001

812

813

Figure 1

Figure 2

10% Ag/SiO₂

SiO₂

Fe₂O₃

ZnO

CeO₂

TiO₂

—●— Unopsonized -▼- Opsonized

Figure 3

10% Ag/SiO₂

Figure 5

Table 1. Materials Investigated, characterization of dry ENM powders

Material	SSA ($\text{m}^2 \text{g}^{-1}$)	d_{BET} (nm)	d_{XRD} (nm)
VENGES Ag/SiO ₂	375	6.7	6.9
VENGES SiO ₂	231	11.8	NA
VENGES Fe ₂ O ₃	131	8.7	NA
Alfa Aesar ZnO	17	63.0	22.3
VENGES CeO ₂	144	5.4	9.5
Evonik TiO ₂	50	28.4	33.0

Properties of ENM powder properties: SSA : specific surface area, d_{BET} : equivalent primary particle diameter, d_{XRD} : diameter by X-ray diffraction.

Table 2. Materials Investigated, colloidal characterization of suspensions in RPMI + 10% FBS.

Material	d_{H} (nm)	PdI	ζ (mV)	σ (mS cm^{-1})	ρ_{EV} (g cm^{-3})
VENGES Ag/SiO ₂	179.2 ± 4.1	0.41 ± 0.05	-9.3 ± 1.3	12.7 ± 0.3	1.103 ± 0.004
VENGES SiO ₂	182.7 ± 3.7	0.57 ± 0.03	-7.3 ± 1.0	12.4 ± 0.1	1.126 ± 0.002
VENGES Fe ₂ O ₃	119.3 ± 1.2	0.44 ± 0.01	-7.5 ± 1.0	11.9 ± 0.7	1.715 ± 0.024
Alfa Aesar ZnO	219.8 ± 79.2	0.75 ± 0.28	-10.5 ± 2.4	12.4 ± 0.1	1.639 ± 0.019
VENGES CeO ₂	127.8 ± 2.3	0.50 ± 0.01	-3.6 ± 5.0	11.2 ± 0.2	2.089 ± 0.114
Evonik TiO ₂	234.9 ± 4.9	0.27 ± 0.01	-6.1 ± 4.8	11.4 ± 0.3	1.606 ± 0.008

ENM suspension properties: d_{H} : hydrodynamic diameter, PdI : polydispersity index, ζ : zeta potential, σ : specific conductance, ρ_{EV} : effective density measured by VCM. Values represent the mean ($\pm$ standard deviation) of triplicate measurements.

Table 3. ENM administered and corresponding mean delivered concentrations.

Material	Administered concentration $\mu\text{g ml}^{-1}$				
	3.125	6.25	12.5	25	50
	Mean delivered concentration $\mu\text{g ml}^{-1}$				
VENGES Ag/SiO ₂	3.44	6.88	13.77	27.54	55.07
VENGES SiO ₂	3.57	7.14	14.29	28.59	57.17
VENGES Fe ₂ O ₃	4.09	8.19	16.38	32.76	65.51
Alfa Aesar ZnO	8.50	17.00	34.00	68.00	136.0
VENGES CeO ₂	5.01	10.03	20.05	40.10	80.20
Evonik TiO ₂	9.42	18.84	37.68	75.35	150.70