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Current understanding of interactions between nanoparticles and the immune system

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Abstract

The delivery of drugs, antigens, and imaging agents benefits from using nanotechnology-based carriers. The successful translation of nanoformulations to the clinic involves thorough assessment of their safety profiles, which, among other endpoints, includes evaluation of immunotoxicity. The past decade of research focusing on nanoparticle interaction with the immune system has been fruitful in terms of understanding the basics of nanoparticle immunocompatibility, developing a bioanalytical infrastructure to screen for nanoparticle-mediated immune reactions, beginning to uncover the mechanisms of nanoparticle immunotoxicity, and utilizing current knowledge about the structure–activity relationship between nanoparticles' physicochemical properties and their effects on the immune system to guide safe drug delivery. In the present review, we focus on the most prominent pieces of the nanoparticle–immune system puzzle and discuss the achievements, disappointments, and lessons learned over the past 15 years of research on the immunotoxicity of engineered nanomaterials.

Graphical Abstract

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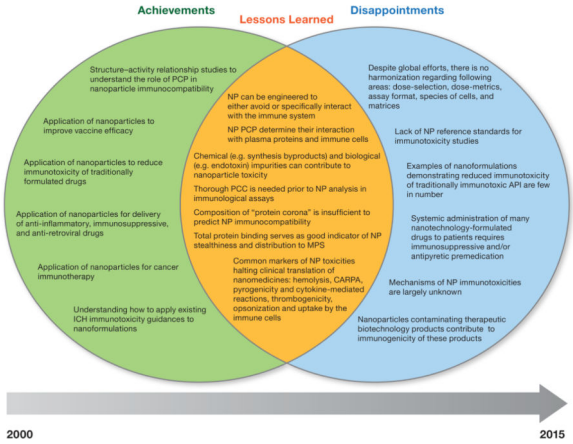

API- active pharmaceutical ingredient; NP – Nanoparticles; PCP – Physicochemical properties, CARPA – Complement activation-related pseudoallergy, ICH – International Conference on Harmonization

Keywords

Nanoparticles; Preclinical; Immunotoxicity; Immunology; Drug delivery

1. INTRODUCTION

The immune system's function in the maintenance of tissue homeostasis is to protect the host from environmental agents such as microbes or chemicals, and thereby preserve the integrity of the body. This is done through effective surveillance and elimination of foreign and abnormal self cells and structures from the body. It is well known that certain environmental contaminants and xenobiotics, as well as other drugs, may alter the immune system's normal function. Therefore, screening for immunotoxicity is a generally accepted step in toxicological research related to both environmental factors and pharmaceutical products (Luebke, 2012).

The interactions between nanoparticles and various components of the immune system have become an active area of research in bio- and nanotechnology because the benefits of using nanotechnology in industry and medicine are often questioned over concerns regarding the safety of these novel materials. The past decade of research has shown that, while nanoparticles can be toxic, nanotechnology engineering can modify these materials to either avoid or specifically target the immune system. Avoiding interaction with the immune system is desirable when the nanoparticles are being used for medical applications not intended to stimulate or inhibit the immune system, as well as when they are used for industrial and environmental applications. Specific targeting of the immune system, on the other hand, provides an attractive option for vaccine delivery, as well as for improving the quality of anti-inflammatory, anticancer, and antiviral therapies (Mallipeddi and Rohan, 2010; Gonzalez-Aramundizet al., 2012; Zaman et al., 2013; Tran and Amiji, 2015). Moreover, nanotechnology-based carriers can be used to reduce the immunotoxicity of traditional drugs (Libutti et al., 2010).

Some nanomaterials, metal colloids and liposomes, for example, were in use more than a decade ago (Gregoriadis et al., 1974), yet most active research in this field began in early 2000, fueled by the attention paid by regulatory agencies, such as the United States Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA), to the rapidly growing number of applications containing various types of engineered nanomaterials. The increase in submissions was expected since innovative research in this area had been progressing for years, culminated by the establishment of several breakthrough technologies that led to the discovery of fullerenes (Benning et al., 1992), carbon nanotubes (Ramirez et al., 1994), dendritic polymers (Tomalia, 1991; Newkome et al., 2002), and quantum dots (Takagahara, 1987). In 2005–2006, many worldwide initiatives were launched to improve the understanding of nanoparticle safety and included, among others, the establishment of the Nanotechnology Task Force by the FDA (http://www.fda.gov/ScienceResearch/SpecialTopics/Nanotechnology/ucm2006658.htm), several nanotechnology research programs by the EPA (http://www2.epa.gov/chemical-research/research-evaluating-nanomaterials-chemical-safety), the E56 committee by the American Society for Testing and Materials (ASTM) International (http://www.astm.org/COMMITTEE/E56.htm), and the TC229 Nanotechnologies technical committee by the International Organization for Standardization (ISO) (http://www.iso.org/iso/iso_technical_committee?commid=381983). In addition to these efforts, the U.S. National Cancer Institute established the Nanotechnology Characterization Laboratory (NCL) to accelerate the translation of nanotechnology-based concepts intended for medical applications in the area of cancer diagnosis and therapy from bench to bedside (http://ncl.cancer.gov/). One of the initial goals of the NCL was to support the nanotechnology community by developing a so-called assay cascade that would include, among other tests, a battery of immunological assays. This assay cascade contributed to the initial understanding of the interactions between nanoparticles and the immune system and created a framework for stimulating discussions in the area of nano-immunotoxicology (Dobrovolskaia and McNeil, 2007; Marx, 2008; Dobrovolskaia et al., 2009a; Pantic, 2011; Smith et al., 2013). Recently, the European Commission has established the European Nanomedicine Characterization Laboratory (EU-NCL), which shares several objectives with those of the NCL (https://ec.europa.eu/jrc/en/news/eu-ncl-launched).

The rapid growth of this field becomes obvious when one compares the number of publications searchable in PubMed using the key words “nanoparticles” and “immune system” between years 2000 and 2015 (Fig. 1). Reviewing these data reveals many advances, as well as disappointments. Moreover, delving into the mechanisms of nanoparticle immunotoxicity uncovered many challenges in material characterization. Due to the wide variety of nanomaterials available, the characterization of their physicochemical properties is directed toward addressing parameters specific to certain type of particles (e.g. porosity is applicable to silicon nanoparticles, but is not informative for liposomes and dendrimers). The grand challenge in the particle characterization that precedes immunotoxicity studies relates to the estimation of immunoreactive contaminants, such as synthesis byproducts (e.g. iron catalysts in carbon nanotubes, cetyltrimethylammonium bromide [CTAB] in gold nanorods), and bacterial endotoxins, as well as excipients (e.g.

Cremophor EL, polysorbate 80), and linkers (e.g. certain linkers used to attach poly(ethylene glycol) [PEG] to the nanoparticle surface) (Crist et al., 2013).

The challenges related to the physicochemical characterization (Clogston and Patri, 2013) and estimation of endotoxin contamination have been recently reviewed elsewhere (Crist et al., 2013; Dobrovolskaia, 2015).

The immunotoxicity of environmental materials has also been reviewed elsewhere (Kagan et al., 2010b).

Herein, we focus on the most prominent pieces of the nanoparticle–immune system puzzle, discussing what worked, what didn't, and what has been learned over the past 15 years of research on nanomaterials engineered for biomedical applications. A summary of achievements, disappointments, and lessons learned is presented in Fig. 2, and is further discussed below.

