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Cytokines as biomarkers of nanoparticle immunotoxicity

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Abstract

Nanoscale objects, whether of biologic origin or synthetically created, are being developed into devices for a variety of bionanotechnology diagnostic and pharmaceutical applications. However, the potential immunotoxicity of these nanomaterials and mechanisms by which they may induce adverse reactions have not received sufficient attention. Nanomaterials, depending on their characteristics and compositions, can interact with the immune system in several ways and either enhance or suppress immune system function. Cytokines perform pleiotropic functions to mediate and regulate the immune response and are generally recognized as biomarkers of immunotoxicity. While the specificity and validity of certain cytokines as markers of adverse immune response has been established for chemicals, small and macromolecular drugs, research on their applicability for predicting and monitoring the immunotoxicity of engineered nanomaterials is still ongoing. The goal of this review is to provide guidelines as to important cytokines that can be utilized for evaluating the immunotoxicity of nanomaterials and to highlight the role of those cytokines in mediating adverse reactions, which is of particular importance for the clinical development of nanopharmaceuticals and other nanotechnology-based products. Importantly, the rational design of nanomaterials of low immunotoxicity will be discussed, focusing on synthetic nanodevices, with emphasis on both the nanoparticle-forming materials and the embedded cargoes.

1. Introduction

Nanomedicines are emerging as potential therapeutics and diagnostics for a wide variety of diseases, and have also found uses in vaccine development, engineering, and materials science applications.[1–4] As a “depot” for various cargoes, they have been successfully used for delivery of hydrophobic and hydrophilic small molecule drugs (*e.g.* anticancer drugs) and biomacromolecules, such as recombinant proteins, enzymes, hormones, peptides, and monoclonal antibodies. In addition, they have been used to deliver nucleic acids of various sizes and structures.[5–8] The macro- and ultra-structures of these nanosized materials can be tailored to accommodate particular therapeutics and to protect them against hydrolytic or enzymatic degradation, provide the appropriate environment for solubility and for gated

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drug release. In addition, they can be equipped with “smart” components (antibodies, peptides, proteins, sugars, aptamers, *etc.*) to aid in their delivery to target organs, tissues and subcellular compartments.[9] Several nanomedicine products are already on the market and in different phases of clinical trials, and many more are still under rigorous investigation, optimization and screening to select the most promising candidates for therapeutic and diagnostic applications.

Nanoparticles can interact with various components of the immune system and either enhance or inhibit its function.[10–13] Modulation of the immune function by nanomaterials can be useful or detrimental, depending on the intended use.[11] Nanoparticles can be designed to be immunomodulatory to serve specific functions (*e.g.* vaccine adjuvants, anti-inflammatory, immunosuppressive drugs). Concerns are raised, however, when an engineered nanomaterial not intended for interaction with the immune system alters its function. It has been established that certain nanomaterials can be immunotoxic, although no standard immunotoxicity assay has been described thus far that is specific to their nano size.[10–15] It is generally agreed that the same set of immunological studies routinely used to assess immunotoxicity of chemicals, medical devices and drugs can be applied to engineered nanomaterials.[13]

Cytokines are proteins produced by various types of cells including immune cells in response to activation. They play a pivotal role in homeostasis by both modulating and regulating immune response. Cytokine functions are pleiotropic, in that they perform multiple actions and often overlap, acting synergistically or antagonizing each other. This is why cytokine interactions are often referred to as a network. Cytokine release can be characterized by fever, hypotension, nausea, headache, chills, vomiting, and muscle pain, and is the cause of infusion reactions commonly associated with antibody-based biotherapeutics[16] which, in some cases, may be life-threatening.[17] Hence, it became a common practice in the pharmaceutical industry to monitor cytokines in preclinical studies to understand, prevent and control undesirable cytokine responses to biotherapeutics.

Since nanoparticles can interact with proteins, and proteins, including antibodies, are often used to target nanoparticles to specific cells and tissues, understanding the use of cytokines as biomarkers of undesirable immunostimulation associated with engineered nanomaterials is emerging as an essential component of nanoparticle safety testing. Evaluation of the immunotoxicity of nanomaterials by measuring the levels of cytokines, in particular the proinflammatory cytokines can be useful tools in evaluating nanoparticle immunotoxicity. High levels of cytokines upon treatment with nanoparticles are usually associated with toxicity, adverse reactions and low therapeutic efficacy, as will be discussed later. Hence, cytokines might be utilized to partially predict the nanoparticle immunotoxicity.

Here, we review the possible interactions between the various components of nanomaterials and the immune system with respect to induction of cytokines. We highlight studies demonstrating the utility of cytokines as biomarkers of both desirable immunostimulation and undesirable immunotoxicity of engineered nanomaterials. In addition, the rational design of nanomaterials with low immunogenicity and high therapeutic efficacy is

discussed, with special attention to chemical modifications to both drugs and the nanoparticle-forming constituents utilized for their delivery.

2. The immune system

2.1 Structure of the immune system

The immune system has a complex architecture comprised of various organs and cell types that interact and communicate *via* chemical conductors to orchestrate an immune response towards a particular event (Box 1 and Figure 1). The parts of the immune system are connected *via* the blood and lymphatic circulatory systems. Bone marrow, thymus, spleen, lymph nodes and mucosa-associated lymphoid tissues are the main organs of the immune system, which are involved in the manufacturing, maturation, differentiation, proliferation and storage of immune cells. The blood is composed of red blood cells and white blood cells, which are suspended in the blood together with other molecules, such as, various complement proteins and immunoglobulins. White blood cells (leukocytes), which play the major role in the immune system, are made up mainly of polymorphonuclear granulocytes (PMN), in addition to monocytes, natural killer cells, B and T lymphocytes. PMN are composed mainly of neutrophils (phagocytic cells), along with eosinophils and basophils. T and B lymphocytes, natural killer cells and PMN are the main cells of the immune system. In addition, dendritic cells and macrophages are essential parts of the immune system that act as scavengers or antigen-presenting cells (APCs). Mononuclear phagocytic system (MPS) is the name given to the part of the immune system that consists of phagocytic cells, such as blood monocytes and macrophages accumulated in lymph nodes, liver, spleen and other tissues (Box 1).

