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REVIEW

Perturbation of Pulmonary Immune Functions by Carbon Nanotubes and Susceptibility to Microbial Infection

Brent E. Walling and Gee W. Lau*

Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL 61802, USA

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Occupational and environmental pulmonary exposure to carbon nanotubes (CNT) is considered to be a health risk with a very low threshold of tolerance as determined by the United States Center for Disease Control. Immortalized airway epithelial cells exposed to CNTs show a diverse range of effects including reduced viability, impaired proliferation, and elevated reactive oxygen species generation. Additionally, CNTs inhibit internalization of targets in multiple macrophage cell lines. Mice and rats exposed to CNTs often develop pulmonary granulomas and fibrosis. Furthermore, CNTs have immunomodulatory properties in these animal models. CNTs themselves are proinflammatory and can exacerbate the allergic response. However, CNTs may also be immunosuppressive, both locally and systemically. Studies that examined the relationship of CNT exposure prior to pulmonary infection have reached different conclusions. In some cases, pre-exposure either had no effect or enhanced clearance of infections while other studies showed CNTs inhibited clearance. Interestingly, most studies exploring this relationship use pathogens which are not considered primary pulmonary pathogens. Moreover, harmony across studies is difficult as different types of CNTs have dissimilar biological effects. We used *Pseudomonas aeruginosa* as model pathogen to study how helical multi-walled carbon nanotubes (HCNTs) affected internalization and clearance of the pulmonary pathogen. The results showed that, although HCNTs can inhibit internalization through multiple processes, bacterial clearance was not altered, which was attributed to an enhanced inflammatory response caused by pre-exposure to HCNTs. We compare and contrast our findings in relation to other studies to gauge the modulation of pulmonary immune response by CNTs.

Keywords: helical carbon nanotubes, lung, inflammation, macrophages, phagocytosis, *Pseudomonas aeruginosa*

*For correspondence. E-mail: geelau@illinois.edu; Tel.: +1-217-333-5077; Fax: +1-217-244-7421

Introduction

Multiple studies have highlighted an association between exposure to ultrafine particles (< 1 micron in diameter) and pulmonary disease (Frampton, 2001), including particles associated with air pollution (Pope *et al.*, 2002; Kan *et al.*, 2008). Of particular interest is the effect of pollution on the host response to pulmonary infections, with data suggesting that air pollutants may be associated with increased severity of respiratory infections (Jakab, 1977; Speizer *et al.*, 1980). Studies in animal models have demonstrated that pollutants from different sources can negatively impact the pulmonary response to multiple pathogens. One study showed that mice exposed to automobile exhaust were more susceptible to infection by *Streptococcus zooepidemicus* (Coffin and Blommer, 1967). In addition, another study demonstrated increased morbidity to group C streptococcus in mice exposed to ambient air from multiple sources and fly ash (Hatch *et al.*, 1985). Moreover, mice exposed to smoke generated by the burning of cordwood were demonstrated to have increased susceptibility to *S. zooepidemicus* infections (Gilmour *et al.*, 2001). Additionally, rats exposed to diesel exhaust particles showed increased susceptibility to infection by both influenza virus and *Listeria monocytogenes* (Hahon *et al.*, 1985; Castranova *et al.*, 2001) which indicates that increased susceptibility to infections is not limited to bacterial pathogens.

The impact of particulate inhalation toxicity is of concern in occupational settings in which workers may be exposed to aerosolized particles. In occupational settings, the inhaled materials are of a more homogenous population. In contrast, smoke, automobile exhaust, and other sources of air pollution are often a heterogeneous mixture of particles (which may vary significantly in size) and chemicals. By the 20th century three particulates had been identified as the cause of most diseases associated with particle pulmonary toxicity: Quartz and the associated pulmonary condition silicosis, asbestos and pulmonary fibrosis and mesotheliomas, and the coal-associated condition identified as pneumoconiosis (Bakand *et al.*, 2012). Exposure to silica has been associated with a high prevalence of pulmonary tuberculosis in South African gold miners (teWaternaude *et al.*, 2006) and South Korean coal workers diagnosed with pneumoconiosis were also found to have a high rate of both tuberculosis and non-tuberculous mycobacterial pulmonary infections (Kim *et al.*, 2009). Control systems have been designed to minimize exposure to the aforementioned particles in occupational