2. ACHIEVEMENTS

2.1. Structure–activity relationship

The physicochemical properties of nanoparticles determine their interactions with proteins in biological matrices (e.g. blood plasma and alveolar fluid) and with the immune cells. The structure–activity relationships between the most prominent physicochemical properties of nanoparticles and their effects on the immune system that lead to the most common types of immunotoxicity are summarized in Fig. 3. Below, we review several examples.

Nanoparticles with cationic surfaces, or those that carry cationic ligands, interact with biological membranes electrostatically. This leads to cellular damage, which triggers hemolysis, platelet activation, and aggregation, and to the induction of leukocyte procoagulant activity (PCA) and disseminated intravascular coagulation (DIC) (Greish et al., 2012; Jones et al., 2012a; Jones et al., 2012b; Ziemba et al., 2012). For example, cationic dendrimers of different architecture and size (generation five [G5] and generation four [G4] poly (propylene imine) [PPI] dendrimers [Bhadra et al., 2005; Agashe et al., 2006], G4 polyamidoamine [PAMAM] dendrimers [Bhadra et al., 2003; Asthana et al., 2005], generation three [G3] PAMAM and G3 PPI dendrimers [Malik et al., 2000], as well as G4 poly-L-lysine [PLL] dendrimers [Agrawal et al., 2007]) were shown to be hemolytic both *in vitro* and *in vivo*. The *in vitro* percent hemolysis varied from 14 to 86% in whole blood from human donors and various animal species, and was dependent on the density of the surface groups. Likewise, cationic PAMAM dendrimers, but not their anionic and neutral counterparts, altered key platelet functions and perturbed plasma coagulation, which culminated with DIC (Greish et al., 2012; Jones et al., 2012a; Jones et al., 2012b). The particle size, surface charge, and conformation of the polymer coating are important determinants of particle clearance by the mononuclear phagocytic system (MPS) in that smaller particles (100–200 nm) with unprotected surfaces and surfaces coated with a hydrophilic polymer in a “mushroom” configuration are primarily cleared by Kupffer cells in the liver; larger particles are eliminated by red pulp macrophages in the spleen. The addition of a hydrophilic polymer coating in a “brush” configuration protects particles from

immune recognition, while increasing the particle size above 300 nm provides no protection, regardless of the polymer conformation (Gbadamosi et al., 2002). Exposure to high aspect ratio particles (e.g. carbon nanotubes, titanium nanobelts, cellulose nanofibers), as well as certain metallic particles (e.g. Si), results in inflammasome activation and the induction of proinflammatory cytokine interleukin (IL)-1β. These particles, as well as certain cationic and carbon-based particles, can exaggerate endotoxin-mediated inflammation (Baron et al., 2015).

The immunotoxicity of a nanoparticle is also influenced by the therapeutic payload it carries. For example, the induction of cytokines and type I interferons, the inflammatory reaction, the prolongation of plasma coagulation time, and complement activation are common dose-limiting toxicities of therapeutic nucleic acids (Levin, 1999). These toxicities are also commonly observed with nanoformulated nucleic acids, and this limits their translation into clinical use (Dobrovolskaia and McNeil, 2015a; Dobrovolskaia and McNeil, 2015b). Cytotoxic DNA-intercalating drugs used to treat cancer (e.g. doxorubicin, daunorubicin, and vincristine) are known to induce PCA and DIC (Wheeler and Geczy, 1990; Swystun et al., 2009; Kim et al., 2011). Formulating these drugs using nanotechnology carriers may help avoiding the toxicity. However, if overcoming these toxicities is not considered during the design and optimization of nanoformulated versions of these drugs, both PCA and DIC may not be resolved.

2.2. Application of nanoparticles to improve vaccine efficacy

The efficacy of nanoparticle-based vaccines depends on the interactions between the particles and the target cells, and is determined by the physicochemical properties of the particles (size, shape, and surface functionalities) because these properties play a key role in particle recognition by the antigen-presenting cells (APCs). The type of nanocarrier is generally selected based on the type of immune response desired from the vaccine. Nanoparticles have been shown to provide a wide variety of advantages over conventional adjuvants. They can improve the solubility of hydrophobic antigens; provide controlled and sustainable release of antigens, therefore reducing the number of required immunizations; target antigens to specific cells and tissues, thus reducing side effects; prevent antigen degradation and deliver multiple antigens concurrently (reviewed in [Xiang et al., 2008; Xiang et al., 2010]). As of today, a wide variety of engineered nanomaterials from different classes (polymeric, chitosanic, magnetic, latex, gold, silica, and polystyrene) have been used successfully as antigen carriers and vaccine adjuvants (Tighe et al., 1998; Pavelic et al., 2001; Weiss et al., 2002; Walsh et al., 2003; Fifis et al., 2004a; Fifis et al., 2004b; Minigo et al., 2007; Mottram et al., 2007).

Depending on their size, particles are internalized by APCs via different pathways, including both pino- and phagocytosis (O'Hagan et al., 2001; Fifis et al., 2004a). Moreover, macrophages utilize multiple routes to take up the same types of nanoparticles (Franca et al., 2011). Several studies reported that smaller particles (20–200 nm) elicit stronger immune responses than their larger counterparts (O'Hagan et al., 2001; Fifis et al., 2004a; Fifis et al., 2004b; Minigo et al., 2007; Mottram et al., 2007; Manolova et al., 2008). For example, Plebanski and her group conducted a series of studies to demonstrate that 40–50 nm

polystyrene particles induce potent CD4+ and CD8+ T-cell responses and do so more efficiently than their larger (> 500 nm) counterparts. In contrast, particles > 500 nm in size were more active in inducing interferon (IFN)-γ and antibody responses (Fifis et al., 2004a; Fifis et al., 2004b; Minigoet al., 2007; Mottram et al., 2007). Several studies demonstrated that small (< 100 nm) nanoparticles quickly travel to the draining lymph nodes (LNs) after intradermal injection and effectively target LN-resident dendritic cells (DCs), B-cells, and macrophages (Manolova et al., 2008),(Reddy et al., 2007b). These data suggested that large particles depend on interaction with and uptake by tissue-resident APCs, while smaller particles utilize both cell-associated migration and lymphatic drainage, thus providing better antigen presentation (Manolova et al., 2008). Manipulation of nanoparticle size, shape, surface chemistry, and charge is generally employed to maximize antigen delivery to DCs. For example, small (< 100 nm) nanoparticles were shown to be taken up more efficiently by DCs, while large (1 μm) particles were preferentially internalized by macrophages (Fifis et al., 2004a). Several other studies have also reported that ~ 50 nm is the optimal nanoparticle size for uptake by DCs (Aoyama et al., 2003; Nakai et al., 2003; Wang et al., 2011).

Particle size was also reported as a primary factor in determining the immunostimulatory profiles of vaccine formulations in that smaller particles (~ 220nm) were more potent in inducing IFN-α responses, while their larger counterparts (~1200 nm) induced tumor necrosis factor (TNF)-α (Rettig et al., 2010). This difference was attributed to the type of the cells that internalized these particles: plasmacytoid DCs engulfed smaller particles, while macrophages preferred larger particles. Moreover, particle size was also suggested to be a key factor in determining the type of immunity induced. For example 40-nm nanoparticles promoted Th1 and CD8+ T-cell responses, while 100-nm particles induced Th2 responses (Fifis et al., 2004a; Fifis et al., 2004b; Mottram et al., 2007).

