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REVIEW

Value of phagocyte function screening for immunotoxicity of nanoparticles in vivo

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Abstract: Nanoparticles (NPs) present in the environment and in consumer products can cause immunotoxic effects. The immune system is very complex, and in vivo studies are the gold standard for evaluation. Due to the increased amount of NPs that are being developed, cellular screening assays to decrease the amount of NPs that have to be tested in vivo are highly needed. Effects on the unspecific immune system, such as effects on phagocytes, might be suitable for screening for immunotoxicity because these cells mediate unspecific and specific immune responses. They are present at epithelial barriers, in the blood, and in almost all organs. This review summarizes the effects of carbon, metal, and metal oxide NPs used in consumer and medical applications (gold, silver, titanium dioxide, silica dioxide, zinc oxide, and carbon nanotubes) and polystyrene NPs on the immune system. Effects in animal exposures through different routes are compared to the effects on isolated phagocytes. In addition, general problems in the testing of NPs, such as unknown exposure doses, as well as interference with assays are mentioned. NPs appear to induce a specific immunotoxic pattern consisting of the induction of inflammation in normal animals and aggravation of pathologies in disease models. The evaluation of particle action on several phagocyte functions in vitro may provide an indication on the potency of the particles to induce immunotoxicity in vivo. In combination with information on realistic exposure levels, in vitro studies on phagocytes may provide useful information on the health risks of NPs.

Keywords: immunotoxicity, phagocytes, cytokines, respiratory burst, nitric oxide generation, phagocytosis

Introduction

Nanoparticles (NPs) are used in many industrial applications and consumer products, and they are also being developed for targeted drug delivery, imaging, and implants in the medical sector. In addition to cytotoxicity, NPs can act on the immune system. Potential immunotoxic effects of NPs are relevant for human health because the immune system is present at all potential portals of entry of NPs and a variety of immunomodulatory actions of NPs has been proposed.[1] The immunmodulatory action of a compound usually describes a desired change in the immune system – for instance, for therapeutic intervention – while "immunotoxicity" is used for adverse immunomodulation indicating nondesired effects on the immune system. Immunotoxicity includes interactions with blood (hemolysis, coagulation, and protein binding), accumulation in the mononuclear phagocyte system (MPS), adjuvant properties, binding of haptens, interference with phagocytosis, and modulation of the Th2/Th1 response to antigens. Epidemiological studies in regions with increased concentrations of ultrafine particles suggested that NPs could influence the immune system. High levels of airborne particles caused worsening of asthma and pneumonia in exposed individuals.[2–5] Ultrafine particles in the atmosphere do not meet the size requirements of NPs because their

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upper size limit is usually 2.5 µm, but the reports stimulated further studies on size-related particle effects and raised the awareness that the large surface area of NPs was the reason for their high biological reactivity and toxicity.[6]

In contrast to cytotoxicity, the role of in vitro immunotoxicity testing is not well established. This is firstly due to general problems in simulating the complexity of the immunological system in vitro, as well as in the extrapolation of in vitro and animal data to human reactions and, secondly, to NP-specific problems. The immune system is redundant and has the capacity to compensate for minor immunotoxicological effects. High interindividual variations of the immune system further complicate the identification of a link between NP exposure and immunotoxicity in humans. Due to the high proliferation rate and compensation capacity of the immune system, only extreme alterations will result in clinical symptoms. On the other hand, decreased immunosurveillance may have long-term consequences, which cannot be directly linked to immunotoxicity. One example of such effects is the three- to fourfold increase in cancer incidence by immunosuppression with cyclosporine A for 5 years.[7]

Engineered NPs, to which humans might be exposed, comprise titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs in consumer products, silver (Ag) NPs in clothing, and silica (SiO_2) NPs in food. For medical products, gold (Au), carbon nanotubes (CNTs), and iron oxide are likely candidates. The main exposure routes are dermal for NPs in consumer products and oral for NPs in food and intravenous for NPs in medical use. The exposure of humans to engineered NPs, due to the different use of these products, is expected to be highly variable. Site-specific composition and reaction of the immune system (lung, skin, blood, etc) affords exposure-specific models because the same NPs might cause no immune effects when applied by the oral and dermal route, but they may induce sensitization after intradermal injection.[8] This creates a high number of different testing scenarios and renders the testing of all variations in vivo ethically and financially problematic. In this situation, prescreening by in vitro assays, similar to cytotoxicity screening for systemic toxicity, would be helpful. Of course, in vitro testing has the limitation that only one or a few cell types can be evaluated. Data produced after exposure to high doses for a short period are not representative for the exposure to most NPs.[9] Furthermore, the protective mechanisms of the body – for instance, mucociliary clearance in the lung and radical scavenging by glutathione in the blood – will mitigate the toxic effect observed in vitro.

According to the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS), immunotoxicity testing of NPs should focus on macrophages, granulocytes, and dendritic cells (DCs), and the testing should use cytokines as readout parameters.[10] Since phagocytes are involved in the unspecific defense, as well as in the specific immune response, impairment of phagocyte function can indicate a decreased reserve of the immune system in NP-exposed individuals.

Therefore, phagocytes appear to be suitable for discriminating between NPs interfering or not interfering with the immune system. Several studies report interference with phagocyte function by iron oxide particles, but the iron oxide NPs, which have been approved for medical use (such as Ferumoxtran-10 [Sinerem®]), did not influence the different aspects of phagocyte function. The secretion of proinflammatory cytokines, oxidative burst, phagocytosis, and chemotaxis was not affected by the exposure to the particles in vitro.[11] The few studies in which the same NPs were assessed by animal exposure and by exposure of cells to Ag and SiO_2 NPs show that impairment of phagocytes function in vitro accords with immune inflammation in vivo.[12,13] Proinflammatory action was seen in vivo as well as in macrophages isolated from animals exposed to TiO_2 and ZnO NPs.[14,15]