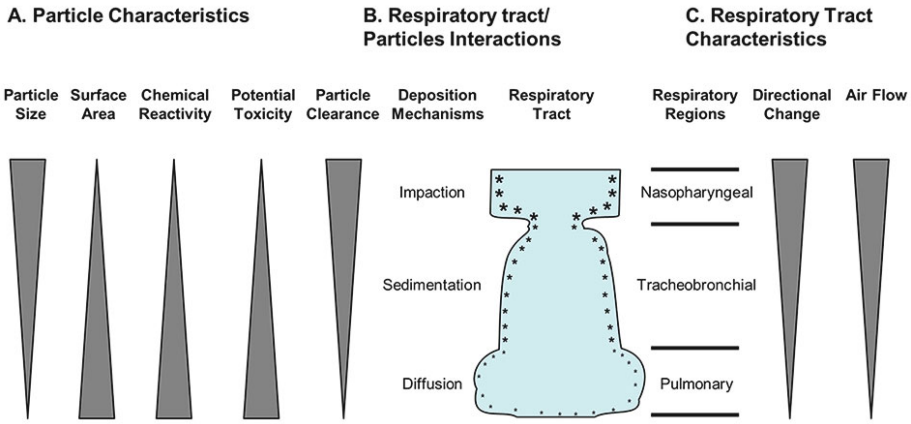

Fig. 1. The interaction of particles with the human respiratory tract. (A) Particle characteristics. (B) Respiratory tract/particles interactions. (C) Respiratory tract characteristics. (Adapted from Bakand *et al.*, 2012).

settings. However, advances in synthesis and manipulation of materials on a nanoscale had led to the development of synthetic vitreous fibers in the 1980s and carbon-based nanoparticles in the 1990s. The field of nanotoxicology soon followed as investigators realized that nanoparticles interacted with biologic systems in novel ways from the better-defined larger particles.

Particle inhalation and clearance

Deposition and clearance of inhaled airborne particles is determined by three factors: respiratory tract anatomy, airflow patterns, and the aerodynamic characteristics of the individual particles (Fig. 1) (Schwab and Zenkel, 1998; Bakand *et al.*, 2012). Physical characteristics of individual particles affect how they travel in the respiratory system and how they interact with the host once they are deposited. Among the different characteristics of airborne particles, size is considered to be most important. The physiology of the pulmonary system provides robust protection from the inhalation of airborne particles, predominantly those > 1 micron in diameter. The nose is the primary filter for large airborne particles. Particles inhaled through the nares encounter warm, humidified air, which builds up on particles, adding to their overall mass. The architecture of the sinuses provides turbulent airflow from sudden directional changes or bifurcations, which leads to particles impacting onto the epithelial surface, particularly at branches of the airways. Particles > 5 microns in diameter are usually trapped by this mechanism in the nasopharyngeal region. The particles become embedded onto the mucous surface and are removed via mucocilliary transport to the oropharynx (Sleigh *et al.*, 1988). Additionally, nasal mucousal contains enzymes, lysozymes, antibodies, phagocytic cells, and cytochrome P-450-dependent monooxygenases which help to neutralize and remove particles (Dahl *et al.*, 1985; Schwab and Zenkel, 1998). If these large particles are inhaled through the mouth, they also experience turbulent conditions at the bifurcations of the bronchi and bronchioles which lead to particles impacting onto the bronchiolar walls and removal by mucocilliary clearance. Smaller particles (1–5 microns), which bypass entrapment in the nares, are usually captured in the tracheobronchial tree primarily by sedimentation. These particles become entrapped in the overlying mucous and, similar to the nares, are exposed to an array of host protective molecules and are removed by the mucocilliary apparatus.