Tuning particle zeta potential is another approach that has been explored in vaccine design. For example, positively charged particles demonstrate greater uptake by DCs (Thiele et al., 2003; Foged et al., 2005; Villanueva et al., 2009) and induction of DC maturation (Thiele et al., 2001; Jilek et al., 2004; Little et al., 2004; Jilek et al., 2005; Reddy et al., 2007a). Cationic poly(D,L-lactic-co-glycolic) (PLG) particles improved the delivery of DNA adsorbed on the particle surface to APCs and induced greater cytotoxic T-lymphocyte responses compared to plain DNA antigen (Singh et al., 2000). More comprehensive coverage of this subject is available elsewhere (Fesenkova, 2013; Xiang et al., 2013).

2.3. Application of nanoparticles for delivery of antiretroviral, immunosuppressive and anti-inflammatory drugs

2.3.1 Antiretroviral—Antiretroviral drug delivery has many assorted challenges, some of which are being effectively overcome using nanotechnology-based carriers. In addition to improving the solubility of antiretroviral drugs, nanoparticles are considered a means to improve drug delivery to tissues and cells serving as viral reservoirs. When antiretroviral drugs are administered using conventional routes and formulations, the concentrations of these drugs in the plasma are usually higher than the concentrations found in the lymphoid tissue, which serves as a major depot for the virus (Fletcher et al., 2014). HIV can replicate in the lymphatic tissue even when the viral load in the peripheral blood is low; therefore, the

need to enhance drug delivery into lymphoid tissue is recognized by many as an effective way of targeting HIV both in systemic circulation and at its depot sites (Fletcher et al., 2014).

The physicochemical properties of nanoparticles that influence lymphatic delivery are: size, charge, molecular weight, lipophilicity, and surface ligands (surfactants, PEG, hyaluronic acid, biotin, peptides, antigens, and lectins) (Cho and Lee, 2014; Singh et al., 2014). Several antiretroviral drugs have been formulated using a wet-bead milling process and showed good stability and the desired tissue distribution. These examples include rilpivirine solid drug nanoparticles (Verloes, 2008; Baert et al., 2009) and a cabotegravir (S/GSK1265744) nanocrystal formulation (Spreen et al., 2013). Other approaches focusing on the use of nanoformulations for oral delivery of these drugs are in progress in order to address another problem related to poor patient adherence to antiretroviral therapy (Prinapori and Di Biagio, 2015). A more comprehensive review of this subject is available in a recent review by Liptrott et al. (Liptrott et al., in press).

2.3.2 Immunosuppressive and anti-inflammatory—Nanoparticles can be immunosuppressive *per se* or used to deliver immunosuppressive drugs. For example, inhaling carbon nanotubes was shown to suppress humoral immune response via a mechanism involving the production of transforming growth factor beta (TGF-β) by alveolar macrophages and subsequent prostaglandin production by spleenocytes leading to the systemic immunosuppression (Mitchell et al., 2009). Other examples of nanoparticles displaying immunosuppressive activities without bearing a therapeutic payload include the imaging agent Resovist, a single intravenous administration of which resulted in suppression of the antibody response to the model antigen (Shen et al., 2011). The water-soluble fullerene derivative polyhydroxy C60 was shown to inhibit type I hypersensitivity reactions to allergens, both *in vitro* and *in vivo* (Ryan et al., 2007). Likewise, allergen-loaded poly(D,L-lactic-co-glycolic) acid (PLGA) particles, chitosan, poly(lactic acid) (PLA), poly[methyl vinyl ether-co-maleic anhydride) nanoparticles, and dendrosomes were used to suppress type I and type II reactions to environmental and food allergens (Royet al., 1999; Scholl et al., 2004; Balenga et al., 2006; Gomez et al., 2007; Gomez et al., 2008), while synthetic peptide dendrimers were reported to block experimental allergic encephalomyelitis (Wegmann et al., 2008).

Other studies have shown the benefits of using nanoparticles for the targeted delivery of immunosuppressive and anti-inflammatory drugs, and to prevent the undesirable immunosuppression of small-molecule drugs (Stinchcombe et al., 2007). For example, using PLGA nanoparticles formulated to deliver glucocorticoids to inflamed joints in the mouse model of arthritis resulted in complete remission of the inflammatory response. The improved efficacy was due to the targeted and controlled release of steroids from the nanocarrier (Higaki et al., 2005). Liposomes loaded with clodronate were used to specifically eliminate macrophages in a swine model to protect animals from endotoxin-mediated lung injury (Gaca et al., 2003). Liposomal and polymeric nanoparticle reformulation of cyclosporine was reported to reduce off-target side effects (e.g. nephrotoxicity) (Freise et al., 1994; Italia et al., 2007). Tacrolimus delivery using lipid nanoparticles resulted in improved skin penetration and tissue deposition, as well as a

reduction in side effects (Pople and Singh, 2012). Polylactide nanoparticles were used for e*x vivo* delivery of cyclosporine A into DCc (Azzi et al., 2010). Reinjection of these drug-loaded DCs into the footpads of mice improved drug delivery to the lymph nodes, where released cyclosporine suppressed T-cell proliferation (Azzi et al., 2010). Delivery of rapamycin by elastin-like polymeric nanoparticles resulted in reduced nephrotoxicity and injection site reactions, while demonstrating comparable efficacy (Shah et al., 2013).

The activity of liposomal formulations of glucocorticoids provides another example of how nanoparticles can alter a drug's tissue distribution so that it provides additional beneficial effects. In this example, the free drug affects T-lymphocytes, while its liposomal counterpart targets macrophages and induces an alternatively activated M2 phenotype, leading to the expression of anti-inflammatory cytokines and, consequently, reduced inflammation (Schweingruber et al., 2011). Nanotechnology is used not only to deliver single drugs, but also to co-deliver anti-inflammatory agents with different mechanisms of action. For example, dexamethasone-loaded PLGA nanoparticles can be combined with siRNA-targeting COX-2 to suppress inflammatory responses (Park et al., 2012). More examples illustrating the benefits of delivering immunosuppressive and anti-inflammatory drugs using nanoparticles have been recently reviewed elsewhere (Ilinskaya and Dobrovolskaia, 2014).

2.4. Application of nanoparticles for cancer immunotherapy

Cancer immunotherapy is another rapidly growing branch of nanomedicine. Drugs used for cancer immunotherapy vary both in structure and by mechanism of action. For example, Iipilimumab (anti-CTLA4) directly interacts with and activates immune cells by removing co-inhibitory signal, while GVAX (GM-CSF tumor vaccine) improves tumor recognition by making the tumor more immunostimulatory (Ali and Lee, 2015; Lipson et al., 2015). A recent study by Fiering et al. demonstrated the use of iron oxide nanoparticles and an alternating magnetic field to induce local hyperthermia in melanoma. Interestingly, besides heat-mediated tumor ablation, this treatment resulted in a potent CD8+ T-cell–dependent response against the tumor, preventing the recurrence of tumor growth (Toraya-Brown et al., 2014). Another study demonstrated that tumor-associated myeloid-derived suppressor cells (MDSCs) characterized by high levels of oxidative reactions may be responsible for the degradation of chemotherapeutic drugs in the tumor environment, and that this degradation could be significantly reduced by drug loading onto functionalized carbon nanotubes (Seo et al., 2015). This example offers an effective way to prolong drug function in the tumor environment by using a nanodelivery approach. Other recent examples include using poly (ethyleneimine) nanoparticles for the delivery of antiPD-1 siRNA (Teo et al., 2015), branched polyethyleneimine-superparamagnetic iron oxide nanoparticles for the enhancement of Th1 cell polarization of DCs (Hoang et al., 2015), 6-thioguanine-loaded polymeric micelles for the depletion of MDSCs (Jeanbart et al., 2015), and a CD4-targeted, oil-in-water emulsion for the co-delivery of IL-2 and TGF-β (McHugh et al., 2015).