This review is focused on plain (not pegylated or formulated) metal and metal oxide NPs, such as SiO_2, iron oxide, Ag, Au, TiO_2, and ZnO NPs, and single-walled CNTs (SWCNTs) and multiwalled CNTs (MWCNTs). These NPs are relevant for humans because they are used in a variety of consumer products and as imaging reagents in medicine. Their classification as non- or low biodegradable NPs is often used to differentiate these particles from the enzymatically degradable NPs, such as liposomes, poly(lactic-*co*-glycolic acid), dendrimers, and so on, which can cause additional effects by their degradation products. However, it should not be forgotten that metal and metal oxide release ions which can interact with proteins and induce inflammation.[16] Nevertheless, the NPs mentioned in this review form a more homogeneous group than nanocarriers for drug delivery, which consist of different materials and possess different surface charges and functionalization. Polystyrene (PS) particles are included in this review because they are often used as model particles for nonbiodegradable NPs.[17]

Role of phagocytes in the immune system

Professional phagocytes are a group of immune cells that share the feature that they can ingest 0.5–10 µm sized particles better than epithelial cells. Since they are key players in the immune defense, they are represented in almost all organs.[18,19]

Mononuclear phagocytes are derived from myeloid progenitor cells in bone marrow and develop into granulocytes and monocytes. Monocytes circulate in the blood and differentiate into macrophages (Mϕ) in the tissue, where they reside as peritoneal Mϕ, alveolar Mϕ, mesangial phagocytes of the kidney, synovial type A cells, bone marrow stromal Mϕ, splenic red pulp and splenic white pulp Mϕ, osteoclasts in the bone, histiocytes in the connective tissue, and as microglia in the brain.[20] DCs are a specific lineage of monocytic phagocytes and are mainly present as myeloid and plasmacytoid DCs in the blood, as interstitial DCs in many organs, and as interdigitating DCs in the lymphatic organs. Based on the history of their discovery, some of them received specific names, such as the DCs in the epidermis (Langerhans cells) and Mϕs in the liver (Kupffer cells). Phagocytes express different surface markers and differ in their optimum size of phagocytosis. Peritoneal macrophages and monocytes in the peripheral blood optimally phagocytose 0.3–1.1 μm particles. The optimal size for phagocytosis by alveolar macrophages is 3–6 μm particles.[21–23] Granulocytes are classified into neutrophilic, eosinophilic, and basophilic granulocytes. The phagocytosis of invading pathogens is the main role of neutrophilic granulocytes. After self-destruction, they are the main component of pus. Compared to neutrophilic granulocytes, eosinophilic and basophilic granulocytes have only a low potential for phagocytosis and act mainly against pathogens by the release of enzymes, as well as toxic and proinflammatory substances.

Macrophages possess a variety of receptors for the binding of bacterial constituents (Figure 1). Complement C3b and the Fc fragment of immunoglobulin (Ig)G enable better uptake of opsonized particles. Distinct adhesion molecules, intercellular adhesion molecule (ICAM)-1 (CD54), ICAM-2 (CD102), lymphocyte function-associated antigen (LFA)-1 (CD11a), and LFA-3 (CD58), together with costimulatory molecules B7.1 (CD80), B7.2 (CD86), or CD40, and processed cytosolic proteins presented by major histocompatibility complex (MHC) I or extracellular proteins presented by MHC II, molecules activate T-cells.[24] Cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon (IFN)-gamma (IFN-γ), as well as their interaction with lipopolysaccharide (LPS)-binding protein, activate macrophages. Phagocytes ingest a variety of pathogens, such as bacteria, mycobacteria, virus, fungi, and nonpathogenic particles (for instance, dyes and dust) in an unspecific way. On the other hand, they fulfill a

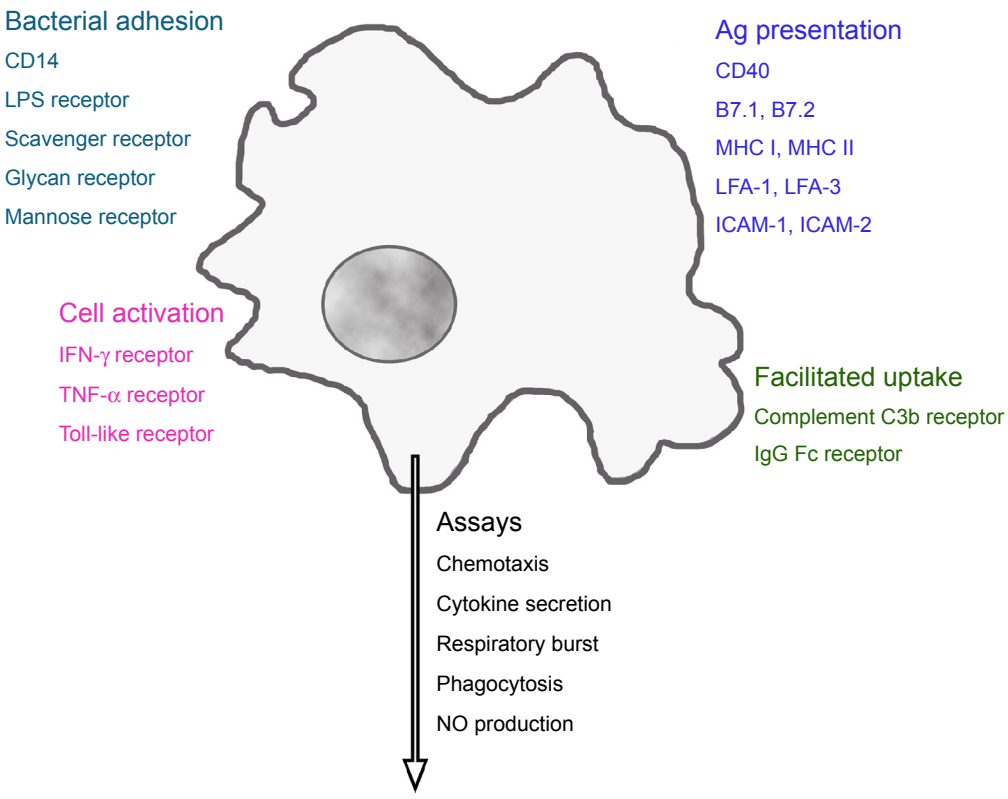

Figure 1 Receptors linked to main functions of phagocytes.
Note: Activation of these receptors regulates macrophage function, which can be evaluated by a panel of in vitro assays.
Abbreviations: CD14, lipopolysaccharide-binding protein receptor; LPS, lipopolysaccharide; IFN, interferon; TNF, tumor necrosis factor; MHC, major histocompatibility complex; LFA, lymphocyte function-associated antigen; ICAM, intercellular adhesion molecule; Ig, immunoglobulin; NO, nitric oxide.

definite function as antigen-presenting cells for the correct function of the specific immune system.