Ultrafine particles and nanoparticles bypass these mechanical defenses and can enter the alveolar spaces. Because of very low airflow, particles diffuse throughout the alveoli, allowing for deposition in the deep airways. From here, clearance may be accomplished by translocation to the blood stream. However, translocation of nanoparticles across the epithelium is most efficient with soluble nanoparticles and only a small fraction of insoluble nanoparticles penetrate the alveolar-blood barrier (Mühlfeld *et al.*, 2008). The alveoli also lack the mucocilliary clearance mechanism found in the bronchioles, and removal of particles relies heavily on phagocytosis by pulmonary macrophages. However, clearance of deposited particles by macrophages is inefficient (Geiser, 2010), especially for particles < 100 microns in diameter (Alexis *et al.*, 2006; Geiser *et al.*, 2008). Additionally, monocytes and macrophages which have been exposed to larger particles, including aggregates of nanoparticles, show limited phagocytosis of small, individual particles (Lundborg *et al.*, 2001; Brown *et al.*, 2007). Those nanoparticles composed of carbon or other metals which have not been functionalized (addition of molecules on the surface to improve solubility), most likely will persist in the alveolar spaces and can serve as a constant source of irritation or injury. The persistent of nanoparticles will also continue to attract the attention of alveolar macrophages and neutrophils.

Carbon nanotubes

Nanoparticles (particles with at least one dimension < 1 micron i.e. diameter) are a relatively new entity and are often considered a subset of ultrafine particles. The term “nanoparticles” is preferentially used when referring to man-made particles, most commonly manufactured particles designed to be less than 1 micron in diameter, although many also include aerosolized byproducts of human activity, blurring the distinction between nanoparticles and ultrafine particles. The carbon nanotube industry has rapidly expanded from a purely academic material over the last decade, with numerous pro-

ducts now incorporating carbon nanotubes (CNTs) as part of their structure. Multi-walled CNTs (MWCNTs) were initially synthesized by wrapping sheets of graphite into a multi-layered stable tube structure with a hollow core (Iijima, 1991). Alternative synthetic methods allowed for the rolling of graphite sheets into single layers using a metal catalyst, resulting in the production of single-walled CNTs (SWCNTs) (Bethune *et al.*, 1993; Iijima and Ichihashi, 1993). Further refinement of methods allowed for the synthesis of additional structures such as nanohorns, nanocapsules, and helical multi-walled nanotubes, with new methods that improved purity and yield. Today, though, most CNTs are of the straight, single-walled or multi-walled variety. Worldwide CNT production has increased 10-fold since 2006 with the most common applications using unorganized CNTs in composites of polymers or thin films (De Volder *et al.*, 2013). CNTs have multiple beneficial mechanical and electrical properties that can be exploited, including high tensile strength and thermal or electrical conductance. The incorporation of CNTs into polymers and resins increases the stiffness and strength, with uses found in bicycle frames, tennis rackets, and boat hulls. They also could be an environmentally-friendly component of paints which can inhibit attachment of barnacles to boat hulls (Beigbeder *et al.*, 2008). MWCNT incorporation into lithium ion batteries also improves battery life cycle (Dai *et al.*, 2012).