2.5. Understanding the role of the immune system in nanoparticle biodegradation

The therapeutic use of biodegradable nanoparticles is accompanied by fewer safety concerns than those of durable nanomaterials. Not surprisingly, the majority of currently marketed nanomedicines are represented by biodegradable, lipid-based materials (Etheridge et al.,

2013). Macrophages are known to play an active role in internalizing and degrading these nanocarriers (Song et al., 2012).

The use of nonbiodegradable nanoparticles is often associated with concern regarding their bioaccumulation and long-term toxicity. In this context, recent studies demonstrating the unique role of activated neutrophils in the enzymatic digestion of durable nanoparticles are very encouraging. Specifically, activated neutrophils were reported to participate in the biodegradation of carbon nanotubes. Myeloperoxidase (MPO)-reactive intermediates and hypochlorous acid (HClO) generated by MPO are believed to contribute to the biodegradation process (Kagan et al., 2010a; Bianco et al., 2011; Shvedova et al., 2012), and the existence of this mechanism was confirmed *in vivo* using MPO-deficient mice (Shvedova et al., 2012). MPO was also found to have a role in the oxidative biodegradation of single-wall carbon nanotubes activated human neutrophils (Kagan et al., 2010a). Moreover, additional research suggests that peroxynitrite ($ONOO^-$)-driven oxidation, resulting from the enzymatic activities of NADPH-oxidase/inducible nitric oxide synthase (iNOS), functions as another carbon nanotube biodegradation pathway in activated macrophages (Vlasova et al., 2012; Bhattacharya et al., 2014; Farrera et al., 2014; Kagan et al., 2014). Recent data demonstrated that tumor-associated MDSCs expressing high levels of arginase, iNOS, NADPH oxidase, and MPO were also able to biodegrade carbon nanotubes via oxidative pathways. These MDSC properties were recently utilized for an interesting nanodelivery approach in which nitrogen-doped carbon nanotube cups (NCNCs) were loaded with therapeutic cargo (paclitaxel), sealed via conjugation with gold nanoparticles (GNPs), and opened by enzymatic oxidative processes within, or in the immediate proximity of, MDSCs. This mechanism was proposed to enhance antitumor immune responses by targeting and inactivating MDSCs using their own highly expressed oxidative enzymatic machinery (Zhao et al., 2015).

2.6. Application of nanoparticles to reducing the immunotoxicity of traditionally formulated drugs

In addition to their use as carriers of novel drugs, engineered nanomaterials are also increasingly being used to reformulate traditional drugs (low-molecular-weight drugs, therapeutic proteins, therapeutic antibodies, and nucleic acids). Reformulation of traditional drugs using nanotechnology has been shown to improve drug solubility and pharmacokinetics, as well as to reduce undesirable side effects. Below, we review several examples demonstrating how reformulation of traditional drugs using nanotechnology resulted in reduced immunotoxicity.

The traditional formulation of the cytotoxic oncology drug paclitaxel relies on the polyethoxylated castor oil excipient Cremophor-EL ®, which is known to induce anaphylaxis in sensitive individuals. The anaphylactic reaction to Cremophor-EL is mediated by its ability to activate the complement system (Weiszhar et al., 2012). Due to this side effect, the Cremophor-EL formulation of paclitaxel (Taxol ®) has to be administered via slow infusion, and after patient premedication with immunosuppressive drugs. In contrast to Taxol, the nanoalbumin formulation of paclitaxel (Abraxane ®) is administered via push injection, and does not require premedication (Gradishar et al., 2005).

Likewise, TNF-α failed in clinical trials because it induces systemic immunostimulation, resulting in fever and hypotension. However, TNF-α immobilized on the surface of PEGylated colloidal gold nanoparticles (Cyt6091) has successfully passed a Phase I trial (Libutti et al., 2010). Therapeutic protein immunogenicity, which leads to the formation of antibodies that neutralize the drug and, in some cases, endogenous proteins, is a common reason for the discontinuation of such drugs (Rosenberg et al., 2012). Liposomes were shown to reduce the antigenicity of recombinant coagulation factor FVIII and to protect encapsulated protein from the antibodies formed in response to the traditional formulation of this therapeutic protein (Ramani et al., 2008). Other success stories demonstrating the reduction of immunotoxicity by reformulation of a traditional drug using nanotechnology-based carriers include the encapsulation of therapeutic antisense oligonucleotides into liposomes to prevent activation of the complement system (Klimuk et al., 2000) and to reduce cytokine-mediated toxicities (Yu et al., 2013), as well as the reformulation of the small-molecule oncology drug 5-fluorouracil, using chitosan nanoparticles to decrease its hematotoxicity (Giacalone et al., 2013; Cheng et al., 2014).

2.7. Understanding the applicability of the existing regulatory framework for immunotoxicity analysis of nanoformulations

Several nanotechnology-formulated drugs have already reached the market. Examples include solid lipid nanoparticles (e.g. Leunesse®), liposomes (e.g. Doxil®), protein-based nanoparticles (e.g. Abraxane), as well as nanocrystals (e.g. Emend®). Regulatory experience gained from working with these formulations, along with data from preclinical reports, has helped to develop a better understanding of how to apply the regulatory framework established and used for small-molecule and macromolecular therapeutics to complex nanotechnology formulations (Tyner and Sadrieh, 2011; Bancos et al., 2013; Cruz et al., 2013; Tyner et al., 2015). The properties shared by nanotechnology-based carriers that have already reached the market are biodegradability (e.g. emulsions and liposomes) and the ability to quickly dissolve in the body (e.g. nanocrystals). Durable nanoparticles (e.g. metals and metal colloids) are expected to accumulate in the body and, as such, raise immunological safety concerns. These concerns stem from the notion that durable materials tend to distribute to the MPS (Sadauskas et al., 2009; Di Gioacchino et al., 2011; Moon et al., 2011; Umbreit et al., 2012). Accumulation of these materials in the MPS may affect the normal function of this system, and therefore this concern should not be ignored.

The FDA applies the same regulatory framework established for all small-molecule and macromolecular therapeutics to the regulation of complex nanotechnology products (Bancos et al., 2013). The manufacture, characterization, and non-clinical safety evaluation of combination products containing nanotechnology platforms have also been covered in several recent regulatory documents, which recommend conducting studies relevant to each component of the complex formulation (CDER, 2006; CDER, 2008). For example, the International Conference on Harmonization (ICH) S8 and S6 guidelines are consulted to estimate the immunotoxicity of small-molecule and macromolecular components, respectively, of any nanotechnology-formulated combination product (CDER, 2001; Bancos et al., 2013).