In vitro assays to study phagocyte function

A panel of in vitro assays of different complexities can assess phagocyte function. Cytokine secretion, chemotaxis, phagocytosis, and respiratory burst can be measured in all phagocytes. Nitric oxide generation is used only for monocytes and macrophages, whereas the detection of the release of myeloperoxidase and elastase is specific for neutrophilic granulocytes.[25] The evaluation of DC function is more complex because it requires interactions with T-cells. Cell isolation, cell exposure, and the detection platform for the performance of the respective assays are described in the core publication and in the supplements of *Current Protocols in Immunology*.[26]

Cytokine secretion

A wide spectrum of cytokines is being used in immunotoxicity studies, and phagocytes isolated from exposed animals or cultures of primary cells, and cell lines are equally suitable for these analyses.[27] In the presence or absence of the test substance, the release of cytokines/chemokines can be analyzed by enzyme-linked immunosorbant assays, enzyme-linked immunosorbent spot assays, antibody array assays, and bead-based assays. To identify proinflammation responses, interleukin (IL)-1, IL-6, IL-8, and TNF-α are routinely used.[28] The classification of allergic responses is based on the type of lymphocyte helper cells that are activated. IL-4 and IL-5 identify T_H2 responses, while marker cytokines for T_H1 responses are IFN-γ and TNF-β.

Chemotaxis

The migration of leukocytes from an upper chamber across a membrane to a lower chamber containing a chemoattractant is termed chemotaxis. Human serum-derived complement 5a, human lymphocyte-derived chemotactic factor, monocyte chemoattractant protein 1, or *N*-formyl-methionyl-leucyl-phenylalanine are commonly used attractants.[29] All leukocytes are able for chemotaxis, but monocytes, either as primary cells or as cell lines, are used most frequently. In conventional assays, membrane-containing inserts separating the upper from the lower chamber are used. The amount of cells that passed the membrane and reached the lower chamber is counted or quantified by viability assays. Alternatively, an impedance-based system (eg, xCELLigence and ECIS/Taxis) can be used.[30,31]

Phagocytosis

The phagocytosis assay evaluates the phagocytic activity of fluorescein-labeled bacteria (*Staphylococcus aureus, Escherichia coli*) in macrophages, monocytes, and polymorphonuclear neutrophils exposed to the test compound.[32]

Respiratory burst (reactive oxygen production)

This assay can be performed in macrophages, monocytes, and polymorphonuclear neutrophils by the detection of reactive oxygen species (ROS), which is produced upon phagocytosis. For the assays, mostly unlabeled *E. coli* is used as the phagocytic stimulus. Either chemiluminescent detection by lucigenin or the oxidation of dyes to fluorescent products (eg, rhodamine 123) can be employed for the quantification of the produced oxygen species.[33]

Nitric oxide (NO) generation

Murine macrophages are routinely used because, when compared to human monocytes, they possess a much higher production of NO.[34] An additional advantage of their use is that, in contrast to human macrophages, they do not need a differentiation step. Differentiation of monocytes with the commonly used phorbol 12-myristate 13-acetate or vitamin D3 cannot reproduce the phenotype of human macrophages in vivo, and it introduces additional variations in the assay.[35] The common and very reliable detection method of NO uses the Griess reagent.[36]

Release of elastase and myeloperoxidase

These enzymes are used as indicators for neutrophilic granulocyte activation.[37] Assays are performed in whole blood or in neutrophilic granulocytes isolated from peripheral blood. These cells only rarely show direct effects to conventional chemicals, but they are activated by particles.[38,39] The relevance of granulocyte activation for immunotoxicity in vivo, however, is currently unclear.

Function of dendritic cells

DCs for testing cannot be obtained directly from the blood in sufficient amounts, but they require differentiation in vitro. $CD14^+$ mononuclear cells isolated from peripheral blood mononuclear cells (PBMCs) are treated with recombinant (rh) granulocyte macrophage colony-stimulating factor and IL-4 for 7 days. Maturation to DCs induced by LPS in the presence and absence of the test compound is verified by the surface expression of CD80, CD83, CD86, and human leukocyte antigen-DR, and by the secretion of IL-12.

DC function requires a mixed lymphocyte culture, which analyzes the ability of T-cells to recognize allogenic cells as not belonging to the organism (nonself) as a result of the presence and proliferation of different MHC class II antigens on their surface. This assay is used to identify sensitizing agents. A DC to T-cell ratio of 1:100 is sufficient to initiate vigorous and optimal responses.[40] Splenocytes or lymph node cells from treated animals (responder cells) with genetically dissimilar cells (stimulator cells) are cocultured. The assay is usually performed in mice, where cells from another strain can be used as stimulators.[41] Stimulator cells are inactivated by irradiation or treatment with a DNA intercalating agent such as mitomycin C. After incubation for several days, proliferation of the responder cells is measured using ^{3}H-thymidine uptake.[42] The reaction can also be performed using human PBMC-derived DCs mixed with allogenic lymphocytes,[43] and the proliferation of the responder T-cells after contact with allogenic lymphocytes is assessed using a viability (formazan bioreduction) assay. Human myeloid leukemia-derived MUTZ-3 cells have the ability to differentiate into DCs,[44] and this assay is in the process of validation as an alternative to the in vivo identification of sensitizing agents.[45]

Specific issues in the assessment of NPs

The specific nature of NPs, mainly linked to their high surface reactivity, complicates their assessment by in vitro assays. The adsorption of molecules (either bacterial proteins or macromolecules from the body to the particle surface) holds importance for the in vivo and in vitro testing of phagocyte function.