The length/width ratio of CNTs, which make them ideal for mechanical, electronic, and biomedical applications, also raises concern regarding their biocompatibility. CNTs are very similar to asbestos fibers, which have been implicated in oxidative stress, injury, and mesothelioma development in the lungs (Pacurari *et al.*, 2010). The risk for occupational exposure to CNTs has increased as mass production has escalated. Production capacity continues to expand exponentially from 3000 metric tons in 2011 to a projected 12,800 metric tons by 2016 (http://www.nanowerk.com/spotlight/spotid=23118.php) while analysts at TechNavio forecast that the global carbon nanotube market will continue growing at an annual rate of 11.5% over the next 5 years (http://www.technavio.com/). In addition, exposure to low ambient concentrations of MWCNTs has been reported secondary to gas combustion emissions (Murr *et al.*, 2005). Several reviews examining potential CNT toxicity both *in vitro* and *in vivo* have been published (Lam *et al.*, 2006; Uo *et al.*, 2011; Bakand *et al.*, 2012). Studies have shown that CNT toxicity depends on multiple factors, including the purity of the nanotubes, dispersion methods, addition of functional groups, and target cells. A few studies have indicated a minimal impact on pulmonary health following exposure to CNTs, especially those which have been altered to allow for better dispersion or solubility. However, most studies using either raw or unmodified CNTs have demonstrated mild to marked cellular cytotoxicity as well as chronic pulmonary lesions including pulmonary granulomas and fibrosis (Shvedova *et al.*, 2005). The varying degrees of pulmonary toxicity suggest that CNTs of different shapes and diameter as well as those with modified surfaces will have to be individually evaluated.

Modulation of pulmonary immunity by nanoparticles and carbon nanotubes *in vitro*

Mechanisms by which pulmonary immunity may be inhibited by inhalation of particles have focused primarily on the alveolar macrophages because of their role in both scavenging and removal of inhaled particles (Palecanda *et al.*, 1999), as well as their importance in the innate immune response to pathogens (Gordon and Read, 2002; Delclaux and Azoulay, 2003). Both smoke and road dust have been shown to impair macrophage phagocytosis (Moores *et al.*, 1993; Ziegler *et al.*, 1994). Exposure to diesel exhaust particles resulted in

Table 1. Compilation of published reports examining the effect of exposure to ultrafine particles or nanoparticles prior to infection with select pathogens in animal models

Particles[a]	Dose; route	Model	Pathogen	Results[b]	Author, Year
Coal dust	15 mg/m^3, 6 h/day, 5 days/week, 3 weeks; inhalation	Guinea pig	*E. coli*	Impaired clearance	Rylander (1968)
Multiple particles	1–100 μg; intratracheal	Mouse	Group C streptococcus	Increased mortality	Hatch *et al.* (1985)
TiO_2 particles	2–20 mg/m^3, 20 h/day, 10 days; inhalation	Mouse	*P. haemolytica*	Impaired clearance	Gilmour *et al.* (1989b)
Silica particles	15 mg/m^3, 6 h/day, 5 days/week, 21–59 days; inhalation	Rat	*L. monocytogenes*	Enhanced clearance	Antonini *et al.* (2000b)
Silica particles (d=1.362 μm)	5 mg/kg or 80 mg/kg; intratracheal	Rat	*L. monocytogenes*	Enhanced clearance	Antonini *et al.* (2000a)
Carbon black	40 μg; intratracheal	Mouse	Respiratory syncytial virus	No effect on clearance	Lambert *et al.* (2003)
SWCNT (d=1-4 nm, L=1-3 μm)	10–40 μg; intrapharyngeal	Mouse	*L. monocytogenes*	Impaired clearance and phagocytosis	Shvedova *et al.* (2008)
TiO_2 rods (d=7.6 nm, L=35.9 nm)	10–100 μg; intratracheal	Rat	*L. monocytogenes*	No effect on clearance	Roberts *et al.* (2011)
SWCNT (d=1-4 nm, L=1-3 μm)	80 μg/day, 2 days; intrapharyngeal	Mouse	*T. gondii*	No effect lung parasite load	Swedin *et al.* (2012)
HCNT (d=200 nm, L=1.9 μm)	50 μg 2X/week, 3 weeks; intranasal	Mouse	*P. aeruginosa*	No effect on clearance; impaired phagocytosis	Walling *et al.* (2013)