Innate and adaptive immunity refer to in-born and acquired immune defense lines, respectively. Innate immunity is rapid and provides a first line defense, while an adaptive response is more involved and takes more time. Phagocytic cells, NK cells and secondary messenger molecules (*e.g.* cytokines, eicosanoids, prostaglandins) produced in response to a pathogen challenge, as well as the complement system, are the major players in an innate immune response. The two main types of adaptive immune responses are cell-mediated and humoral- or antibody-mediated immunity, and they are mediated mainly by T and B lymphocytes. The interaction between innate and adaptive immunity is regulated through mediator molecules such as complement proteins and cytokines.

Phagocytic cells (*e.g.* monocytes, neutrophils, dendritic cells, B cells, platelets and macrophages) can be found in the blood, skin, mucous membranes and in various organs, such as, the liver, spleen, lymph nodes, lung and brain (Box 1). Some of them (*e.g.* monocytes and neutrophils) roam through the body and act as scavengers to attack and engulf foreign particles, remove pathogens, old or dead cells, and synthesize complement proteins and cytokines that are essential for synchronization of the various parts of the immune system. These cells may attack and engulf particles that are tagged by opsonins (adsorbed molecules that enhance or accelerate clearance by the immune system) or non-opsonized particles.

APCs engulf and digest foreign antigens and present fragments of the antigens on their surface-bound receptors, major histocompatibility complexes (MHC), to other cells of the immune system such as T cells and B cells. There are two types of MHC: MHC I that are found in most of the body tissues, and MHC II that are only present in the APCs. The MHC II receptors wrap the antigen and present it to T cell receptors (TCR), resulting in activation of T cells. T lymphocytes confer cell-mediated immunity and cooperate with B cells, enabling them to secrete antibodies for specific antigens. T lymphocytes include T helper (T_H) cells, suppressor T cells and cytotoxic T cells, with the categorization depending on the expressed surface protein. T_H cells express CD3 and CD4, whereas the suppressor and cytotoxic T cells express CD3 and CD8 receptors. The T cells also express other receptors. For example, TCR, which can identify a broad range of specific antigens presented on MHC molecules. The $CD4^+$ cells recognize antigens presented on the MHC II by the APCs, whereas $CD8^+$ cells recognize antigens on the MHC I. $CD4^+$ cells are T_H0 cells that differentiate into either T_H1 or T_H2 cells. T_H1 cells participate in cell-mediated immunity and regulate the steps of inflammation “T inflammatory cells”, while T_H2 cells induce proliferation of mast cells and eosinophils and favor the differentiation of B cells to produce IgG and IgE, thereby promoting humoral immunity.[18,19] T_H1 cells secrete large quantities of interferon (IFN)-γ, interleukin (IL)-2, IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF) and small quantities of tumor necrosis factor (TNF). T_H2 cells secrete large quantities of IL-3, IL-4, IL-5, IL-6, IL-10, and small quantities of GM-CSF and TNF. The cytokines produced by specific T_H cells (*e.g.* IFN-γ from T_H1 cells and IL-10 from T_H2 cells) usually inhibit the action of other type of T cells and thereby potentiate a particular pattern of immune response. IFN-γ and IL-4 are the main markers of T_H1 and T_H2 cells, respectively. It is accepted that the main function of T_H1 cells is to fight viruses and intracellular pathogens, while that of T_H2 cells is controlling humoral response and extracellular pathogens. More recently, other subsets of T cells were discovered which are distinct from T_H1 and T_H2 cells. They are T_H9 cells, which produce IL-9 and are involved in fighting parasites, T_H17 cells, which produce IL-17 and are involved in autoimmune response, regulatory T cells (Tregs), characterized by the presence of FoxP3 and antigen-experienced follicular helper T cells, identified by expression of CXCR5.[20,21] The TCR and B cell-bound antibodies recognize antigens and initiate an immune response by recruiting other immune cells, cytokines and complement proteins.

2.2 Proinflammatory cytokines

The T_H1/T_H2 hypothesis, proposed more than 20 years ago, suggested that naïve $CD4^+$ T cells can differentiate into distinct subsets performing various functions to protect the host from certain pathogens.[22] The initial studies which provided the background for the T_H1/T_H2 hypothesis were performed in mice and were later adopted to human cells. The balance between the T_H1 and T_H2 cytokines was thought to be clinically significant. Some studies have suggested that it should be evaluated[23], because this balance is also of particular importance to prevent the occurrence of several diseases (*e.g.*, arthritis, diabetes, asthma, cancer).[23–28] However, not all immune responses can be described through T_H1/T_H2 theory. For example, some substances (*e.g.* omega-3 fatty acids) have considerable effects on inflammatory and autoimmune conditions without significant shift in T_H1/T_H2 balance, while other substances (*e.g.* melanine, probiotics and zinc) affect T_H1/T_H2 balance

but do not cause inflammatory and autoimmune diseases.[26] The limitations of this theory were further clarified by the discovery of other types of T effector cells (T_H9, T_H17, Tregs). However, in spite of this, the type of immunostimulation and its outcome to the host is still often judged by the T_H1 and T_H2 type cytokines, and most commercially available assays are aimed at the T_H1/T_H2 panel.

Many cytokines including IL-1, IL-6 and TNF-α activate functions of inflammatory cells during acute inflammatory responses. These cytokines increase the vascular permeability and thus cause swelling and redness associated with inflammation. IL-1 and IL-6 are responsible for fever reactions, TNF-α stimulates endothelial cells and is responsible for hypotension, IL-8 is a chemokine which plays a key role in the activation of neutrophils and their recruitment to the site of inflammation. Many cytokines act together to initiate and regulate the inflammation process. For example, IFN-γ plays a significant role in the inflammatory process, as it can attract macrophages to sites where antigens are present.[29] Although T_H cells are the major source of cytokines that regulate the immune response, other cells, such as keratinocytes, can produce cytokines that serve as mediators of inflammatory and immunologic reactions in skin exposed to irritants.[30–32] These cytokines can also affect the proliferation and differentiation of keratinocytes and control the production of other cytokines.[33] These proinflammatory cytokines have been well studied and characterized as participants in the basic inflammatory process and mediators of cellular infiltration.[30–32,34–36]