3. DISAPPOINTMENTS

3.1. Harmonization of testing is still in progress

Ten years ago, international standards development organizations ASTM International and ISO established the E56 and TC229 committees, respectively, to lead the development of standard test methods for nanomaterials. This effort resulted in the development of three standardized test methods—ASTM E56-2524-08(2013), ASTM E56-2525-08(2013), and ISO 29701:210—for the analysis of the hemolytic properties of nanoparticles, the effects of nanoparticulate materials on the formation of mouse colony-forming unit granulocyte/macrophage colonies, and of potential endotoxin contamination in nanomaterials, respectively (http://www.astm.org/COMMIT/SUBCOMMIT/E5603.htm; http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_tc_browse.htm?commid=381983). While these harmonized methods are important to support immunotoxicity studies, they are obviously insufficient to address the broad spectrum of end-points that are indicative of nanoparticle immunotoxicity. Despite global efforts, there is no harmonization in the following areas: dose selection, dose metrics, assay format, cell species, and matrices. Recently, an integrated approach was proposed to estimate relevant *in vitro* doses of tested nanomaterials in terms of total mass, surface area, and particle number (Cohen et al., 2014). While the results of this study are encouraging, adaptation of this approach by other laboratories and harmonization efforts are needed.

3.2. Lack of nanoparticle reference standards for immunotoxicity studies

Reference standards are well-characterized materials with known properties. These materials can be used to validate toxicology protocols and ensure the quality of measurements specific to a given protocol measuring given end points. It is generally acknowledged that the lack of nanoparticle reference standards limits the validation of instruments, protocols, and materials used to assess exposure to nanomaterials and understand their biocompatibility. While many types of nanomaterials have been linked to certain types of immunotoxicity (e.g. cationic dendrimers are thrombogenic; PEGylated liposomes induce anaphylaxis), there are no standard reference materials to use for these and other types of immunotoxicity studies. Stefaniak et al. conducted a literature review and identified 25 nanomaterials that were considered to be candidate nanoparticle reference materials by standards development organizations worldwide (Stefaniak et al., 2013). Interestingly, this study found a limited consensus regarding the types of candidate nanoparticles between various organizations involved in the development of reference materials: the U.S. National Metrology Institute, the U.S. National Institute of Standards and Technology, the REFNANO project funded by the UK government, the Organization for Economic Cooperation and Development, and several NanoImpactNet projects (NanoImpactNet, NanoSustain, and NanoValid) funded by the European Commission (Stefaniak et al., 2013).

In the absence of consensus on and availability of relevant, well-characterized reference materials, immunotoxicity studies are conducted using traditional positive controls, such as lipopolysaccharide for cytokine induction, phytohemagglutinin for leukocyte proliferation, Triton X-100 for hemolysis, and cobra venom factor for complement activation (Dobrovolskaia, 2015). Preclinical studies often rely on nanomedicines with known clinical

immunotoxicities (e.g. Doxil or Taxol for complement activation and anaphylaxis tests) (Dobrovolskaia, 2015). Despite the thorough characterization of their physicochemical properties, the main limitation in using these products as reference materials is their expense and limited accessibility to the nanotechnology research community.

3.3. Examples of nanoformulations demonstrating reduced immunotoxicity of traditionally immunotoxic APIs are fewer than expected

Despite clear examples demonstrating the potential of nanotechnology for reducing the immunotoxicity of traditional active pharmaceutical ingredients (APIs), the systemic administration of many nanoformulations to patients still requires immunosuppressive and/or antipyretic premedication. For example, the complexing of therapeutic nucleic acids with a nanocarrier is commonly based on electrostatic interactions. As such, the lipid and polymeric carriers used to formulate therapeutic nucleic acids tend to be cationic. As discussed earlier, cationic particles are known to be cytotoxic to a variety of immune cells, induce cytokine secretion, exaggerate endotoxin-mediated toxicities, activate complement, bind plasma proteins, trigger pro-coagulant activity, and may also affect protein conformation and function (Pantic, 2011; Boraschi et al., 2012; Dobrovolskaia and McNeil, 2013a). These data raise safety concerns. Indeed, several studies have demonstrated that base modifications were insufficient to reduce the immunostimulatory activity of certain nucleic acids (e.g. siRNA). Furthermore, lipid nanocarriers were shown to contribute to the drug's immunostimulation (Abrams et al., 2010). Examples of immunostimulatory lipids used to prepare nanocarriers for therapeutic nucleic acids include 2-(4-[(3β)-cholest-5-en-3-yloxy]butoxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (CLinDMA) and PEG-dimyristoylglycerol (Abrams et al., 2010), protamine (Li et al., 1999), trimethyl ammonium propane-cholesterol (Kim et al., 2007), and 1,3-dioleoyl-3-trimethylammonium propane (DOTAP) (Li et al., 1999; Vasievich et al., 2011). Clinical studies investigating the safety of such formulations are often designed to prevent adverse reactions by premedicating patients with immunosuppressive cocktails containing immunosuppressive agents (e.g dexamethasone), antipyretic agents (e.g acetaminophen), histamine H1 receptor blockers (e.g. diphenhydramine), and histamine H2 receptor blockers (e.g. ranitidine) (Coelho et al., 2013). It is clear that the advantages of tuning the physicochemical properties of nanoparticles for the purpose of reducing drug immunotoxicity have not yet been fully explored. The continuing evolution of the framework for evaluating nanoparticle immunotoxicity is the likely reason for this observation.

3.4. The mechanisms of nanoparticle immunotoxicity are largely unknown

The majority of studies reported in the past decade were focused on understanding the structure–activity relationship between the physicochemical properties nanoparticles and immunotoxicity. Uncovering the mechanisms of nanoparticle immunotoxicity is still a work in progress. Several interesting mechanisms have been recently described. For example, the inhibition of phosphoinositol-3-kinase (PI3K) by cationic PAMAM dendrimers has been suggested to contribute to the exaggeration of lipopolysaccharide (LPS)-induced leukocyte PCA in human peripheral blood mononuclear cells by these particles (Ilinskaya et al., 2014). The activation of mitogen-activated protein kinase (MAPK) p38 by gold nanoparticles

functionalized with α-lipoyl-ω–methoxy poly(ethylene glycol) was proposed as a mechanism for exaggeration of LPS-triggered nitric oxide and IL-6 secretion in murine macrophages (Liu et al., 2012). The binding of colloidal gold nanoparticles to high-mobility group B-1 was implicated in the attenuation of nuclear factor-kappaB (NF-kB) signaling, the phosphorylation of JNK, and the secretion of TNF-α triggered by TLR9 activation by CpG oligonucleotides (Tsai et al., 2012).

The induction of oxidative and nitrosative stress by zinc oxide nanoparticles was described as a mechanism through which these particles activate redox-sensitive NF-κB and MAPK signaling pathways, leading to an inflammatory response in human monocytes (Senapati et al., 2015). The oxidative stress induced by the nanoemulsion Cremophor-EL was also suggested to trigger IL-8 production by human monocytes via a mechanism that bypasses gene expression (Ilinskaya et al., 2015).