[a] A brief description of the particle including diameter (d) and/or length (L) is included if provided in the study.
[b] Results do not include any histological changes or alterations in BALF leukocytes or cytokines.

immune suppression secondary to inhibition of on LPS-stimulated production of IL-1 and TNF-α, impairment of phagocytosis, as well as decreased nitric oxide production by alveolar macrophages (Yang *et al.*, 1999, 2001; Yin *et al.*, 2002). Studies have also shown that harvested alveolar macrophages could respond to the presence of particles by increasing secretion of multiple proinflammatory mediators including TNF-α, IL-β, and IL-6, as well as generation of reactive oxygen species (ROS) (Ishii *et al.*, 2004; Shoenfelt *et al.*, 2009; Fenoglio *et al.*, 2012), all of which could contribute to increased morbidity during a concurrent pulmonary infection. However, these effects may have been influenced by trace metals associated with particles and not directly from the particles themselves (Pulskamp *et al.*, 2007). *In vitro* studies using the immortalized macrophage cell lines RAW 264.7 and J774.2 have also demonstrated this phenomenon. RAW 264.7 macrophages responded to the presence of TiO_2 particles by increasing expression of IL-6 and TNF-α (Palomaki *et al.*, 2010). Both TiO_2 particles and MWCNTs enhanced ROS production in RAW 264.7 macrophages (Sohaebuddin *et al.*, 2010). Additionally, pre-exposure to both carbon black and TiO_2 particles inhibited phagocytosis of latex beads by J774.2 macrophages (Renwick *et al.*, 2001). Using alveolar macrophages harvested from healthy human patients, it was demonstrated that preloading of aggregated carbon black could subsequently inhibit phagocytosis of amorphous silica particles, *Candida albicans*, and *Cryptococcus neoformans* opsonized with fresh serum (Lundborg *et al.*, 2006). These authors also showed that phagocytosis was inhibited regardless of the receptors used for binding the target.

Thus far, only a few studies have been published that showed altered phagocytic capacity by macrophages when exposed to CNTs. Exposure to SWCNTs inhibited phagocytosis of apoptotic Jurkat cells by RAW 264.7 macrophages (Witasp *et al.*, 2009). Amorphous carbon, silica particles, SWCNTs and MWCNTs inhibited the phagocytosis of latex beads by alveolar macrophages harvested from healthy guinea pigs (Jia *et al.*, 2005). In the case of MWCNTs, this was shown to be both size and diameter-dependent (Wang *et al.*, 2009). These *in vitro* observations raise the question of whether exposure to aerosolized CNTs may alter or inhibit the pulmonary immune response to subsequent infection by pulmonary pathogens.