3. Nanomaterials

The structure and composition of nanoparticles can be tailored to carry drugs of various sizes and solubilities (Box 2 and Figure 2).[4] Nanoparticles can also be engineered to carry charged macromolecules (*e.g.* amino acids, nucleic acids or enzymes). Attachment or encapsulation of these drugs into nanoparticles can improve their stability, protect them from systemic exposure and recognition by the immune system and control their release. For certain types of engineered nanomaterials, coating with hydrophilic polymers may impart further protection against the external environment and increase blood circulation times. The coating is a non-ionic hydrophilic flexible shell which prevents the adsorption of opsonins, thereby limiting uptake by the MPS and prolonging the circulation half-life of the encapsulated drug. Prolonged circulation times allow for passive targeting of the nanoparticles into tumors *via* the enhanced permeation and retention (EPR) effect (Figure 3). The EPR effect is explained by the leaky vasculature and impaired lymphatic drainage at tumor sites, which results in the deposition of colloidal particles in tumor peripheries. Once deposited in tissues, the hydrophilic corona facilitates transport of the nanomedicine through the extracellular matrix. Nanoparticles have also been decorated with targeting ligands to allow specific cellular uptake or direct the delivery system to a diseased site, and to circumvent various physiological barriers (Figure 3).[2] They can also be equipped with pH- or thermo-responsive (*i.e.* smart) components to allow release of the drug only in diseased tissues, thereby reducing the toxicity to healthy tissues.[4,38]

Nanoparticles of different structures and compositions have been developed for biomedical delivery applications, such as, polymeric, lipidic, metallic and graphite-based nanoparticles

(Box 2 and Figure 2).[1,4,39–42] Much focus has been given to the design of nanoparticles with tailored properties that can accommodate drugs of various sizes, structures and physicochemical properties, and to allow them to circulate for long times in the blood and to circumvent the various physiological barriers encountered on the way to the target sites, and to overcome cellular and subcellular barriers.[4] In addition, careful design of the various components of these nanosized particles to reduce toxicity and to enhance biocompatibility of nanoparticles has been shown to significantly enhance the safety of these formulations. For instance, the use of neutral hydrophilic layers on the surface of various nanostructures (*e.g.* nanocrystals, polymeric or lipidic drug complexes) has been shown to reduce the toxicity of these formulations by limiting the interactions of core components and biomacromolecules.

The benefits of using nanotechnology platforms for drug delivery are often challenged by concerns regarding the safety of these materials. Nanoparticle interactions with components of the immune system represent one category of safety concerns. The potential immunomodulatory effects of nanoparticles need to be understood in order to estimate whether or not nanomaterials are capable of inducing adverse reactions and complications. Information regarding nanoparticle immunotoxicity is also needed to ensure that interactions with the immune system will not affect the therapeutic efficacy of these nanotherapeutics.[43,44] Interactions between the various types of nanoparticles (organic and inorganic) and various components of the immune system, including plasma proteins have been described elsewhere.[10–15] However, at the time when these reports were prepared, the role of cytokines in nanoparticle-mediated immune responses had not been investigated in depth. This review presents a more recent and comprehensive overview of the currently available literature on this subject, with a focus on engineered nanomaterials.

4. Cytokines as biomarkers of immunomodulatory properties of nanomaterials

The ability of nanoparticles to induce an immune response (immunogenicity) is a product of the nanoparticles' physicochemical properties (size, charge, hydrophobicity, *etc.*). It is also influenced by other factors such as the surface targeting moieties and therapeutic payload, the animal model, and the route of administration. Nanoparticles can reach the systemic circulation *via* different sites, where the route of administration plays a pivotal role in dictating the fate of the nanomaterials and their immunotoxicity (Figure 4). The immune system may recognize many of the components of nanoparticles (*e.g.* shell, core, surface-decorating moieties, and cargoes) as foreign, and initiates an immune response through a complex process.

There are several markers and measures, which can be utilized to study and predict the immune response of biomaterials, such as, lymphocyte proliferation, cell surface markers, morphological and histopathological examination. Soluble mediators, such as, antibodies (*e.g.* IgG, IgE, IgM), complement proteins (*e.g.* C3a, C5a), and cytokines (*e.g.* T_H1 and T_H2 cytokines) have also been exploited to evaluate the immunogenicity of various therapeutics. This review focuses on utilizing cytokines as biomarkers for nanoparticles immunotoxicity. However, the selection of the functional assay and biomarkers depends on several factors,

such as, the structure and composition of the medical device, route of administration and therapeutic application. Combination of several markers might also be useful to understand the underlying mechanisms of immunotoxicity induced by nanomaterials.

4.1 Proinflammatory cytokines as biomarkers of nanoparticle immunotoxicity

The proinflammatory cytokines are often measured to predict immunomodulatory effects of nanomaterials and the possibility of inflammation-mediated toxicity. They may also alter therapeutic outcomes of the active pharmaceutical ingredients delivered through nanomaterials. The administration of nanoparticles may polarize the balance between the $T_H1$and T_H2 cytokines towards one specific pathway.[14,70–72] For instance, administration of poly-hydroxylated metallofullerenol polarized the cytokine balance towards T_H1 cytokines *via* decreasing the production of T_H2 cytokines (IL-4, IL-5 and IL-6), and increasing the production of T_H1 cytokines (IL-2, IFN-γ and TNF-α) in the serum of the treated mice.[71] In another study, systemic administration of cationic protamine DNA lipoplexes to mice resulted in production of large amounts of proinflammatory cytokines (TNF-α, IL1-β, IL-12 and IFN-γ).[44] These cytokines were associated both with toxicities in animals and inhibition of transgene expression (short duration of gene expression and refractoriness to repeated dosing at frequent intervals). The inhibition of gene expression was tested by preinjection of the vehicle and was correlated with the levels of cytokines production. In addition, intraperitoneal injection of dexamethasone suppressed cytokine production and led to higher levels of transgene expression. As commonly recognized, the unmethylated CpG motifs, especially when combined with cationic vehicles are responsible for immunomodulatory effects. It was reported recently that polyethyleneimine (PEI) (linear *in vivo*-jetPEI), a commonly utilized cationic polymer for nucleic acid delivery, is a selective TLR5 agonist and can elicit the production of TLR5-inducible cytokines in a dose-dependent manner, whereas no secretion of these cytokines were found in the *Tlr5*$^{-/-}$ mice.[36] Intraperitoneal administration of PEI to mice induced significant and selective upregulation of the chemokine KC (the mouse functional homolog of human IL-8[32,35]) 2-h after injection.[36] In another study, increased levels of IL-6 and G-CSF were detected after treatment with PEGylated PEI (branched PEI, molecular weight = 25 kDa).[34] However, further studies are still required to understand the detailed mechanisms of these immunomodulatory patterns and the particular specificity to PEI. The release of proinflammatory cytokines has also been observed with inorganic nanoparticles of various types and morphologies (titanium dioxide (TiO_2), nanodiamond and nanoplatinum) in addition to other manifestations of immunotoxicity, such as dendritic cell maturation and activation and proliferation of naïve T cells.[73,74]