It is also important to note that the effects of nanomaterials on immune cells may result in both suppression of the immune effector cells and activation of the immune regulatory (immunosuppressive) cells. Therefore, any discussion of nanoparticle immunotoxicity should consider specific immune cell subsets. For instance, airborne carbon nanotubes have recently been reported to induce rapid accumulation of pulmonary MDSCs in mice, which was associated with the accelerated growth of lung carcinomas *in vivo* (Shvedova et al., 2013). Further analysis of the mechanism revealed that carbon nanotubes may presensitize MDSCs to produce the immunosuppressive cytokine TGF-β, which contributes to the observed immunosuppression and, as a consequence, tumor growth (Shvedova et al., 2013). Thus, both uncovering the immunomodulatory properties of nanomaterials and understanding the molecular and cellular mechanisms of their activities are important for the clinical translation of these materials and potential minimization of any undesirable immunoreactivity.

Further research is clearly needed to uncover additional mechanisms and to link them to the physicochemical properties of nanoparticles.

3.5. Accidental nanoparticulate contaminants contribute to the immunogenicity of therapeutic proteins

The observation that particulate materials between 0.1 and 10 μm in size can contaminate recombinant protein therapeutics and contribute to their immunogenicity has generated increasing levels of concern (Carpenter et al., 2010). The mechanism by which this occurs has not been fully investigated; however, several factors, such as nanoparticle-triggered protein aggregation (Mire-Sluis et al., 2011; Van Beers et al., 2012); the adsorption of proteins on the particle surface, leading to the formation of highly immunogenic repeated-protein structures (Mire-Sluis et al., 2011; Van Beers et al., 2012); and the exaggeration of inflammatory responses triggered by trace amounts of endotoxin (Dobrovolskaia and McNeil, in press) may play a role. Tungsten microparticles originating from the tungsten pins used in the manufacturing process were shown to induce protein aggregation and increase the immunogenicity of a recombinant protein product (Liu et al., 2010).

Another study suggested that hydrophobic metal, glass, and polystyrene particulates adsorb protein on the particle surface and contribute to protein aggregation and immunogenicity (Van Beers et al., 2012). Several other materials were named among common particulates found to contaminate therapeutic proteins, including cellulose and glass fibers, silicon oil, rubber, stainless steel, fluoropolymers, and plastics (Carpenteret al., 2010; Liu et al., 2010; Van Beers et al., 2012). Cellulose fibers were shown to exaggerate the production of endotoxin-induced pro-inflammatory cytokines (Dobrovolskaia and McNeil, in press). Gowns and other materials used in clean rooms during the manufacturing of therapeutic proteins—closures, filling pumps, containers, vial stoppers, etc.—serve as sources of these particulate contaminants (Carpenter et al., 2010). These data led regulatory agencies to require the detection and characterization of particulate materials that can contaminate therapeutic proteins; however, there is no general agreement as to what methods should be used (Carpenteret al., 2010). The major challenge in understanding the mechanism of therapeutic protein immunogenicity in the presence of contaminating particles is the limited quantities of these particulate materials. Even when a particulate contaminant is detected and isolated from the protein product, the quantity of the isolate is usually insufficient to conduct a follow-up mechanistic study (Carpenter et al., 2010). It is obvious that more research is needed to address this important issue.

4. LESSONS LEARNED

4.1. Nanoparticles' physicochemical properties are the keys to determining particle interaction with the immune system

It is now well established that nanoparticles can be engineered to either avoid or specifically interact with the immune system. The tuning of nanoparticles to attain desirable attributes can be achieved by manipulating the physicochemical properties (size, charge, hydrophobicity, shape) of the particle that determine its interaction with plasma proteins and immune cells. This subject has been extensively reviewed elsewhere (Smith et al., 2013). We have also discussed many examples underlining this point in the achievement section of this paper.

4.2. Chemical (e.g. synthesis byproducts) and biological (e.g. endotoxin) impurities can contribute to nanoparticle toxicity

It is very important to distinguish nanoparticle-mediated immunotoxicities from those triggered by chemical and biological impurities. The presence of traces of CTAB used as a stabilizing agent in the synthesis of gold nanorods was implicated in the cytotoxicity of these particles (Leonov et al., 2008). Iron and nickel used to catalyze reactions involved in the synthesis of carbon nanotubes were shown to trigger inflammatory reactions in response to nanotube exposure (Madani et al., 2013). Bacterial endotoxin is a common biological impurity affecting over 30% of preclinical-grade nanomaterials (Crist et al., 2013; Dobrovolskaia and McNeil, 2013b). If not properly identified in and eliminated from nanoformulations, endotoxin can confound the results of both nanoparticle immunotoxicity and efficacy studies. The elimination of endotoxin from nanoparticles was shown to reduce their immunotoxicity (Vallhov et al., 2006). Moreover, some nanomaterials, while not inflammatory themselves, were able to potentiate endotoxin-mediated inflammation. Silica-

and carbon-based nanomaterials, as well as some metal oxides, have been shown to exaggerate endotoxin-mediated inflammation in the lungs (Inoue et al., 2006; Inoue et al., 2007; Shi et al., 2010; Inoue, 2011; Inoue and Takano, 2011), while cationic PAMAM dendrimers were reported to exaggerate endotoxin-induced leukocyte PCA (Dobrovolskaia et al., 2012; Ilinskaya et al., 2014). Strategies for endotoxin detection have been discussed elsewhere (Dobrovolskaia, 2015).

4.3. Thorough physicochemical characterization is needed prior to nanoparticle analysis in immunological assays

Since the physicochemical properties of nanoparticles are the keys to determining particle interaction with the immune system, thorough particle characterization is needed prior to immunotoxicity studies (Clogston and Patri, 2013). Several examples have shown that instability of particle surface coatings results in inflammatory reactions, yet partial loss of surface coating is undetectable by dynamic light scattering and zeta potential analysis, traditionally used for particle characterization (Clogston and Patri, 2013; Crist et al., 2013). Certain processes and reagents commonly involved in nanoparticle research (e.g. sterilization procedures and inhibitors used for signal transduction studies) may also affect nanoparticle integrity (Zheng et al., 2011; Dobrovolskaia and McNeil, in press). Missing such details may lead to misinterpretation of the study results and faulty conclusions. Another important lesson has been learned from using nanoparticles for drug delivery: Drug conjugation to a nanotechnology-based carrier may change the drug's original properties. For example, celastrol conjugated to a dendrimer carrier retained its ability to suppress LPS-induced nitric oxide release, but lost its ability to inhibit production of pro-inflammatory cytokines (Boridy et al., 2012).

4.4. Total protein binding serves as good indicator of nanoparticle stealthiness and its distribution to the MPS

Proteins bind to a nanoparticle surface instantaneously upon entry of the particle into the bloodstream. Some of these proteins stay on the surface as long as the particles circulate in the bloodstream, while others dissociate from the particle surface or get replaced by proteins with a higher binding affinity. Protein binding was shown to affect nanoparticle hydrodynamic size and charge (Dobrovolskaia et al., 2009b), and was also suggested to influence the way cells and tissues interact with and process the particles, ultimately guiding cellular uptake, clearance route, and tissue distribution (Goppert and Muller, 2005; Michaelis et al., 2006; Nagayama et al., 2007; Zensi et al., 2009). It is now well established that total protein binding can serve as an indicator of particle “stealthiness.” Stealthy particles tend to stay in circulation longer. In contrast, particles with proteins bound to their surface are cleared by the cells of the MPS.

