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From Immunotoxicity to Nanotherapy: The Effects of Nanomaterials on the Immune System

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The potential for human exposure to the diverse and ever-changing world of nanoscale materials has raised concerns about their influence on health and disease. The novel physical and chemical properties of these materials, which are associated with their small size, complicate toxicological evaluations. Further, these properties may make engineered nanomaterials (ENMs) a prime target for interaction with the immune system following uptake by phagocytes. Undesired effects on antigen-presenting cells and other phagocytic cells are of concern due to the high likelihood of ENM uptake by these cells. In addition, ENM interactions with lymphocytes and other cell types can contribute to a varied spectrum of possible effects, including inflammation, hypersensitivity, and immunomodulation. Furthermore, the mast cell (a type of immune cell traditionally associated with allergy) appears to contribute to certain inflammatory and toxic effects associated with some ENMs. Although incidental exposure may be undesirable, nanomedicines engineered for various clinical applications provide opportunities to develop therapies that may or may not intentionally target the immune system. The interaction between ENMs and the immune system and the resulting pharmacokinetic and phenotypic responses are critical factors that dictate the balance between toxicity and clinical efficacy of nanotherapeutics.

Key words: **nanomaterials; immune system; immunotoxicity; mast cells; drug carrier agents.**

Don't eat that… it contains "toxic" nanoparticles! What are nanomaterials anyway? And why should we care about them? They're so small… how can they be toxic? What do you mean are they "different" than larger particles? Wait… can they actually be beneficial? What's the immune system have to do with any of this, anyway? Sound familiar? These statements and questions, and many others like them, are increasingly being asked in the midst of today's global engineered nanomaterial (ENM) "frenzy." The fact is, in recent years, there has been considerable research examining the potential utility of nanoscale materials and nanostructures in commercial and biomedical applications. So what do we make of this attention, and how can the scientific community effectively answer such questions and concerns? This forum article covers a symposium of the same title presented at the 52nd Annual Meeting of the Society of Toxicology, held in March 2013, in San Antonio, Texas, which sought to bring attention to issues surrounding ENM toxicity evaluations, ENM-immune system interactions, and important considerations related to the use of ENMs in medicine.

SMALL MATERIALS, LARGER ISSUES

Broadly defined, ENMs are a set of substances with at least one critical dimension less than 100 nm. Because of their small size, ENMs are believed to possess novel chemical and physical properties that make them useful in various applications. However, these same properties can also make their interactions with biological systems difficult to predict and evaluate in traditional toxicity models (Balbus *et al.*, 2007; Hoet *et al.*, 2009). Further complicating matters is the fact that ENMs are not a single "class" of agents that can be treated as a single entity, due to considerable diversity in size, composition, surface modifications, and physicochemical properties. Even within a given "class" there is variability among what otherwise may appear to be "similar" ENMs. For example, nanotubes differ by tube status (single, double, and multi-walled), tube length and diameter, surface modifications, and metal composition (as a synthetic byproduct during synthesis; Donaldson *et al.*, 2006). This

diversity makes the translation of toxicological findings from one ENM to another problematic.

Another issue related to assessing the safety of ENMs is the relevance of the "tested agent." Currently, there is limited information on specific agents with known human exposure, as well as the characteristics of ENMs to which people are exposed. Consequently, the choice of a specific agent to evaluate may be based on pragmatic decisions such as commercial availability, instead of known human exposure. Further, the physicochemical properties of an ENM may change within the test system. For example, it is known that proteins adsorb to the surface of ENMs in the body (Cedarvall *et al.*, 2007; Monopoli *et al.*, 2012), forming a protein corona that fundamentally changes the physical and chemical characteristics of the ENM that had been so carefully characterized. How do these coronas influence extrapolation from *in vitro* to *in vivo* studies, and are the differences of any toxicological consequence, or are they simply phenomenological?