Nanoparticle-induced triggering of the NLRP3 inflammasome results in secretion of IL-1β, which is a key pro-inflammatory cytokine.[75–78] Inflammasomes are multiprotein complexes that act as a major mediator for inflammatory responses. Among the various inflammasomes, NLRP3 inflammasomes are being the most studied and their activation has been linked to exposure of the biological system to nanomaterials of various compositions. For instance, double-walled carbon nanotubes enhanced the release of the pro-inflammatory cytokine IL-1β from human monocytes *via* NLRP3 inflammasome activation pathway.[78] Production of IL-1β has also been observed upon incubation of human monocytes with

silver nanoparticles, and the cytokine release was enhanced upon reducing the size of the particles.[76] It was found that the silver nanoparticles induced inflammasome formation which triggered the release of the IL-1β.

4.2 Route of administration

Intravenous route—In general, parenteral administration is associated with stronger immune response than oral administration, may be due to the lower bioavailability from the oral route, in contrast to the readily available immune cells to interact with the nanoparticles administered *via* the parenteral routes. Poly(D,L-lactide-*co*-glycolide) (PLGA) particles loaded with pertussis toxoid and filamentous haemagglutinin were able to induce potent T cell and antibody response to higher extent when administered parenterally, as compared to the oral immunization, and hence lower dose and less frequent administration were required.[79] Nanoparticles introduced into the body *via* an intravenous (i.v.) route are exposed to a complex environment of blood cells and proteins immediately upon injection. Most immediately adsorb blood proteins on their surfaces. The adsorption of plasma proteins to nanoparticle surfaces, composition of the protein corona and interactions with blood cells determine biodistribution and therapeutic efficacy of the nanoparticles, and may also contribute to immunotoxicity.[80,81] Opsonization is considered to be one of the major barriers to nanoparticle stability and delivery *in vivo*. The most common components of the nanoparticle protein corona are immunoglobulins, complement proteins, albumin, apolipoprotein and fibrinogen.[10] Attachment of immunoglobulins and complement to the surface of nanoparticles tags them for attack by the MPS.[81] Blood cells exposed to certain nanoparticles may produce cytokines, but the mechanisms of cytokine induction are poorly understood. Unlike traditional antigens (lipoproteins, polysaccharides, DNA, RNA *etc.*), which induce cytokines after triggering TLRs, it is unknown whether unfunctionalized nanoparticles are recognized by the immune cells through a specific TLR, a combination of TLRs, or by other receptors. It is also unclear whether unfunctionalized nanoparticles are recognized by receptors on the cell surface or upon internalization.

Subcutaneous and dermal routes—Most nanoparticles do not cross intact skin barriers, but can be engineered for dermal or transdermal delivery. Skin is an immunologically active site rich in immune cells such as the epidermal Langerhans' cells and dermal dendritic cells. This is why delivery of nanoparticles *via* skin can initiate an inflammatory response. Several studies have demonstrated that small (<50nm) nanoparticles administered subcutaneously distribute through lymphatic drainage into draining lymph nodes where they stimulate antigen-presenting cells and lymphocytes.[82,83] Lymphatic drainage is thought to promote an adaptive immune response through several mechanisms involving local complement activation and stimulation of T-cells.[82,83] This property has been used in the development of nanoparticle-based vaccines, however, there is no study which demonstrates a similar mechanism for nanomaterials which are not intended for subcutaneous delivery. Subcutaneous administration of PLGA nanoparticles loaded with antigens (tyrosinase-related protein 2 and 7-acyl lipid A) in mice activated dendritic cells and transmitted the immune response to the draining lymph nodes and spleen, as demonstrated by the secretion of IFN-γ at lymph nodes and spleen.[84] The levels of the proinflammatory cytokines at the tumor tissues of the treated mice (bearing melanoma B16

tumors) were higher than the control group (mice immunized with empty nanoparticles). The route of administration not only influences the extent of immune response, but also the type of response. Intraperitoneal and intramuscular administration of pertussis toxoid and filamentous haemagglutinin in PLGA microparticles resulted in T_H1 response, whereas parenteral and oral immunization of the antigens in solution or loaded into PLGA nanoparticles resulted in T_H2 or mixed T_H1/T_H2 responses.[79] The size, surface area, availability of antigens in various formulations and route of administration might explain this difference in responses. In this study, immune response following subcutaneous immunization was inferior to the other parenteral routes of administration (*i.e.* intraperitoneal and intramuscular).