Modulation of pulmonary immunity by nanoparticles *in vivo*

Studies using *in vivo* models of particle exposure have yet to achieve a consensus regarding the impact on pulmonary infections. To date, a small number of studies have been published examining the impact of particle exposure on mortality or phagocytosis/clearance of pathogens following infection (Table 1). In an early study, mice exposed to coal dust showed impaired clearance of infection by *Escherichia coli* (Rylander, 1968). Another robust study found that a diverse range of particles, including TiO_2 and carbon, which can be found in occupational settings, increased the mortality of mice exposed to group C streptococcus (Hatch *et al.*, 1985). Similarly, mice exposed to aerosolized TiO_2 also showed impaired clearance of aerosolized *Pasteurella haemolytica* both immediately and 10 days after cessation of TiO_2 exposure (Gilmour *et al.*, 1989a, 1989b). Alveolar macrophages from rats exposed to TiO_2 also showed impaired phagocytosis of iron particles (Warheit *et al.*, 1997). However, not all particles impair macrophage phagocytosis or pulmonary clearance. Some particles might require the addition of chemical or functional groups to impair host immunity. For example, although concomitant exposure to carbon black and formaldehyde inhibited phagocytosis by alveolar macrophages, carbon black alone had no effect (Jakab *et al.*, 1992). Likewise, binding of acid sulfate to carbon black was required for inhibiting Fc-mediated phagocytosis by alveolar macrophages (Clarke *et al.*, 2000). Clearance of respiratory syncytial virus in mice was not affected by carbon black exposure alone (Lambert *et al.*, 2003). Additionally, exposure to TiO_2 rods, which are similar in dimension to CNTs, did not affect clearance of subsequent *Listeria monocytogenes* infection in rats (Roberts *et al.*, 2011). And, contrary to what would be expected, rats exposed to silica demonstrated increased pulmonary clearance of *L. monocytogenes* (Antonini *et al.*, 2000a). This unexpected outcome was attributed to an elevated pulmonary immune response, including an increased influx of neutrophils, NK cells, T lymphocytes, and activated macrophages as a result of pre-exposure to silica (Antonini *et al.*, 2000a). Again, these results listed above reveal the difficulty in establishing a consensus regarding particulate toxicity due to the different pulmonary responses to specific particulates. Nevertheless, the *in vitro* data discussed in previous section strongly suggests that CNTs interfere with macrophage function, and individuals exposed to aerosolized CNTs may be at higher risk for developing more severe pulmonary infections. Impairment of the pulmonary immunity by CNTs could also result in establishing infections by pathogens which are normally cleared by healthy individuals without developing clinical symptoms.

Modulation of pulmonary immunity by carbon nanotubes *in vivo*

With the rapid growth of the CNT industry and research showing impairment of phagocytosis by CNTs *in vitro*, studies exploring CNT-mediated toxicity and modulation of pulmonary defenses *in vivo* became necessary. This question was first explored by Shvedova *et al.* (2008) using a combination of SWCNT and *L. monocytogenes*. In the study, mice were exposed to a single intrapharyngeal dose of 10 or 40 μg of SWCNT. After 3 days, mice were intrapharyngeally infected with 10^3 CFU of *L. monocytogenes*. Clearance of *L. monocytogenes* was quantified 3 and 6 days post-infection. At both time points, mice receiving 40 μg SWCNTs showed significant inhibition in bacterial clearance. At 6 days post-infection, these mice also had significantly elevated numbers of neutrophils in the BALF compared to infected mice which had received 0 or 10 μg SWCNT or uninfected mice given 10 or 40 μg SWCNT. A duplicate experiment from the same study examining the percentage of alveolar macrophages that phagocytized a fluorescent-tagged *L. monocytogenes* demonstrated inhibition of phagocytosis at both 10

and 40 μg SWCNTs. *In vitro* experiments also confirmed SWCNT-mediated inhibition of *L. monocytogenes* phagocytosis by immortalized macrophages (Shvedova *et al.*, 2008). This was the first published study which directly linked an inhibited immune response to infection following exposure to CNTs.

A second study using a similar pre-exposure protocol sought to examine the effect of SWCNTs on the host response to *Toxoplasma gondii* infection (Swedin *et al.*, 2012). *T. gondii* is an obligate intracellular protozoan which, after infection, can disseminate widely throughout the host. *T. gondii* may cause a variety of clinical manifestations, including pneumonia in immunosuppressed individuals. Results from the study showed that pre-exposure to SWCNTs did not alter the parasitic load in the lungs following intrvenous infection of *T. gondii.* Analysis of bronchoalveolar lavage fluid (BALF) showed no differences in total leukocytes, macrophages, neutrophils, or eosinophils between SWCNT + *T. gondii* treated mice compared to just *T. gondii* alone. Levels of IL-1β, IL-6, IL-10, INF-γ, and TNF-α in the BALF were also not significantly different, although MCP-1 and TGFb2 were significantly higher in those *T. gondii* infected mice which had received SWCNTs compared to just *T. gondii* infected mice. The authors acknowledged that inhalation in not the normal route of *T. gondii* infections, and indicated that SWCNT deposition may have a different impact on the immune response to pathogens depending on the route of infection and dissemination. This concept is further supported by noting, that the dosage of SWCNTs used in this study, 160 μg (80 μg/day administered on two consecutive days), is higher than that used by Shvedova *et al.* (2008). One would expect that an increased exposure level of SWCNTs would negatively impact the immune response to primary pulmonary pathogens. There are two relevant topics arising from this study. First, as acknowledged by the authors, the study only examines the early immune response and did not explore the chronic phase of infection. Potentially, SWCNT exposure may have a greater impact on chronic infections given the slow clearance of CNTs from the lungs. Development of a chronic infection model, including systemic infections, during or following CNT exposure is of upmost importance as studies have shown that aspirated or inhaled CNTs can modulate systemic immunity by suppressing T-cell or NK cell activity (Mitchell *et al.*, 2007; Tkach *et al.*, 2011). Additionally, as CNTs are being considered for carriers of biologics to systemic targets, intravenous administration of CNTs may alter the host immune response to *T. gondii* or other systemic pathogens in a different manner than inhaled CNTs.