Defining how best to characterize the "dose" further complicates ENM toxicological assessments. Indeed, comparing the toxicity and potency of materials based on simple mass-based dose metrics (e.g., mg/kg) may not be appropriate (Oberdorster *et al.*, 2007). For example, in some metal oxides, where the biological effects of materials are driven by properties related to the surface area of a material, it is more appropriate to compare different-sized materials basing the dose administered on the total surface area, rather than on mass. The issue of dose extrapolation between *in vivo* and *in vitro* exposure is also of concern. At present, there is little correlation between *in vitro* and *in vivo* toxicity profiles for nanoparticles (Sayes *et al.*, 2007). As noted by Teeguarden *et al.* (2007), differences in diffusion and settling of nanoparticles—or "particokinetics"—in solution during *in vitro* studies can lead to differences in ranking of ENMs despite similar mass doses. Mathematical models of particle behavior and dosimetry *in vitro* are also being considered in order to relate dosage to cells with specific adverse outcomes (Hinderliter *et al.*, 2010). Further, the absorption, distribution, metabolism, and elimination kinetics of ENMs can be affected by particle size and surface coating/chemistry. For example, a clear size dependency was reported in the translocation of polystyrene microspheres (50, 100, 300, 500, 1000, and 3000 nm) across the gastrointestinal tract following oral administration to female Sprague Dawley rats, with increased translocation associated with decreased particle sizes (Jani *et al.*, 1990).

ENMS AND THE IMMUNE SYSTEM

Phagocytic cells of the immune system, including macrophages, neutrophils, and dendritic cells, are responsible in part for ENM uptake (Patri *et al.*, 2007). ENM interactions with cells of the immune system, including phagocytes, lymphocytes, and mast cells, can be altered by ENM physicochemical properties, including size and surface modification (Fischer and Chan, 2007). For example, there are reports of both increased and abrogated uptake of single-walled carbon nanotubes, depending upon the surface functional group (Antonelli *et al.*, 2010; Singh *et al.*, 2006), suggesting a role for surface modifications in mediating immune recognition and/or uptake of ENMs. Further, ENM size has been correlated with activation of the complement system (Pedersen *et al.*, 2010).

Current predictive and validated immunotoxicity testing strategies, Tkach *et al.* (2011) using a tiered approach, have been well established (Luster *et al.*, 1988) but have been used infrequently to evaluate ENMs. Some well-established assays include the mononuclear phagocytic system (MPS) assay (Munson *et al.*, 1970) for innate immunity, the plaque assay (Jerne *et al.*, 1963), the enzyme-linked immunosorbent assay for T-dependent antibody responses (humoral immunity), and the delayed-type hypersensitivity assay (Smith and White, 2010; White *et al.*, 2012) for cell-mediated immunity (CMI). Contact hypersensitivity responses are regularly assessed using the local lymph node assay (LLNA); however, many ENMs cannot penetrate the stratum corneum of intact skin. In such cases, the lymph node proliferation assay (LNPA; Weaver *et al.*, 2005), which utilizes subcutaneous (s.c.) administration, may be a useful alternative (Dobrovolskaia *et al.*, 2009), although it has not been validated for assessing ENM hypersensitivity. In fact, it is currently unclear whether any of the "traditional" immunotoxicology methods are sensitive and predictive of ENM-mediated immunotoxicity, as well as how they can/should be modified to better predict ENM immunomodulation.

ENM MODULATION OF IMMUNE FUNCTION

Consider a handful of studies that have evaluated immune effects of titanium dioxide nanoparticles. A recent series of studies evaluated the immunotoxicity of anatase nano-TiO_2 using various routes of exposure (dermal, s.c., and oral gavage) at doses up to 250 mg/kg (Auttachoat *et al.*, 2013). Dermal exposure for three days produced irritancy but not contact hypersensitivity (LLNA), although the LNPA demonstrated significant increases consistent with either inflammation/irritation or a possible hypersensitivity response. The authors also reported that oral exposure for 28 days produced no immune effects (innate, humoral, and CMI). Contrasting with the negative findings for the oral immunotoxicity studies by Auttachoat *et al.* (2013), another recent study evaluating oral exposure to TiO_2 nanoparticles reported increased mast cell activation within the stomachs of young rats (Wang *et al.*, 2013b). Whereas the Auttachoat *et al.* studies did not specifically evaluate mast cells, these findings further the concern as to whether or not the current immunotoxicity testing strategies are sufficiently sensitive and predictive or even appropriate for ENM immunotoxicity evaluations. Clearly, immunotoxicity testing of ENMs is anything but straightforward. Table 1 provides some examples of the va-

TABLE 1
Examples of Nanomaterial-Immune Interactions for Selected Engineered Nanomaterials