In vivo and in vitro – binding of endotoxin

NPs may bind endotoxin, an LPS and pyrogenic compound of the wall of Gram-negative bacteria. Endotoxin is a strong stimulant of the immune response and causes a pyrogenic reaction in the human body.[46] Endotoxin contamination of metal and metal oxide NPs and CNTs is less expected because synthesis often includes steps that kill bacteria. However, contamination is often difficult to exclude because endotoxin can be present in distilled water.[47] Due to the strong stimulation of endotoxin, its presence in the sample does not allow for the identification of NP effects. The detection of endotoxin is usually achieved by evaluation in the limulus amebocyte lysate assay, one of the accepted alternatives to the in vivo endotoxin detection assays.[48] This assay can be performed in different formats, generally as clotting tests and by colorimetric detection.[49] Unfortunately, several NPs interfere with this assay. While for some NPs (TiO_2, Ag, $CaCO_3$, SiO_2 NPs), interference with the gel-clotting assay was more prominent,[50] for other particles (Au NPs), interference with the colorimetric limulus amebocyte lysate assay has been reported.[51] The release of inflammatory cytokines (IL-6, IL-8, IL-1) from PBMCs produced variable results and it has been suggested that NPs and endotoxin compete against each other in the induction of cytokines.[52]

In vivo and in vitro – protein corona

High surface activity leads to the binding of macromolecules to the particle surface once they get into contact with physiological solutions. This coating consists mainly of proteins and has been termed "protein corona".[53] It is hypothesized that the composition of the protein corona determines the trafficking and biological effects of NPs. For a description of the composition and variability of the protein corona, the reader is referred to reviews focusing on this topic.[54,55] The physicochemical parameters of the NPs and the composition of the biological fluid are the main factors determining the composition of the protein corona. As a general rule, hydrophobic particles bind more proteins than do hydrophilic particles,[56] and abundant proteins in the incubation solution are bound faster on the NP surface than the low abundant proteins.[57] Dependence on size and shape, as well as surface charge, has been reported in the following way: Au and SiO_2 NPs >10 nm bound more proteins than particles <10 nm; more proteins were attached to TiO_2 nanospheres than to nanorods and nanotubes; and binding to positively charged Au, PS, and carbon black particles was higher than to particles without charged groups.[58–62] While the composition of the inner coating (hard corona) appeared to be more stable, the composition of the outer part (soft corona) was dynamic and changed in its composition when the particle was transferred from one medium to the other.[63] The passage through various media left a fingerprint of the protein composition of the previous media on the NP.[64] NPs retained the protein corona during endocytosis; the coat was subsequently removed in lysosomes.[65]

The role of the protein corona composition for biological effects is still not entirely clear. The reduction of toxic effects, such as cytotoxicity and hemolysis, by protein coating of NPs has been observed in several studies of nonphagocytic cells.[66–70] This decreased effect was linked to reduced cellular uptake. Bovine serum albumin (BSA) bound to the surface of carboxyl-functionalized PS, quantum dot (Qdot), and Au NPs decreased cell uptake. The opposite was observed for BSA

bound to these types of NPs when they were functionalized with amine groups instead of carboxyl groups.[71] All BSA-coated NPs displayed the same effective surface charge, but apparently the BSA structure was influenced by the binding in such a way that different groups were visible for the cells. As a result, BSA-coated carboxylated NPs bound to the albumin receptor, while BSA-coated amine-functionalized NPs were ingested after binding to the cellular scavenger receptor.

Protein-coated NPs are expected to produce more pronounced immunological effects because coating with serum increased the uptake by phagocytes.[72] The secretion of proinflammatory cytokines by DCs was higher for spherical-than sheet-shaped ZnO NPs, which also bound more proteins on their surface.[73] While increased protein binding might have caused the higher secretion of cytokines, the opposite behavior has also been observed: coating of SiO_2 NPs with serum decreased cytokine secretion of murine macrophages.[74] The presence of complement in the protein corona plays a specific role because the binding of complement C3b and IgG increases uptake by phagocytes by binding to the complement and Fc-receptors. Responses to complement binding were variable; firstly, complement proteins could be activated or inactivated by the binding, and secondly, increased uptake could lead to the activation or inhibition of phagocytes.[75,76] Changes in protein conformation appear to be the reason for the different effects; binding of fibrinogen to negatively charged poly(acrylic acid)-conjugated Au NPs induced activation of the Mac-1 receptor on THP-1 monocytes, resulting in a proinflammatory response.[77] While these studies support a specific role of the bound proteins, other studies do not support the hypothesis of a protein corona-specific effect because the composition of the protein corona did not correlate with hemocompatibility.[78]

In vitro – cellular doses

Dose-dependent effects are more difficult to identify for NPs than for conventional compounds because cellular uptake is influenced by the diffusion and sedimentation of the single NPs and agglomerates of the NPs. Several mathematical models have been developed to calculate the deposition of particles suspended in liquids on adherent cells.[79,80] Particle-dependent minimal deposition was seen between 50–200 nm, while larger and smaller particles deposited at higher rates.[79] Small changes in the dispersion factor caused considerable variations in the deposited dose.[80] The differences are due to the formation of agglomerates, but the extent of agglomeration and its effect on deposition are difficult to quantify by mathematical models. The measured deposition of 50–1,000 nm plain PS particles on macrophages increased over time and showed a minimum for 100 nm particles.[17] Carboxyl PS particles of 20–1,000 nm showed the cellular uptake of 25%–40% in macrophages with a minimum at 100 nm.[81] The cellular dose of the same type of particles with sizes of 20–500 nm in endothelial cells increased from 4.6% to 28.4%, demonstrating higher particle uptake by phagocytic cells, as compared to nonphagocytic cells, in general.[82] When adherent cells were cultured upside-down, they ingested much less NPs than the cells cultured in the standard orientation.[83] Further complications arise when cells are exposed to aerosolized NPs because cell contact is dependent on the used exposure system, as well as on the variations in the size and concentration of the aerosol; great variations in deposition rates between 0.037% and 30% of the applied dose per well for different particles have been reported.[84–88] Furthermore, the influence on flow has to be considered when assessing NP uptake from the systemic blood circulation.[89] Endothelial cells best ingested Qdots and SiO_2 NPs at a shear stress of 0.05 Pa, which corresponds to postcapillary venules and peripheral arteries.[89]

In vitro – assay interference

The interference of NPs with several assay systems can strongly influence the results (Table 1). The absorbance of NPs could lead to false-negative results (absence of cytotoxicity is detected, although the NP is cytotoxic) because the metabolic activity (according to absorbance) is estimated to be higher than it actually is.[90] Enzyme inhibition by NPs could also cause false-negative results. Lactate dehydrogenase (LDH) is released into the supernatant of cells when the plasma membrane integrity is lost. Its enzymatic activity correlates to the amount of damaged cells. If LDH activity is inhibited by NPs, a lower degree of cell damage will be determined.