4.5. Composition of protein corona is insufficient to predict nanoparticle immunocompatibility

In contrast to total protein binding, the composition of the protein corona has less predictive value. Complement and fibrinogen are abundant plasma proteins, and they have been reported as part of the so-called “protein corona” for many engineered nanomaterials.

However, the presence of these proteins on the particle surface *per se* does not mean that the proteins will be activated (Salvador-Morales et al., 2006; Salvador-Morales et al., 2008; Deng et al., 2009; Deng et al., 2011; Deng et al., 2012a; Deng et al., 2012b). The particle concentration needed to deplete these proteins to a level that would affect their function must be very high. Achieving such concentration *in vitro* is possible for some, but not all, nanoparticles. Moreover, protein levels *in vivo* vary from donor to donor, and even within the same donor, due to homeostasis. Therefore, the identification of the composition of a nanoparticle's protein corona cannot reliably serve as a predictor of particle immunotoxicity (Salvador-Morales et al., 2006; Salvador-Morales et al., 2008; Deng et al., 2009; Deng et al., 2011; Deng et al., 2012a; Deng et al., 2012b; Dobrovolskaia et al., 2014).

4.6. Common markers of nanoparticle immunotoxicities halting clinical translation of nanomedicines

The toxicities that represent the most common safety concerns and reasons for nanoparticle failure in the preclinical stage include erythrocyte damage, thrombogenicity (platelet aggregation, plasma coagulation, DIC, and leukocyte PCA), cytokine-mediated inflammation and cytokine storming, pyrogenicity (mainly due to bacterial endotoxin contamination), and anaphylaxis and other complement activation-mediated reactions, as well as recognition and uptake by the cells of the MPS (Dobrovolskaia, 2015). As such, screening for these toxicities early in preclinical development helps in eliminating potentially toxic candidates. Fig. 3 can be consulted as a guide to selecting the most appropriate screening method based on the immunotoxicity endpoint, which can be suggested by nanoparticle physicochemical properties.

5. CONCLUSION

The past decade has been full of exciting discoveries: unraveling trends in nanoparticle immunocompatibility, understanding the role of particle characterization, and finding therapeutic applications for variety of nanocarriers. Since the mechanisms of nanoparticle immunotoxicity are not completely understood, the next decade of research should focus on identifying mechanisms and mapping them to the physicochemical properties of nanoparticles.

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Highlights

- Achievements, disappointments and lessons learned over past decade are reviewed
- Areas in focus include characterization, immunotoxicity and utility in drug delivery
- Future direction focusing on mechanistic immunotoxicity studies is proposed

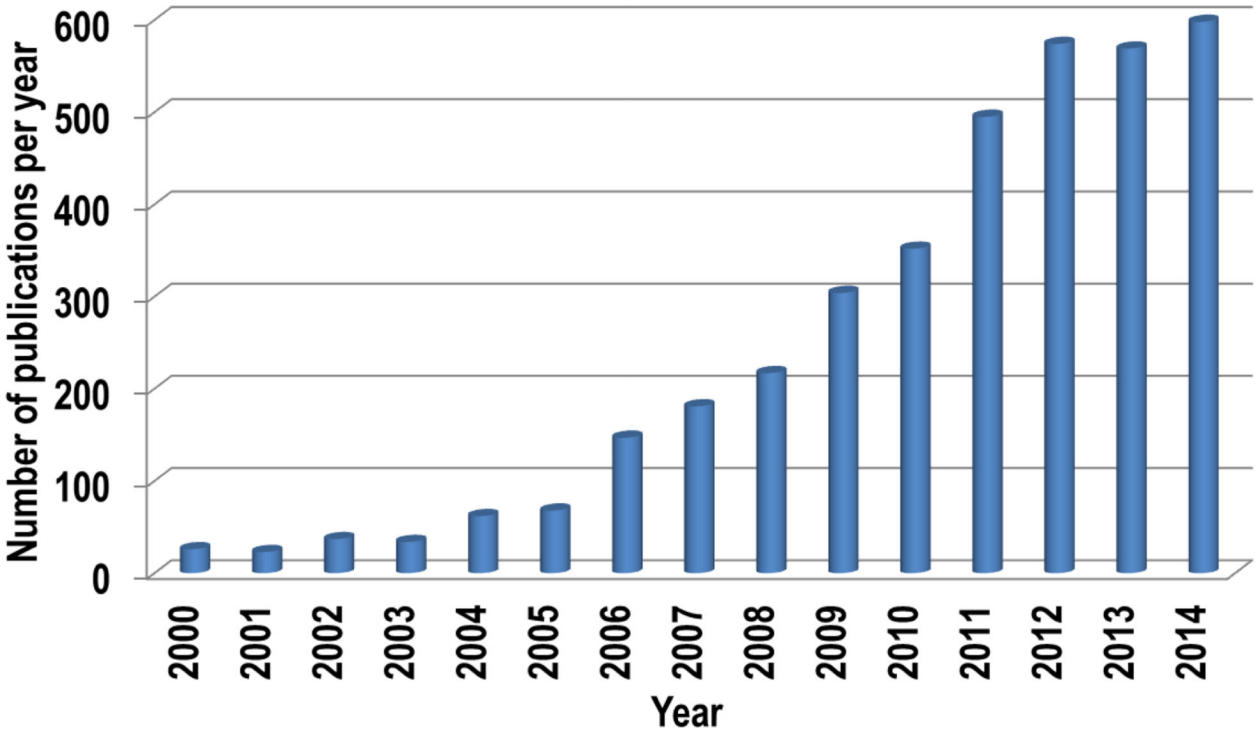

Fig. 1.

Publications statistics. The PubMed data base was searched using the keywords “nanoparticles” and “immune system” for the years 2000–2015. The data for 2015 were excluded from the analysis because the publication year was incomplete at the time of the search. Each bar shows the total publication number per year.