Engineered nanomaterial	Target immune cell(s)	Administration route(s)	Immune effects	References
TiO_2	Multiple cell types	Dermal and subcutaneous	Demonstrated route-specific effects on hypersensitivity responses	Auttachoat *et al.* (2013)
	Neutrophils	Oropharyngeal instillation	Increase in numbers of neutrophils in lung	Gustafsson *et al.* (2011)
	Neutrophils	Intragastric	Decrease in numbers of neutrophils in the blood	Sang *et al.* (2012)
	Dendritic cells	*In vitro*	Upregulated expression of MHC-II, CD80, and CD86	Winter *et al.* (2011)
	T cells	Intragastric	Decreased the proliferation of both $CD4^+$ and $CD8^+$ T cells, as well as the ratio of CD4+ to CD8+ cells in the liver	Duan *et al.* (2010)
	B and NK cells	Intraperitoneal	Delayed B-lymphocyte development; decrease in numbers of spleen resident NK cells, specifically $CD11b^-$ NK cells	Moon *et al.* (2011)
	B cells	Intratracheal installation	Increase in numbers of B cells in the spleen and whole blood as well as increased production of IgE in lung lavage fluid	Park *et al.* (2009)
	NK cells	Intratracheal installation	Increase in numbers of NK cells in lung as well as a transient increased expression of the NKR-P1A receptor	Gustafsson *et al.* (2011)
	NK cells	Intragastric	Decrease in whole blood NK cell numbers	Sang *et al.* (2012)
	Mast cells	*In vitro*	Activating mast cells to release histamine through membrane L-type $Ca2^+$ channels	Chen *et al.* (2012)
	Mast cells	Oral gavage	Reported increased mast cell activation in stomachs of young rats	Wang *et al.* (2013b)
Carbon nanotubes	T and NK cells	Inhalation	Immunosuppression of T-cell-dependent antibody responses and NK cell activity	Mitchell *et al.* (2007)
	Macrophages	*In vitro*	MWCNTs induce COX-2 production through a MAPK-dependent mechanism and iNOS production through a MAPK-independent mechanism	Lee *et al.* (2012)
	Macrophages	*In vitro*	Increases in cytokine release including IL-1β, IL-6, and IL-8 from human macrophage cell line	Murphy *et al.* (2012)
	Dendritic cells	Oropharyngeal aspiration	Increased lung inflammation, but systemic immunosuppression of dendritic cells leading to decreased T-cell proliferation	Tkach *et al.* (2011)
	T cells	Intravenous	Increases in both $CD4^+$ and $CD8^+$ T cells in the spleen of C57BL/6 mice dependent on nanomaterial (comparison of graphene and carbon nanotubes)	Wang *et al.* (2013a)
	Mast cells	Oropharyngeal aspiration	IL-33, released from damaged lung epithelial cells by MWCNT exposure, subsequently activated mast cells resulting in adverse cardiopulmonary responses	Wang *et al.* (2011) Katwa *et al.* (2012)
	Splenocytes	Oropharyngeal aspiration	Single-walled carbon nanotubes (SWCNTs) do not impact the early immune response to *Toxoplasma gondii* in mice.	Swedin *et al.* (2012)
Fullerenes	Basophils	*In vitro*	Inhibit IgE dependent activation of peripheral basophil activation	Ryan *et al.* (2007) Norton *et al.* (2010)
	Mast cells	Intraperitoneal	A significant reduction in the mast-cell-dependent anaphylactic-induced drop in core body temperature as well as behavioral responses that accompany anaphylactic shock	Norton *et al.* (2010)

riety of NM-immune interactions that have been reported for TiO_2 nanoparticles, carbon nanotubes, and carbon fullerenes.

In addition to concern that ENM exposure can modulate normal immune function, ENM-immune cell interactions may result in unforeseen activation of one or more immune cell types. The implications of activating immune cells may be significant and may have toxicological impact on other critical systems. In fact, there is increasing evidence that one immune cell in particular—the mast cell—may play a role in the initiation of both pulmonary and cardiovascular events following ENM exposure (Shannahan *et al.*, 2012). Mast cells have long been recognized for their role in the genesis of allergic inflammation in diseases such as asthma, atopic dermatitis, allergic rhinitis, allergic eye disease, and anaphylaxis (Brown *et al.*, 2008). In-

deed, the activation of mast cells by ENM may pose a significant hurdle to the development of safe nanotechnologies to avoid development of these allergic conditions, but also to prevent any unwanted anaphylactic-type responses that could be associated with nanotherapeutics.