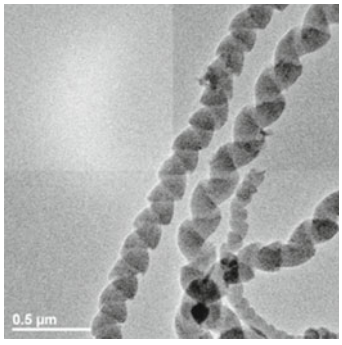

Fig. 2. A representative scanning electron microscopic image of helical carbon nanotubes used in the study by Walling *et al.* (2013).

In our laboratory, we selected to examine the impact of CNT exposure on *Pseudomonas aeruginosa* infection, a known respiratory pathogen most commonly associated with chronic pulmonary diseases including cystic fibrosis, chronic obstructive pulmonary diseases, as well as ventilator-associated infections. The host immune response to *P. aeruginosa* infection relies on a robust inflammatory response composed primarily of neutrophils, without which, mortality is significantly elevated (Koh *et al.*, 2009). We chose to study helical CNTs (HCNTs, Fig. 2), a type of MWCNT which are larger than the SWCNTs used by Shvedova *et al.* (2008). Our protocol called for exposing mice to 50 μg HCNTs twice/week for 3 weeks prior to infection. Total lung burden over 3 weeks would approach ~150 μg/mouse as approximately 50% of intranasally administered materials reach the lungs (Southam *et al.*, 2002). The CNT exposure level in our study as well as that of Shvedova *et al.* (2008) and Swedin *et al.* (2012) are relevant to those which may be found in CNT production facilities with various controls to limit airborne exposure (Porter *et al.*, 2010; Kuempel, 2011). Results from our study indicated that HCNTs inhibit phagocytosis of *P. aeruginosa* by alveolar macrophages, similar to the results observed by Shvedova *et al.* (2008). However, clearance of *P. aeruginosa* from the lungs was not inhibited by pre-exposure to HCNTs (Walling *et al.*, 2013). Examination of the BALF from mice showed a significantly higher infiltration of neutrophils and activated macrophages in HCNT-exposed mice following *P. aeruginosa* infection compared to HCNT-unexposed + infected mice. Histologic analysis of the lungs confirmed this observation. Our results led us to postulate that HCNT exposure primes the lung for a more robust inflammatory response, similar to the results observed in a previous study involving silica exposure (Antonini *et al.*, 2000a, 2000b). Additionally, the elevation of leukocytes in HCNT-treated mice may have compensated for the decreased phagocytic function of alveolar macrophages.