False-positive results (cytotoxicity is detected although the NP is not toxic) are detected when the fluorescent signals of dihydrofluorescein (the detection of oxidative stress) or of propidium iodide (the disruption of membrane integrity) are enhanced by NPs.[91–95] Depending on the assays used, the masking of toxic effects and the identification of nonexistent toxicity by NPs can occur simultaneously. Increased absorbance by colored NPs will result in a higher signal of LDH (indicating more dead cells) and in the MTT assay (indicating more viable cells). The use of multiple assays, therefore, helps to reveal assay interference. The addition of protein, mostly BSA, could prevent interference, but it also could increase it. While false-negative results by the inhibition of LDH activity by Si, Au, and CdSe NPs[96] was avoided, the addition of BSA caused false-negative effects in protein

Table 1 Mechanisms of interference between nonbiodegradable NPs and in vitro assays

Mechanism	Assay(s)	Particle(s)	Reference(s)
Absorbance	Hemolysis	Au, C_{60}	128–130
	LAL	Au, C_{60}	131
	MTT	Au, CB, C_{60}, Qdots, Ag, AgO, iron oxide, SWCNT	90, 92, 93, 129, 132–136
	WST-1	Iron oxide	137
	MTS	Al, Qdots	51, 97, 138
	LDH	Au, SiO_2, iron oxide, CeO_2	51, 98, 135, 139
Light scattering	MTT, ATP	Carbon nanodiamonds	140
Dye absorption	MTT	SWCNT, CB, Al, iron oxide	95, 141–144
	NR	CB, SWCNT, C_{60}, Si, TiO_2	90, 134, 141, 145–148
	AB, AK	SWCNT	146
	CB, WST-1	SWCNT	145
Enzyme inactivation/inhibition	LDH	Cu, Ag	149, 150
	LDH	ZnO	135
	LDH	Si, Au, Qdots	96
	LDH	Au	151
	AK	PS	105
Enzyme adsorption	LDH	Cu, Qdots, TiO_2	93, 95, 133, 149, 152, 153
Reduction/enzymatic activity	MTT	Si, SWCNT, C_{60}, TiO_2	91, 95, 129, 145, 148, 152, 154, 155
	AB	Si	148
	AK	SWCNT	146
Prevention of reduction	MTT	Zn	93
Oxidation	H_2DCF	CB	135
	Hemoglobin	Iron oxide	128
Protein binding	Cytokines	CB, SWCNT, iron oxide, Cu, SiO_2, Al_2O_3, CeO_2, NiO_2, TiO_2	93, 135, 141, 146, 156–159
Increase enzymatic activity	LAL	Iron oxide	131
	Hemoglobin	PS, SWCNT	129, 146
Physical interaction	TB, calcein AM, live/dead	SWCNT, C_{60}, CB, Qdots	90
	COMET	Ge	160
Quenching of fluorescence	H_2DCF	CB, SiO_2, SiO_2-iron	161, 162
	LDH	CB, SWCNT, C_{60}, Qdots	90
	Calcein AM/EthD-1	CB, SWCNT	90
Increase of fluorescence	H_2DCF	Au, iron oxide, TiO_2, C_{60}, SiO_2, CB, SWCNT	91–95
	PI	Qdots, PS	95
	Calcein AM/EthD-1	CB, iron oxide	91
	AB	CB, SWCNT, TiO_2, Qdots	90, 97, 145
	Resazurin	CoO	51
	COMET	TiO_2, CuO	163
Increase of luminescence	Phagocytosis	Qdots	130
	ATP	SiO_2	139
Quenching of luminescence	Phagocytosis	Au	129, 131
Aggregation	Platelet aggregation	Au, C_{60}	129

Abbreviations: NPs, nanoparticles; Au, gold; C_{60}, C_{60} fullerenes; LAL, limulus amebocyte lysate; CB, carbon black; Qdots, CdSe quantum dots; Ag, silver; SWCNT, single-walled carbon nanotube; WST-1, water soluble tetrazolium salt; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); Al, aluminum; LDH, lactate dehydrogenase; SiO_2, silica; CeO_2, cerium oxide; ATP, adenosine triphosphate; NR, neutral red; TiO_2, titanium dioxide; AB, alamarBlue; AK, adenylate kinase; Cu, copper; ZnO, zinc oxide; PS, polystyrene; Zn, zinc; H_2DCF, dihydrodichlorofluorescein; Al_2O_2, aluminum oxide; NiO_2, nickel oxide; TB, trypan blue; AM, acetoxymethylester; Ge, germanium; EthD-1, ethidium homodimer 1; PI, propidium iodide; CoO, cobalt oxide; CuO, copper (II) oxide.

detection via the Bradford reagent.[97] For the identification of potential assay interference, the incubation of NPs with the assay compounds alone (in the absence of cells) and with cells alone (in the absence of assay compounds) can be used. These controls are, however, only useful when the NPs interact with assay compounds and with the readout; assay interactions by Au NPs, which increased the detected amount of dead cells by shuttling the indicator dye, propidium iodide, into the cells, would not have been revealed.[98,99] Similarly, the more global effects of NPs on cultured cells, such as the depletion of nutrients by SWCNTs,[100] would go unnoticed. Interference can show dose dependency; dye (acridine) fluorescence is increased by low concentrations of Ag NPs and quenched at high concentrations of NPs.[101]

Some general rules may help identify and prevent the false interpretation of results. The use of low NP concentrations reduces the problem of interference, but the removal of NPs by centrifugation is generally not recommended because analytes adsorbed to the particles might be removed. Assay interference of the colored CNTs, carbon black, C_{60} fullerenes, and Au NPs, and of the fluorescent Qdots, may occur more frequently than interference with noncolored Si, SiO_2, TiO_2, and ZnO NPs. Testing of NPs with several assays based on different detection methods can reduce the risk of misinterpretation.[90,102] In this regard, immunotoxicity testing poses more problems than cytotoxicity testing because a lower number of assays for a given immunological function are usually available. On the other hand, compared to cytotoxicity testing, NPs are usually studied at much lower NP concentrations, reducing the risk for interference.