Increasing evidence suggests that mast cells may be recruited by ENM exposure and can contribute significantly to ENM toxicity. For example, a study by Murray *et al.* (2009) found that dermal exposure to single-walled carbon nanotubes resulted in increased epidermal thickening and activation of dermal fibroblasts, which the authors suggested was due to accumulation of neutrophils and mast cells in the skin. A more recent study using multi-walled carbon nanotubes (MWCNTs), which have well documented pulmonary and cardiovascular toxicity (Wang *et al.*, 2011), has begun to elucidate mechanisms by which mast cells may contribute to ENM toxicity (Katwa *et al.*, 2012). In this study, oropharyngeal aspiration of MWCNTs elicited a robust release of IL-33 into the bronchoalveolar lavage fluid and suggested damage to the epithelium (Katwa *et al.*, 2012). IL-33, which signals through the ST2 (IL-1RL1) receptor (Ali *et al.*, 2007; Ho *et al.*, 2007; Iikura *et al.*, 2007) has recently been described as a significant mechanism of mast cell activation, leading to production of several pro-inflammatory cytokines under acute exposure conditions (Ho *et al.*, 2007; Iikura *et al.*, 2007). The pathologies associated with MWCNT inhalation—including inflammation, pulmonary fibrosis, airway functional changes, and adverse cardiovascular events (Wang *et al.*, 2011)—appeared to be mast cell dependent in this model, as MWCNT failed to induce these toxicities in $Kit^{W\text{-}sh}$ mice (Katwa *et al.*, 2012). Overall, these findings demonstrated a lack of adverse toxicological responses to MWCNT when there was a deficiency of mast cells or when the mast cells were unable to respond to IL-33. In contrast to MWCNTs, work by Ryan *et al.* (2007) has demonstrated that another carbon-based ENM, fullerene C60, inhibits mast-cell-mediated allergic responses, suggesting that the aspect ratio of MWCNTs likely contributes to immune activation and further illustrating the connection between physicochemical properties of ENMs and the resulting responses of the immune system.

The role of mast cells in mediating ENM toxicity is concerning, particularly in light of the inability to evaluate these cells using the current validated immunotoxicity testing battery. Many questions remain, including "if" and "how" ENMs promote allergic responses through development of antigen-specific IgE, activation of basophils, and exacerbation of underlying allergic conditions. Indeed, more effort needs to be focused within the general area of allergic responses to ENMs, as this may represent a likely immune outcome following exposure.

NANOTHERAPEUTICS AND THE IMMUNE SYSTEM

Carrier-mediated agents consist of nanoparticles, nanosomes (nanoparticle-sized liposomes), and conjugates. Theoretical advantages of carrier-mediated drugs include increased drug solubility, prolonged duration of exposure, selective delivery of entrapped drug to the site of action, and improved therapeutic index. The disposition of encapsulated drug is dictated by the composition of the carrier, thus altering the pharmacokinetic (PK) profile of the drug. The phagocytes of the MPS are one proposed clearance pathway of nanotherapeutic agents. Studies suggest there is a bidirectional interaction between nanosomal agents and the MPS. However, potential factors associated with the clearance and disposition of carrier agents in patients and preclinical animal models have not been extensively evaluated.

A phase I study has been reported in which the relationships between the disposition of the carrier-mediated agent S-CKD602 (PEGylated liposomal CKD-602, a camptothecin analogue) and changes in monocytes and absolute neutrophil counts were evaluated (Zamboni *et al.*, 2010). Results of this evaluation of the PK and pharmacodynamic (PD) relationships between a liposomal anticancer agent and immune cells in patients suggested that monocytes were more sensitive to S-CKD602 than neutrophils. The increased sensitivity appeared to be related to the liposomal formulation and not the released drug from the liposome or the small molecule formulation. Thus, there is preliminary evidence suggesting that factors associated with the MPS may contribute to PK and PD variability of nanotherapeutics. As such, there is a compelling need to identify the relevant factors associated with MPS function in order to improve the preclinical and clinical studies of ENMs. Further, it will be essential to test whether the PK of ENM can be scaled across species using various measurements and surrogates of the MPS, such as monocyte and macrophage activity or function, genetics, complement, or cytokines.