Nasal and oral routes—Delivery of nanoparticles *via* nasal and oral routes brings them in contact with the mucosa-associated lymphoid tissues and again may cause interaction with the residing macrophages or lymphocytes with possibilities of subsequent transportation of cells or the immune products to the circulation and other body tissues. Singh *et al.*[85] demonstrated that the intranasal administration of PLGA-*b*-poly(ε-caprolactone) nanoparticles-loaded with diphtheria toxoid induced higher production of the IL-6 and IFN-γ, as compared to the intramuscular administration of the same nanoparticles. However, this difference cannot be generalized to other types of nanomaterials as it depends on the characteristics of the nanoparticles and the nature of the antigenic payloads.[79,86,87] In addition, the site of administration and the type of the APCs that first present the antigen and the cytokine environment at the activation site can all contribute to the type and extent of immune response and to the profile of the induced cytokines.[79,87–90]

Intraperitoneal—Some researchers consider this route for nanoparticle-mediated drug delivery into tumors and it has been utilized for the treatment of other diseases. However, peritonitis (inflammation in the peritoneum) due to stimulation and recruitment of immune cells as well as systemic distribution of the particles and related off-target toxicities are common concerns. Several studies have demonstrated that certain nanomaterials can distribute systemically, cause inflammation and cytokine responses upon intraperitoneal administration. For example, Liu *et al.*[91] has recently reported that mesoporous hollow silica nanoparticles administered *via* the intraperitoneal route cause induction of proinflammatory cytokines (IL-1β, TNF-α), liver damage and activation of Kupffer cells. Another study demonstrated that nanosized TiO_2 nanoparticles caused oxidative stress, neutrophil activation and inflammation in lungs 4 hours after intraperitoneal administration into mice. Analysis of the bronchoalveolar fluid from these animals has also revealed high levels of inflammatory cytokines TNF-α, IL-1β, and macrophage inflammatory protein (MIP)-2, the functional homolog of the human chemokine IL-8. Elevated gene expression of TNF and IL-1β was also found in lung tissues of animals exposed to TiO_2 particles *via* intraperitoneal route.[92] Other examples of off-target toxicity associated with induction of inflammatory cytokine responses by nanomaterials administered *via* the intraperitoneal route include brain inflammation and activation of microglial cells by co-administration of nano-sized TiO_2 particles and low levels of endotoxin[93] and allergic sensitization and lung inflammation caused by co-administration of TiO_2 nanoparticles and ovalbumin.[94]

Distribution of nanoparticles from the peritoneum to other compartments and across the blood brain barrier is interesting. The mechanisms of such traffic are poorly understood, but this strategy is being explored for therapeutic applications. For example, intraperitoneal administration of a C60 fullerene derivative into mice 9 hours after challenge with gram negative bacteria reduced inflammatory cytokine levels and protected mice from bacterial meningitis.[95] Similarly, intraperitoneal administration of dendrimer antisense oligonucleotide conjugates reduced cytokine secretion associated with infection of Japanese encephalitis.[96] Another application is vaccine delivery. For example, antigen-loaded solid lipid nanoparticles were efficient in eliciting T_H1 type immune responses and cytokines in mice upon intraperitoneal administration [97]

4.3 Endocytic pathways

The endocytic pathway, surface decorating moieties on the nanoparticles and engagement of specific cellular receptors in the endocytosis process dictate the immune response. Phagocytosis is a receptor-mediated endocytosis restricted to professional phagocytes (*e.g.* macrophages, dendritic cells, platelets). There are four main phagocytic routes, mannose-receptor (MR)-, complement receptor (CR)-, Fcγ receptor (FcγR) and scavenger receptor (SR)-mediated phagocytosis. Mannose, complement proteins, and immunoglobulins are opsonins targeting the MR, CR and FcγR routes, respectively.[98] SRs are thought to react with negatively charged surfaces.[98] Phagocytic cells can also utilize other routes to engulf and eliminate foreign materials. Some of these pathways are receptor-independent and include various forms of pinocytosis: macropinocytosis, clathrin-dependent, caveolin-dependent and clathrin/caveolin-independent pinocytosis. In addition to phagocytic receptors, immune cells utilize many other surface and transmembrane proteins to sense and endocytose foreign materials. One type of such receptors are Toll-like receptors (TLRs). TLRs are pattern recognition receptors which can either be associated with the membrane surfaces (*e.g.* TLRs 1, 2, 5, 4 and 6) or localized into the endosomes (*e.g.* TLRs 3, 7, 8 and 9). Each of these receptors can act alone or in cooperation with other members of this protein family to effectively recognize so-called Pathogen Associated Molecular Patterns (PAMP). Examples of common PAMPs are bacterial lipoproteins, lipopolysaccharides, unmethylated cytosine-phosphate-guanine (CpG) motifs, and single- and double stranded RNA.[99,100] Activation of TLRs by their respective PAMPs results in production of various cytokines, initiates inflammation and coordinates cellular and humoral immunity.[100,101] There are also other components of the immune system that regulate the immune response, such as cytoplasmic immunoreceptors (*e.g.* retinoid inducible gene-1 protein, NOD-like receptors (NLR), and RIG-like receptors).[102] Activation of these receptors initiates receptor-linked intracellular signaling pathways (*e.g.* nuclear factor-kappaB (NF-κB), interferon responsive factors (IRF), activating protein-1 (AP-1), interleukin-1 receptor-associated kinase, *etc.*).[101,103]

Various surface functionalization strategies have been undertaken to create nanoparticles with “pathogen-like” surface properties. For example, it has been demonstrated that nanoparticles can be engineered to target specific receptors (*e.g.* MR-mediated phagocytosis) and induce cytokine responses by introducing mannosylated chitosan residues onto their surface.[104] In another study, di-mannose and galactose, employed to modify the

surface of polyanhydride nanoparticles, resulted in an increase in mannose receptor expression on the surface of alveolar macrophages, which was accompanied by an increased uptake of the particles (presumably through MR-mediated phagocytosis). Binding to the receptors initiated downstream signaling pathway that end up with the production of proinflammatory cytokines (IL-1β, IL-6 and TNF-α), mainly through the activation of NF-κB. The production of the cytokines was higher for the targeted nanoparticles than for the non-targeted ones.[105] Likewise, "grafting" the particle surface with C-type lectins was shown to increase their uptake into bone marrow derived dendritic cells and induce inflammatory cytokine secretion.[106] Another strategy involves conjugation or encapsulation of specific PAMPs to nanoparticles. For example, it has been demonstrated that TLR ligands such as poly-IC, R848 and lipid A are effectively delivered into immune cells and act as adjuvants through the induction of cytokine secretion.[8,84,107] All these strategies are employed to generate deliberate cytokine responses to improve vaccine efficacy. However, nanoparticles unintended to be immunostimulatory (not designed to be vaccine adjuvants), but inducing cytokine responses, do not carry mannose or other ligands which would target the pro-inflammatory pathways. The mechanism of cytokine induction by this category of nanoparticles is less clear. For example, one recent study has shown that polyacrylic acid-conjugated gold nanoparticles interact with the integrin receptor Mac-1 and induce secretion of inflammatory cytokines through a mechanism dependent on the binding and unfolding of fibrinogen.[108] Interestingly, not all particles capable of binding plasma fibrinogen shared this property.[108] It was proposed that activation of the Mac-1 receptors increases NF-κB activity by increasing the degradation of IκB, which allow the translocation of NF-$_{\kappa}$B into the nucleus and upregulate the expression of the proinflammatory genes. The fibrinogen-bound PAA-gold nanoparticles induced the release of IL-8 and TNF-α, although fibrinogen and PAA-gold nanoparticles could not induce the release of these cytokines. Inhibition of the NF-κB pathway reduced the release of these cytokines.