Immunotoxicological data from NP exposure

In vivo exposure includes voluntary inhalation and oral application, forced inhalation (intranasal and intratracheal instillation, oropharyngeal administration), forced oral (intragastric/gavage) application as well as noninvasive dermal and invasive (intradermal injection) dermal applications. Parenteral applications include intravenous and intraperitoneal injection. Table 2 shows the general reaction pattern of the immune system after in vivo exposure to NPs.

Systemic immune effects

Effects in the respiratory tract with only a thin epithelium were more pronounced than effects after dermal or oral ingestion exposure, where a horny layer or a thick mucus layer separated NPs from epithelial and immune cells.[103]

Inflammation in the lung is one of the most frequently reported effects of respiratory exposure to NPs.[104] Since cytokines are produced by several cell types, it is not clear whether the reported increases in cytokine secretion and subsequent inflammation were due to specific activation of immune cells, or if they were a consequence of cytotoxic action on alveolar epithelial cells. Heavy metal-containing NPs reacted in a similar manner as PS particles.[105,106] Given that heavy metal-containing NPs show ROS generation, and since they are expected to have greater cytotoxicity, the similarity of the reaction does not support the hypothesis of cell death (induced by more cytotoxic heavy metal-containing NPs) as a main inductor of inflammation.

Only a few studies have reported the absence of immunological effects, which could be due to restricted access to

Table 2 Effects of NPs after inhal, IN, IG, IP, IV, oral, oroph, and SC application, and ID and IT in normal animals and in animal models (Model)

Particle	Size (nm)	Application	Model	Effect	Reference(s)
Polystyrene	25–50	IT		Lung inflammation	164
	68	IT	Coupled antigen	Stimulation of antigen response	165
	40	ID	OVA	Increased sensitization	166
	58	IT	OVA	Increased sensitization	167
	100	SC	OVA	Increased sensitization	168
	20, 50, 100	ID	Atopic dermatitis	Aggravation of inflammation	169
SWCNT	1–2	Oroph		Decreased lung clearance of bacteria	170
	1–2	IT		Proinflammatory cytokine secretion	171
	1–2	IT	OVA	Increased sensitization	172
	1–2	Inhal, SC	OVA	Increased sensitization	113
	1–2	IT	LPS	Aggravation of inflammation	173
DWCNT	2	IN		Proinflammatory cytokine secretion	174
MWCNT	20–30	SC		Proinflammatory cytokine secretion	175
	10–20	Inhal		Proinflammatory cytokine secretion	176
	12.5–25	Oroph		Lung inflammation	177
	20–30	IT		Proinflammatory cytokine secretion	178
	25	IV		Proinflammatory cytokine secretion	179
	90	IP		Proinflammatory cytokine secretion	180
	67	IT	OVA	Increased sensitization	181
	10–50	Inhal, SC	OVA	Increased sensitization	113
	30–50	Inhal	OVA	Increased sensitization	182
	67	IT	LPS	Aggravation of lung inflammation	173
	10–50	IT	LPS	Aggravation of inflammation	183
Ag	10	ID		Erythema	8

(*Continued*)

Table 2 (*Continued*)

Particle	Size (nm)	Application	Model	Effect	Reference(s)
	18	Inhal		Lung inflammation	184
	18	Inhal		Lung inflammation	185
	20	IV		Suppressed immune response to KLH immunization	187
	22, 42, 71	Oral		Increased TGF-β levels	186
	52	IT		Proinflammatory cytokine secretion	12
	33	IN	OVA	Increased sensitization	188
Au	50	IT		Lung inflammation	189
	50, not 10	IP		Proinflammatory cytokine secretion	190
	21	IP		Anti-inflammatory action in adipose tissue	108
	15	Oroph	TDI	Aggravation of asthma	191
	5, 15	IP	IL-1β inflammation	Decrease of inflammation	109
Iron oxide	5.3	IT		Lung inflammation and allergic response	193
	20	IV		Proinflammatory cytokine secretion	194
	36	IT		Lung inflammation and cytokine secretion	192
	58	IV		Decreased OVA-specific antigen production	195, 196
	43	IT	OVA	Increased sensitization	197, 198
	35	IT	OVA	Increased sensitization	199
SiO_2	10	IT		Lung inflammation	200
	12	IP		Proinflammatory cytokine secretion	13
	30, 70	IP		Proinflammatory cytokine secretion	201
	15	IV		Proinflammatory cytokine secretion	202
	70	IV		Proinflammatory cytokine secretion	203
	30, 70, 100	ID	Atopic dermatitis	Aggravation of inflammation	204
	10–20	IT	OVA	Increased sensitization	205
TiO_2	2–5	Inhal		Lung inflammation	207
	5	IP		Proinflammatory cytokine secretion	215
	5.5	IG		Infiltration of immune cells in spleen	213
	8–10	IN		Lung inflammation	206
	20	ID, not oral		Immune activation	8
	20	Inhal		Lung inflammation	208
	20	IT		Lung inflammation	209
	25	IT		Proinflammatory cytokine secretion	210
	25	IT		Lung inflammation	211
	15, 28	IT		Lung inflammation	212
	30–40	IT		Lung inflammation	200
	66	Oral		Proinflammatory cytokine secretion	14
	20	IG		Proinflammatory and allergic cytokine secretion	214
	14, 29	Inhal	OVA	Increased sensitization	217
	15	Oroph	TDI	Aggravation of asthma	191
	20	IP	LPS	Aggravation of lung inflammation	218
	28	Inhal	OVA	Increased sensitization	216
ZnO	10	Inhal		Lung inflammation	219
	<10	IT		Lung inflammation	200
	21	Oral		No effect on oral tolerance to OVA	107
	21	IP	OVA	Increased sensitization	114
	55	IP	OVA	Increased sensitization	115