Finally, it is important to emphasize that timely consideration must be given to the effects of nanotherapies on the many components of the immune system well in advance of the clinical trial stage. Indeed, in initial design stages, the carrier ENM should carefully be selected to avoid undesired immune effects. Here, a key point emerges. If suppression or stimulation of an immune response is the desired result, then a certain degree of immunotoxicity of the carrier ENM and/or the delivered drug may be appropriate. For example, if an ENM carrier were used to deliver vaccines, then stimulation of the immune system by the ENM carrier would be desired. Some ENMs have been shown to possess potent adjuvant effects, capable of augmenting immune responses directed against antigens (de Haar *et al.*, 2006; Dobrovolskaia and McNeil, 2007; Zolnik *et al.*, 2010). Indeed, the number of publications detailing investigations of ENM vaccines has increased exponentially since 2007.

Recently, it has been shown that physicochemical properties play an important role in the adjuvant-like responses elicited by ENMs (Sun *et al.*, 2013). In the study by Sun *et al.* (2013), the use of aluminum oxyhydroxide nanomaterials with varying shape (rods, plates, or polyhedrons) and crystallinity resulted in improved adjuvant capacity compared with alum in both *in vitro* (dendritic cell activation) and *in vivo* (IgG and IgE responses

to ovalbumin) studies. In addition to adjuvant responses, it has recently been demonstrated that nanoparticle shape influences antibody and cytokine production (Niikura *et al.*, 2013). In this study, the shape and size of gold nanoparticles (AuNP) coated with West Nile virus envelope protein influenced the production of specific antibodies in mice, with a spherical AuNP resulting in the highest level of antibody production whereas a rod-shape AuNP produced half the level of antibody (Niikura *et al.*, 2013). Further, the cytokine profile produced by macrophages and dendritic cells varied depending on the shape, with the rod-shaped AuNP resulting in inflammasome activation and subsequent IL-1β and IL-18 release, whereas the spherical AuNPs induced TNF-α, IL-6, IL-12, and GM-CSF (Niikura *et al.*, 2013).

Whereas most traditional immunotoxicological studies have focused on immunosuppression, most nanoparticle studies have examined their inflammatory properties. However, other studies have shown that nanoparticles can be used to deliver immunosuppressive drugs. Cyclosporine A-conjugated polymeric nanoparticles have been shown to suppress T-cell function through downregulation of priming by dendritic cells (Azzi *et al.*, 2010). In addition, local delivery of an immunosuppressive drug may be advocated, if local suppression of an undesired immune response is required. The feasibility of such therapy was demonstrated by McLoughlin *et al.* (2011) and McLoughlin (2012) in studies where an immunosuppressive drug was incorporated into nanofibrous biomaterials. Following s.c. implantation in mice, this biomaterial exhibited sustained drug release, and a therapeutic window was identified, in which local immunosuppression was achieved without systemic immunotoxic effects. Ultimately, the interpretation of effects that nanotherapies may exert on the immune system is dependent on the intended application.

FUTURE RESEARCH DIRECTIONS

Many key questions remain unanswered. If ENM exposure has the potential to modulate immune function, and modulation of the function of certain immune cells (e.g., mast cells, lymphocytes, and phagocytic cells) in turn may contribute to other toxicities, how can ENM toxicity be evaluated, and can this snowball effect be avoided? Furthermore, given the interaction of ENMs with the cells of the immune system, is it possible to find or engineer ENMs, having certain desired physical and chemical characteristics, that can be used to engage and direct the immune system, without undesirable adverse toxicity, for therapeutic/medicinal purposes? If so, can these nanotherapeutics be controlled or directed? What cell(s) should they be designed to target? Are the PK and PD profiles of nanotherapeutics similar to those of more conventional medicinal therapies? And how can this be evaluated in order to establish appropriate first-in-man doses? These questions and many others must be considered in future research as nanotherapeutics are increasingly explored.

CONCLUDING SUMMARY

Interactions between ENMs and the immune system are unavoidable, and concerns about the possibility of ENM-mediated immunomodulation promote a growing need to evaluate the effects of these novel materials on the many facets of the immune system. Conversely, ENM toxicity that is mediated by one or more types of immune cells adds a further complication. However, by investigating the bidirectional ENM-immune cell interactions, the resulting toxic effects, and the mechanisms by which these effects occur, we can better characterize the hazards these materials pose in order to select—with knowledge and forethought—appropriate nanotherapeutics for use in medicinal applications.

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