4.4 The role of the nanocarriers: Physicochemical characteristics

The extent of opsonization, destabilization and clearance depends mainly on the nanoparticle characteristics.[4] The composition of the nanoparticle, the size, charge, morphology, and most importantly, surface chemistry, dictate the toxicity and immune response induced by nanoparticles. The ability of nanoparticles to move into specific regions (*e.g.* lymphoid tissues) and to activate APCs greatly affects their immunomodulatory effects. Hence, it can be seen how the selective uptake and biodistribution of nanoparticles can greatly affect their immunogenicity. The stronger the interaction between the nanoparticle and cells, the higher the expected immune response. Hence, it is expected that cationic carriers administered *via* certain routes (*e.g.* subcutaneous or intradermal) will induce higher immunotoxicity and serve as better adjuvants (*i.e.* materials not antigenic *per se* but capable of enhancing the immune response to the antigen). Cationic liposomes were shown to induce the expression of T_H1 cytokines, most notably TNF-α, IFN-β and IL-12, which is associated with tumor static effects.[109] Cytokine induction was reported for other (non-cationic) nanomaterials as well. For instance, exposure of dendritic cells to zinc oxide nanoparticles with negative zeta potentials (−31 to −36 mV) upregulated the expression of CD80 and CD86 and stimulated the release of proinflammatory cytokines (IL-6 and TNF-α), although there was no observable cytotoxic effects.[110] CD80 and CD86 are proteins that can be found

on activated B cells, monocytes, and dendritic cells and macrophages that work as co-stimulators for T cells activation (*i.e.* acting as ligands for proteins on T cell surface), and they are also well-known markers for dendritic cells activation and maturation.

Size—It has been reviewed previously in detail how the size of nanoparticles can determine their cellular uptake, intracellular trafficking pathways, biodistribution and *in vivo* fate.[4,111] It has been generally found that the strength and type of immune response depends also on the size of the nanoparticles.[112,113] Although it is still controversial, it has been reported several times that the nanometer sized particles are more toxic than micrometer sized particles, probably due to the increased surface to volume ratio.[112,114–119] Hussain *et al.* have found that even small differences in the size of the particles can cause significant changes in the inflammatory responses.[112] The immunotoxicity of carbon black and TiO_2 particles with average sizes of (13, 21 and 95 nm) and (15 and 25–75 nm), respectively, were tested at concentrations that are not cytotoxic to cells. The smallest nanoparticles of both types resulted in the highest induction of the GM-CSF cytokine.[112] The same study demonstrated also a dose- and cellular uptake-dependent induction of the inflammatory cytokine.

It has been demonstrated that the size of a nanomaterial affects both the type and the strength of the immune response to an antigen. For example, in an interesting study examining the effect of particle size on immune response, nanometer and micrometer (220, 500 and 1200 nm) RNA/protamine particles were utilized.[120] It was concluded that the smaller particles elicited viral-like responses by triggering the release of IFN-α, whereas the larger particles developed bacterial-like immune responses and induced the release of TNF-α, whereas neither of the nanoparticle components (RNA or protamine) could increase the level of any of the tested cytokines (IFN-α and TNF-α) in human peripheral blood mononuclear cells. It was suggested that the immune system distinguishes the size of the particles associated with the antigen (*i.e.* single stranded RNA) to trigger antiviral and antibacterial/antifungal immune responses for the nano- and micro-sized particles, respectively. In other studies, it was observed that only small nanoparticles (about 50 nm) conjugated to a model antigen could induce T_H1 response, whereas antigen conjugates to larger particles tend to promote a T_H2 response.[121–123]

Shape and hydrophobicity—The effect of shape of zinc oxide nanoparticles (spherical *vs.* sheet) of approximately similar specific surface area was studies. Although the higher cellular association of the spherical particles, there were no significant differences in the generation of the reactive oxygen species and in stimulating the secretion of proinflammatory cytokines (IL-6 and TNF-α).[110] The secretion of TNF-α was higher in RAW 264.7 mouse macrophages treated with spherical zinc oxide nanoparticles, as compared to the sheet zinc oxide particles. In contrast, the opposite pattern was observed in mouse primary dendritic cells, where the sheet-like nanoparticles induced higher release of TNF-α than the spherical ones. Hence, the cell type is an important factor in dictating the immune response to nanoparticulates. The hydrophobicity is among the factors that affect the immunotoxicity of nanoparticles. For instance, immune response to poly(ε-caprolactone) nanoparticles following both intramuscular and intranasal administration was increased, as

compared to PLGA, due to the lower hydrophobicity of the latter.[85] The same trend (greater immune response for nanoparticles with higher hydrophobicity) was observed with other particles of varying degrees of hydrophobicities.[124]

Composition and Surface modifications—The effects of surface modification on the inflammatory effects of silica nanoparticles (30–1000 nm) have been studied by Morishige *et al*. both *in vitro* and *in vivo*.[125] The smaller particles (30 and 70 nm) induced higher production of TNF-α than did larger particles *in vitro*, and stronger inflammatory responses upon intraperitoneal administration. The mechanism involved in the immune toxicity was likely through the production of reactive oxygen species and the activation of mitogen activated protein kinases (MAPKs). Surface modification of the particles with carboxyl groups reduced the activation of MAPKs and subsequently the inflammatory responses both *in vitro* and *in vivo*. Partial modification (15%) of cationic shell crosslinked knedel-like nanoparticles with histamine (instead of primary amines) was found to significantly reduce the toxicity and immunotoxicity of the nanoparticles, probably due to the lower charge density (lower primary amine content) which was confirmed by the lower zeta-potential value and lower cellular binding/uptake.[126] The immunotoxicities of the 0%- and 15%-Histamine modified particles were studied by measuring the levels of 23 cytokines upon treatment of RAW 264.7 mouse macrophages with the nanoparticles for 24 h. Generally, lower secretion of the cytokines was observed from cells treated with the histamine-modified nanoparticles. A similar trend was observed for most of the tested cytokines, although the differences between the levels of the secreted cytokines were significant for 12 cytokines, IL-3, IL-6, IL-9, IL-10, IL-12(p40), IL-13, Eotaxin, RANTES, monocyte chemotactic protein (MCP)-1, MIP-1β, KC and TNF-α.