Abbreviations: NPs, nanoparticles; inhal, inhalation; IN, intranasal; IG, intragastral; IP, intraperitoneal; IV, intravenous; oroph, oropharyngeal; SC, subcutaneous; ID, intradermal; IT, intratracheal instillation; OVA, ovalbumin; SWCNT, single-walled carbon nanotube; LPS, lipopolysaccharide; DWCNT, double-walled carbon nanotube; MWCNT, multiwalled carbon nanotube; Ag, silver; TGF, transforming growth factor; KLH, keyhole limpet hemocyanin; Au, gold; TDI, toluene diisocyanate; IL, interleukin; SiO_2, silica; TiO_2, titanium dioxide; ZnO, zinc oxide.

immune cells.[107] The absence of immune effects after the oral ingestion of and exposure to ZnO and TiO_2 NPs could be explained by the hindered assessment of the particles to the cells by mucus.[8,107] On the other hand, the low reactivity of intraperitoneally applied Au NPs appears to be due to their high biocompatibility given that few studies have reported on the adverse cellular effects of Au NPs.[108,109] This statement is supported by a lack of immunological interference in the cellular assays showing no increased cytokine secretion,[110,111] and no effect on DC maturation and activation.[94,112]

When NPs were applied to diseased animals, the pathology of the disease was aggravated. This aggravation was seen

in asthma models, as well as in atopic dermatitis (Table 2). Aggravation of asthma is unlikely to be caused by cytotoxicity of the NPs because exposure by the respiratory tract and by other routes (subcutaneous, intraperitoneal), where no direct contact with the alveolar epithelium occurred, caused the same effects.[113–115] The mechanisms for amplifying pre-existing pathologies have been proposed through the following mechanisms:[116] pre-existing inflammation in the respiratory tubes could be amplified by enhancing the levels of inflammatory factors or humoral immunity. Second, NPs within the size range of <100 nm were able to stimulate and enhance hypersensitivity, which is primarily mediated by Th2 cells.[116]

In vitro and ex vivo effects

Phagocyte function after in vitro (cells exposed in wells) and ex vivo (cells harvested from exposed animals) exposure is summarized in Table 3. To evaluate the potential of screening in phagocytes, first, data obtained from ex vivo and in vitro studies have to be compared. Second, the similarity of ex vivo and in vitro exposures to in vivo exposure has to be tested. In vitro data on cytokine secretion and chemotaxis corresponded to the respective ex vivo data (Table 3). NPs showed a similar pattern of interference with phagocyte functions; proinflammatory cytokine secretion (mostly IL-6, IL-1β, and TNF-α) and respiratory burst increased, while phagocytosis and chemotaxis decreased. The degranulation of neutrophilic granulocytes has been shown for a few particles.[81,117] The influence on DC maturation and function varied markedly between the particles. MWCNTs inhibited maturation, Au and iron oxide showed no prominent effect, and SiO_2 and TiO_2 activated DCs.[94,112,118,119] The different results could be due to the use of different readouts (maturation and activation).

Table 3 Immune effects in isolated phagocytes, either after in vivo treatment with nanoparticles (ex vivo) or by in vitro treatment

Particle	Effects	
	Ex vivo	**In vitro**
Polystyrene		Proinflammatory cytokine secretion[81]
		Increased respiratory burst[81]
		Neutrophilic granulocyte activation[81]
MWCNT		Proinflammatory cytokine secretion[220]
		Inhibition of DC maturation[118]
SWCNT		Decreased chemotaxis[221]
		Decreased phagocytosis[221]
Ag	Proinflammatory cytokine secretion after IT application[8]	Proinflammatory cytokine secretion[222–224]
	Proinflammatory cytokine secretion after inhalation[225]	Decreased phagocytosis[12]
	Proinflammatory cytokine secretion after oropharyngeal application[226]	Increased respiratory burst[227]
	Proinflammatory cytokine secretion after oral application[186]	Decreased NO production[228]
		Neutrophilic granulocyte activation[117]
Au		Proinflammatory cytokine secretion[112,229,230]
		No increased cytokine secretion[110,111]
		No effect on DC maturation, no activation[94,112]
Iron oxide	Proinflammatory cytokine secretion after IT application[199,231]	Proinflammatory cytokine secretion[232]
	Upon LPS challenge, decreased cytokine secretion after IT application[233]	Decreased phagocytosis[234]
		Increased NO production with and without LPS challenge[233,234]
		No effect on DC maturation[94]
SiO_2	Increased NO production after IT application[235]	Proinflammatory cytokine secretion[13,236,237]
		Activation of DC[119]
TiO_2	Proinflammatory cytokine secretion after IT application[235,238,239]	Proinflammatory cytokine secretion[240]
	Proinflammatory cytokine secretion after IG application[10]	Decreased chemotaxis[241]
		Decreased phagocytosis[12]
	Increased NO production after IT application[235,239]	Increased respiratory burst[242]
	Decreased chemotaxis after IT application[235,239]	Activation of DC[119]
ZnO	Decrease of cytokine secretion after oral application[116]	Proinflammatory cytokine secretion[243–245]
	Proinflammatory cytokine secretion after IT application[199]	Decreased chemotaxis[241]
	Proinflammatory cytokine secretion after inhalation[11,219]	Decreased phagocytosis[12]
		Increased respiratory burst[246]

Abbreviations: MWCNT, multiwalled carbon nanotube; DC, dendritic cells; SWCNT, single-walled carbon nanotube; Ag, silver; IT, intratracheal instillation; NO, nitric oxide; Au, gold; LPS, lipopolysaccharide, SiO_2, silica; TiO_2, titanium dioxide; IG, intragastral; ZnO, zinc oxide.