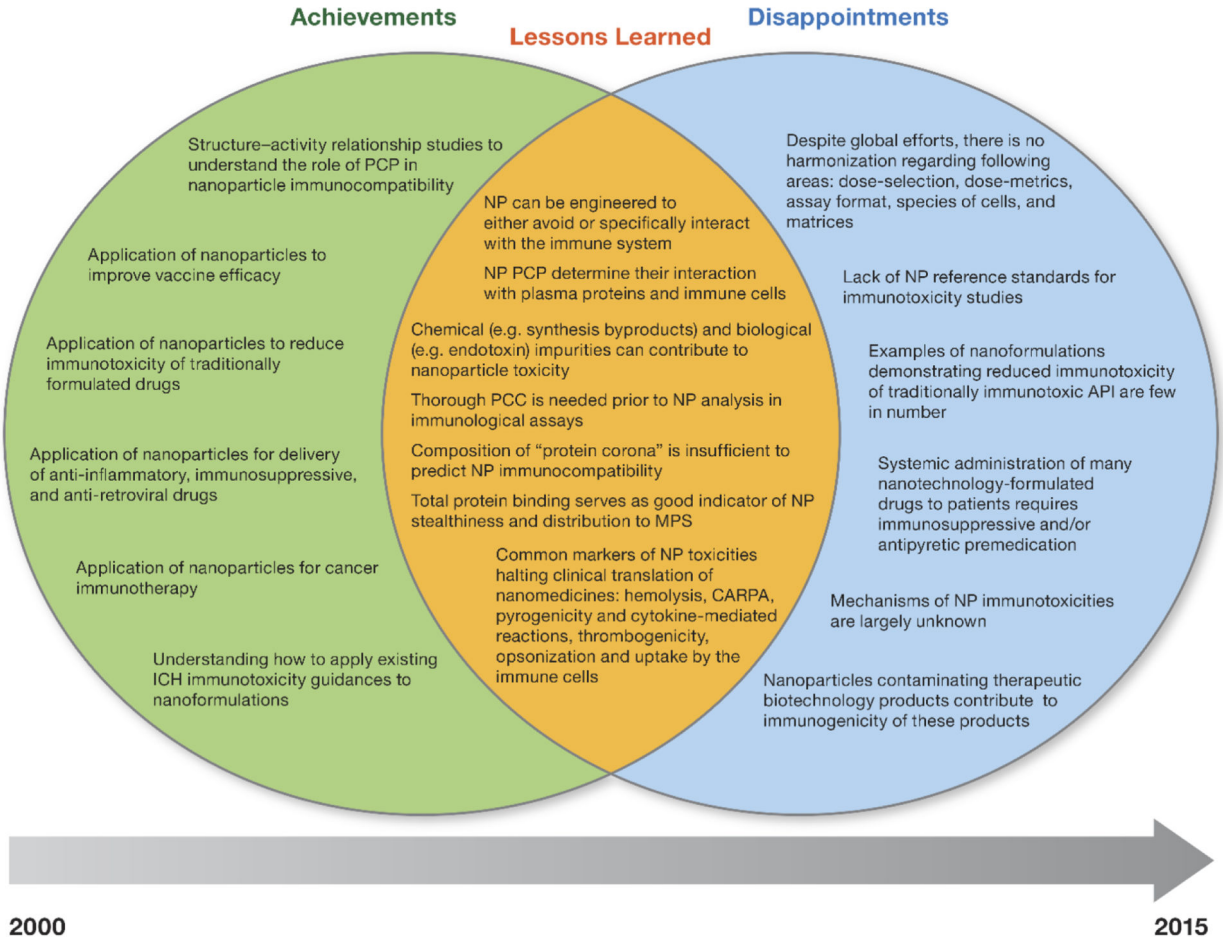

Fig. 2.

Achievements, disappointments, and lessons learned from the characterization of engineered nanomaterials over the past decade. This diagram outlines achievements (left circle) and disappointments (right circle) based on the studies dedicated to investigating nanoparticle immunotoxicity over the past decade. The overlapping area shows the lessons learned from these studies. API- active pharmaceutical ingredient; NP – Nanoparticles; PCP – Physicochemical properties, CARPA – Complement activation-related pseudoallergy, ICH – International Conference on Harmonization.

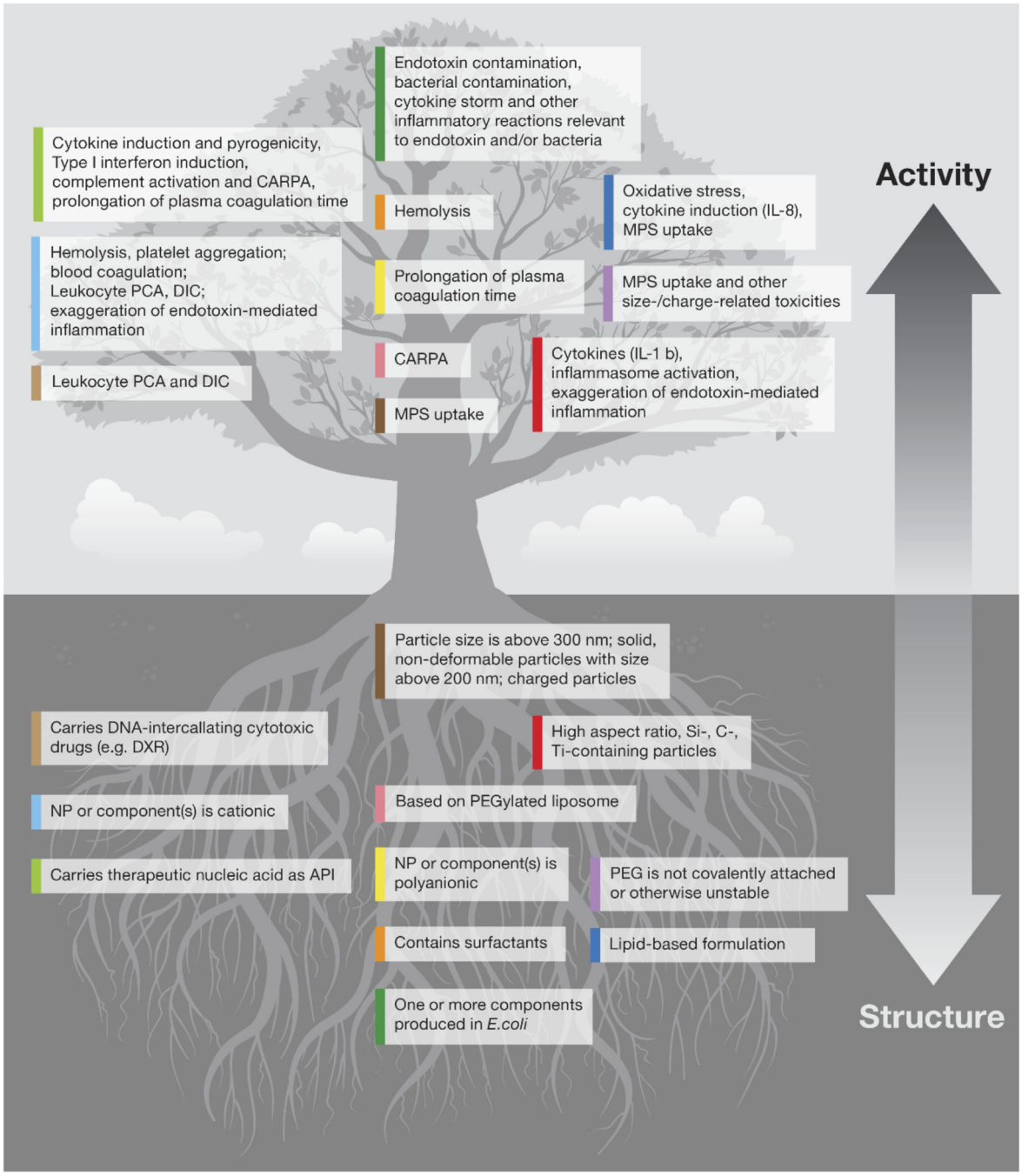

Figure 3.

Structure–activity relationship summary. Shown are the structure–activity relationships between nanoparticles and their effects on the immune system. Each block listed in the bottom (structure) part of the figure is color-coded. To find what toxicity is related to the given structure block, please find the block in the top (activity) part of the figure marked with the color matching that of the structure block. PCA – Procoagulant activity, DIC – Disseminated intravascular coagulation, CARPA – Complement activation-related pseudoallergy, MPS – Mononuclear phagocytic system, IL – Interleukin, PEG –

Polyethylene glycol, NP – Nanoparticle, DXR – Doxorubicin, API – Active pharmaceutical ingredient, DNA – Deoxyribonucleic acid.