4.5 The contribution of the payloads

The cargo, not only the nanoparticle carrier composition, is of great importance. Several examples will be given in this section to emphasize the effect of the payload on the immunotoxicity of nanoparticles, and will include nucleic acids and taxanes.

The administration of lipoplexes of pDNA or antisense oligonucleotides is usually associated with toxicity. Cationic lipoplexes (composed of DOTAP, cholesterol, protamine and plasmid DNA (pDNA)) stimulated the expression of CD80/CD86 on dendritic cells, and induced the release of TNF-α.[109] It was concluded that both DNA and the cationic lipid (DOTAP) are required for full immunostimulation. The immunostimulatory sequences in the plasmid or smaller DNA structures (*i.e*. bacterial CpG motifs) can activate the immune system *via* the endosomal TLR9.[127,128] The immune stimulation triggers the proliferation of the B cells and natural killer cells and the release of inflammatory cytokines (*e.g.* IFN-γ, IFN-α/β, IL-6, IL-12, GM-CSF and TNF-α), which may lead to serious local and systemic inflammatory reactions.[44,129] It has been reported in clinical and preclinical studies that the administration of pDNA lipoplexes results in mild “flu-like” inflammatory syndrome in humans and increased levels of inflammatory cells and cytokines within the lungs of mice.[43,130,131] It is commonly observed that the DNA lipoplexes induce the secretion of inflammatory cytokines by T_H1 cells in a CpG-dependent manner[132,133] and this immunostimulation is usually associated with reduction of gene expression in a dose-

dependent manner.[43,134] Although this opens up the possibility of taking advantage of immunostimulatory effects of CpG motifs for vaccination, it might complicate the situation and may result in uncontrollable adverse reactions. In addition, they will be burdensome to the use of these vehicles as platforms for other biomedical delivery applications where immunostimulation is not desirable.

The systemic administration of RNA is often associated with immune stimulation both *in vitro* and *in vivo*, predominantly due to the interaction with TLRs 3, 7 and/or 8, and sometimes due to recognition by other immunoreceptors which recognize RNA and subsequently release pro-inflammatory cytokines.[135–137] When formulated with lipids, RNA can trigger both the lipid- and RNA-sensing TLRs and cytoplasmic immunoreceptors.[101,137–140] As with DNA, the use of cationic lipids for siRNA delivery may result in inflammation and anaphylactic reactions.[137,141,142] It has been reported that the systemic administration of lipid siRNA complexes triggers the release of proinflammatory cytokines and enhances the level of serum transaminases in mice at high doses.[139,142–144] Examples of the cytokines that are usually induced upon systemic administration of siRNA include IL-1α, IL1-β, IL-6, IL-10, IL-12, keratinocyte-derived cytokine, TNF-α, IFN-α, IFN-γ and MCP-1.[101,140,142,144]

Immune responses to chemotherapeutic agents are also well-established, and were reported to contribute significantly to their anticancer activity.[145] For instance, taxanes are mitotic inhibitors that include paclitaxel and docetaxel. Their anticancer activity is achieved through binding to tubulins, and inhibition of cell mitosis at the G2/M phase through stabilization of the microtubules, which triggers apoptosis.[146] However, the immunostimulation induced by taxanes contributes significantly to their anticancer activity.[147] It has been reported several times that the biological and immunostimulatory effects of taxanes are similar to those of lipopolysaccharides (bacterial components that elicit strong immune response).[147,148] Taxanes activate macrophages to initiate cytotoxicity against tumor cells. Activated macrophages also secrete cytokines (*e.g.* IL-1β, IL-8, IL-12, GM-CSF and TNF-α), which in turn stimulate the cytotoxic lymphocytes and natural killer cells against cancer cells (tumoricidal activity).[147,149]

5. Mechanisms of nanoparticle immunotoxicity

The exact mechanism of nanoparticle immunotoxicity and correlation with the endocytic pathways have not been clarified yet, due to the biological variability and dependence of the results on the exact composition of nanomaterials, cell type, cell cycle, animal model, disease status, *etc*. In addition, most of the studies utilize the various markers to predict the possible immunomodulatory effects of nanomaterials, rather than investigating the exact mechanisms beyond the immunotoxic effects and/or the differences in immune responses to nanoparticles of different size, shape, surface chemistry and composition. Sometimes, the induction of both proinflammatory cytokines (*e.g*. IL-6 and TNF-α) and anti-inflammatory cytokines (*e.g.* IL-10) due to unregulated innate immune response (*i.e.* cytokine storm) makes it harder to understand the underlying mechanisms of immunotoxicity.[150,151] Dissection of the mechanism(s) of the proinflammatory response is often complicated by the presence of endotoxin in the nanoparticle formulations. Such contamination is common,[152]

undesirable and often overlooked. Removal of endotoxin from nanoparticles has been shown to eliminate cytokine response and fever reactions.[153,154] Of interest is the increasing data demonstrating that some nanoparticles *per se* do not induce cytokine response, but significantly enhance a cytokine response initiated by low concentrations of endotoxin. In some cases the mechanism involves the NLR3 inflammasome.[155,156] For example, fibrous, TiO_2 nanoparticles did not induce cytokines, but when these particles were combined with low, unreactive amounts of endotoxin they resulted in a strong induction of cytokines of the IL-1 family (IL-1β, IL18, IL33) through a cathepsin B-mediated mechanism.[156] Cytokine storms and exaggeration of inflammatory reactions to endotoxin were also reported for several environmental nanoparticles.[157–160] Another common culprit in undesired cytokine induction by engineered nanomaterials are chemical impurities (*e.g.* synthesis by-products and metal catalysts). For example, early studies on carbon nanotubes demonstrated that these particles can induce proinflammatory cytokine responses[161], but when these same particles were purified from iron contaminants, they were later shown to be non-immunostimulatory and did not induce cytokines.[162,163] Although all these complications in studying the mechanisms of immunotoxicity, mechanisms that have been mostly involved in the induction of cytokines release due to treatment of cells or animals with nanomaterials will be briefly discussed in this section.