The secretion of proinflammatory cytokines was increased by all NPs when applied by in vitro exposure, and after the ex vivo respiratory exposure, to NPs. The lower sensitivity of phagocytes by the oral route was confirmed in an ex vivo study.[120]

Uptake of NPs by phagocytes

When NPs are coated with proteins in biological fluids, they are well ingested by phagocytes.[121] Phagocytosis of NPs by primary cells, cell lines, macrophages, monocytes, and monocyte-derived macrophages indicated accumulation in the MPS and showed a good correlation to the accumulation of particles in the MPS of the spleen and liver in vivo.[122] Due to the crucial function of macrophages and DCs in the specific immune response, the accumulation of NPs in the MPS could result in immunotoxicity. The indication of uptake by the MPS or accumulation in lymphatic organs, however, was not correlated to adverse effects on the immune system in vivo or in vitro.[81,123] Accumulation in the spleen was only observed for 30 nm Au particles, while adverse effects on the immune system according to increases in relative spleen weight and immune cell numbers were seen for 5 nm, 10 nm, and 60 nm Au particles.[123] Small carboxyl PS particles were ingested in much higher numbers than 1,000 nm particles by macrophages.[81] While the 1,000 nm large particles induced oxidative burst and cell damage, particles in the size range between 40 nm and 500 nm were taken up without obvious interference with cell viability and function. Taken together, these data suggest that the uptake of NPs may not result in impaired phagocyte function.

Guidelines for sample preparation and exposure

Physiologically relevant testing is based on sample preparation, as well as on the use of dispersant and intended exposure routes. Most NPs form stable solutions in distilled water, which cannot be used for in vitro studies. The presence of ions and protein in the physiological solution leads to NP agglomerates, which may increase in size, but they may also disintegrate. The surface coating of NPs determines their penetration of barriers, cellular uptake, and immune response.[124] The Office of Economic Co-operation and Development (OECD) guidelines for sample preparation and dosimetry had advised that dose should be indicated in terms of mass, surface area, and particle number at a minimum.[125] To get information on the stability of the dispersion, repeated measurements are recommended with the documentation of agglomeration and ion release. The dispersants should

Table 4 Overview of nanoparticle actions on phagocyte functions

Particles	Cytokine secretion		Chemotaxis		Phagocytosis		Respiratory burst		NO generation		Degranulation		DC maturation	
	Ex vivo	In vitro	Ex vivo	In vitro	Ex vivo	In vitro	Ex vivo	In vitro	Ex vivo	In vitro	Ex vivo	In vitro	Ex vivo	In vitro
Polystyrene		←						←				←		
MWCNT		←											→	
SWCNT				→		→								
Ag	←	←				→		←		→		←		
Au		←											–	
Iron oxide	←	←				→				←			–	
SiO_2		←							←				←	←
TiO_2		←	→	→		→		←	←					
ZnO	← →	←		→		→		←						

Notes: Red arrow: increase; black arrow: decrease; –: no change.
Abbreviations: NO, nitric oxide; DC, dendritic cell; MWCNT, multiwalled carbon nanotube; SWCNT, single-walled carbon nanotube; Ag, silver; Au, gold; SiO_2, silica; TiO_2, titanium dioxide; ZnO, zinc oxide.

preferentially contain macromolecules that are present in the target tissue. For exposure with aerosols, and in addition to the NP parameters, the mass median aerodynamic diameter and aerosol concentration should be determined. Guidelines for sample preparation for nanoscale TiO_2 are already available,[126] and existing guidelines for exposure by spontaneous inhalation, oral gavage, and dermal application are applicable for NP exposure. Moreover, the additional effects of intravenous exposure (for instance, behavior in the syringe) have to be considered.

Freshly prepared solutions from stock solutions prepared in water, diluted in cell culture medium, and treated by sonification should be added to the cells. In the case that no route-specific surfactants, such as 1,2 dipalmitoyl-*sn*-glycero-3-phosphocholine for pulmonary exposure, are used, BSA appears to be a good choice because this zwitterionic molecule prevents the binding of protein from the solution.

Conclusion

Due to the complexity of the immune system, in vivo testing will remain the gold standard. However, intraindividual variations in the immune system, as well as its compensatory abilities, are major limitations. As has been observed in environmental studies of airborne particles, individuals with impaired immune function were affected by small particle doses, while no effects were observed in the healthy population.[2–5]

This overview on a variety of carbon, metal, and metal oxide NPs shows that these particles caused relatively similar patterns of immunotoxicity in vivo, which involved inflammation and immunosuppression in healthy animals and aggravation of the pathology in animals with pre-existing diseases. This suggests that the classification of particles as more or less immunotoxic by in vitro screening might be helpful. The extent to which such screening could lead to valid results was studied by comparing data obtained by in vivo exposure, in vitro testing and in vitro data (Table 4). This analysis showed that the results obtained in cells isolated from NP-exposed animals were similar to the data obtained of cells, which were exposed to NPs in vitro. Secondly, NPs that inhibited phagocyte functions in vitro reacted in an immunotoxic manner in vivo (Tables 2 and 4). The data suggest that the in vitro testing of phagocytes might predict the typical immunotoxicity pattern of NPs in vivo. Cellular assays may also be suitable to identify disease-related alterations in the immune reaction to NPs because comparison between reactions of PBMCs from healthy and allergic donors showed that the cells exhibited disease-related differences upon challenge.[118]

Due to the specific composition of the immune system at different portals of entry, exposure-specific coculture models including immune cells could serve as a possibility to assess immunotoxicants in vitro. Alveolar epithelial cells and alveolar macrophages in cocultures released inflammatory cytokines at lower concentrations of TiO_2 NPs than did the respective monocultures.[127] At the expense of greater complexity, these systems could increase the sensitivity of immunotoxicity in vitro screening and enable exposure-specific testing. However, until a correlation of these findings in these systems to data obtained in humans has been shown, their value remains elusive.

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Disclosure

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