Results from our study and that of Shvedova *et al.* (2008) indicate that different types of CNTs can similarly inhibit phagocytosis of pathogens but differ on the outcome and clearance of pathogens from the lungs. The divergent results can be partially explained by two separate but related variables of the studies. The first is the pathogen used. An effective immune response to *L. monocytogenes*, a Gram-positive intracellular pathogen, requires macrophages phagocytosis and expression of antigens for T-cell activation (Shen *et al.*, 1998). Accumulation of CNTs within the cytoplasm of macrophages could potentially interfere with the expression of pathogen receptors, uptake, or presentation as CNTs have been demonstrated to interact and bind to F-actin (Holt *et al.*, 2010). In contrast, *P. aeruginosa* is a Gram-negative extracellular pathogen and neutrophils are essential in the immune response to pulmonary infection by the bacterium. Failure to do so results in elevated mortality following infection (Scarff and Goldberg, 2008; Koh *et al.*, 2009). Clearance of any pulmonary infection relies on the combined efforts of leukocytes for both the release of anti-bacterial mediators such as nitric oxide and neutrophil extra-

cellular traps as well as phagocytosis. The relative contribution and importance of these mediators and actions may depend on whether the pathogen is extracellular or intracellular. Macrophages are important for the recruitment of neutrophils during acute *P. aeruginosa* infection (Hashimoto *et al.*, 1996; Kooguchi *et al.*, 1998). However, the contribution of *P. aeruginosa* clearance by macrophage phagocytosis remains unclear.

Second, an elevated inflammatory response to pathogens and subsequent clearance may depend on the size of CNTs. Results from ovalbumin-sensitized mice suggest that longer MWCNTs induce a stronger proinflammatory response than their shorter counterparts (Erdely *et al.*, 2009; Nygaard *et al.*, 2009). Our HCNTs, similar in length to long MWCNTs used by Erdley *et al.* (2009) and Nygaard *et al.* (2009), presumably induced a stronger inflammatory response to *P. aeruginosa* infection compared to unexposed mice, and the elevated inflammatory response (e.g., elevated influx of neutrophils and activated macrophages) induced by our HCNTs may have compensated for decreased internalization by resident alveolar macrophages. However, a separate study using shorter HCNTs would be necessary to support this assertion. Even if the different types of CNTs provoked a similar inflammatory response, the intracellular lifestyle of *L. monocytogenes* may provide some protection from the extracellular antibacterial mediators.

Finally, the route of infection may also contribute to some of the disparate results observed in the literature. As seen in the study by Shvedova *et al.* (2008) and Swedin *et al.* (2012), both of which used the same type of CNTs, pre-exposure to nanotubes was inhibitory to direct pulmonary infection by *L. monocytogenes* while showed no discernible effect during intravenous infection of *T. gondii* with subsequent dissemination to the lungs. This is most likely due to minimal transfer of CNTs from the lungs to systemic circulation following intrapulmonary exposure, limiting CNT interactions to alveolar macrophages and epithelial cells. What would be of interest is how CNTs administered intravenous or intraperitonally might affect the immune response to blood-borne or systemic pathogens. This is an area urgently in need of clarification as CNTs are being considered as novel drug-delivery devices (Yang *et al.*, 2008; Liu *et al.*, 2009).

Conclusion

In summary, pulmonary exposure to CNTs results in a proinflammatory response similar to those of other airborne nanoparticles. Additionally, CNTs can impair the phagocytic function of alveolar macrophages and, in the case of *L. monocytogenes*, can inhibit clearance of pathogens. However, this phenomenon is not universal, as observed from our studies with *P. aeruginosa*, which reflect similarly divergent conclusions following TiO_2 exposure. One may conclude that different types of CNTs, much like different types of particles, will have a different impact on the pulmonary response to infection. Furthermore, how CNTs or other nanoparticles affect the immune response will also depend on the pathogen, which differs depending on the type of pathogen used (bacterial, viral or fungal), pathogen lifestyles in the host (intracellular versus extracellular), as well as the route of infection. Clearly, determining any potential interference with pulmonary immunity will require testing of specific CNTs. Also, additional studies should consider utilizing known respiratory pathogens.

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