Recognition of PAMPs derived from various pathogens by TLRs and/or cytoplasmic immunoreceptors (*e.g.* retinoid inducible gene-1) in the various immune cells stimulates the innate immune response and initiates signaling pathways that activate the transcription factor NF-κB and other pathways (*e.g.* p38/AP1, PI3K and interferon regulatory factor 3/5/7), which lead to production of proinflammatory cytokines.[101,109,164–166] Activation of NF-κB can occur *via* various mediators, such as, proinflammatory cytokines, TLRs, reactive oxygen species and others.[103,164,165] The NF-κB controls the expression of several proinflammatory cytokines and upregulation of costimulatory molecules on dendritic cells, which are required for activation of T cells. In addition, it plays a pivotal role in coordinating innate and adaptive immunity.[164–166] This transcription factor presents in the cytoplasmic inactive form that bound to inhibitory proteins called I_kBs. Activation of NF-κB upon cell stimulation, which can occur through multiple pathways, phosphorylates and degrades the IκBs, where the free NF-κB translocates from the cytoplasm to the nucleus to control the transcription of several cytokines. The detailed mechanisms of the NF-κB activation and subsequent induction of secreting inflammatory cytokines have been reviewed elsewhere.[164–166] Activation of TLRs can occur through the NF-κB and MAPKs and stimulates the production of proinflammatory cytokines, maturation of dendritic cells and expression of costimulatory molecules (*e.g.* CD80 and CD86) on their surfaces.[36,74,109,110] The activated dendritic cells can then migrate to the local lymphoid tissues for antigen presentation. It was reported in one study that cationic liposomes (DOTAP) stimulated the expression of CD80 and CD86 on dendritic cells without inducing the release of TNF-α, due to NF-κB independent pathway.[109] Another study suggested the presence of multiple pathways for induction of toxicities and release of cytokines. In this study, pretreatment with inhibitors of multiple pathways (*e.g.* PI3K, mTOR, p38/AP1 and NF-κB) partially inhibited the release of cytokines induced by siRNA-lipid nanoparticles.[101]

Recognition of immunostimulatory lipids by TLR2 and TLR4 on the cell surface of macrophages and other cells initiates proinflammatory transcriptional programs, including induction of several cytokines.[142,167] In one study, several cytokines (IL-1α, IL-1β, IL-6, KC, IL-10, IFN-γ and TNF-α) were released in the plasma of mice treated with lipid nanoparticles and inflammatory reactions were mainly attributed to the lipid components.[142] The mice treated with the same lipid (cationic lipid, CLinDMA), but formulated in emulsion, caused a similar cytokine release response in mice. Pre-treatment with intraperitoneal injection of dexamethasone (glucocorticoid receptor agonist) inhibited the cytokine release and inflammation in multiple tissues in a dose-dependent manner. Usually, activation of this cytoplasmic nuclear hormone receptor inhibits the transcriptional activity of NF-κB and can inhibit multiple pathways of inflammatory reactions.[165,168,169]

In several other studies, it was reported that both lipids and nucleic acids are required for immunostimulation, which can be explained by several hypotheses.[109] One of the possible reasons is that cationic complexes enhance cellular uptake of DNA/RNA. For instance, induction of cytokines and cellular influx in the lung airway were observed following intratracheal administration of an *N*-[1-(2–3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride/cholesterol/plasmid positively charged complexes in mice.[132] The cytokine release was enhanced by complexing the plasmid to the cationic lipid. Another hypothesis is that the synergistic effect is due to separate responses to the nucleic acid and lipid, which results in two different sets of cytokines. In addition, the released cytokines due to treatment with one component may induce the secretion of other cytokines. A potential mechanism could also be the protection afforded by the electrostatic complexation against enzymatic degradation, which sustain the immunostimulatory effect of nucleic acids. Furthermore, the cationic carriers facilitate delivery of DNA/RNA to local lymphoid tissues, where they can activate APCs.

Nanoparticles may also generate large quantities of reactive oxygen species that can trigger the release of proinflammatory cytokines through the activation of NF-κB.[110,125] The production of reactive oxygen species might be higher for nanoparticles than microparticles due to the enhanced surface area and surface reactivity.[125] Carbon black, TiO_2 nanoparticles induced the release of cytokines in bronchial epithelial cell line *via* oxidative stress.[112,170] The inflammatory effect and release of cytokines were inhibited by catalase, an enzyme that protect against oxidative stress by catalyzing the decomposition of hydrogen peroxide into oxygen and water. Crystalline silica nanoparticles generated reactive oxygen species that triggered TNF-receptors, which activated the cellular NF-κB transcription factor and resulted in inflammatory response.[34]

6. Relationship between cytokines and adverse reactions

Modification of the immune system functions can lead to various consequences ranging from mild adverse reactions to serious and fatal immune complications. Lymphocyte proliferation in response to simulation by an antigen or mitogen is a direct reflection of cellular immunity, and is usually associated with the production of antibodies or cytokines.[23] *In vitro* and *in vivo* studies are confirming that nanomaterials can interact with the immune system and stimulate the production of proinflammatory cytokines, which are

capable of recruiting inflammatory cells including basophils, macrophages, dendritic cells, T cells, neutrophils and eosinophils.[10,11,29,37,71,171] The immune response against the nanoparticles or their payloads may be beneficial or detrimental depending on the intended use of the given nanoformulation, its mode of action and the route of administration.[11] For example, inflammation and granuloma formation have been observed in tissues exposed to unfunctionalized multiwalled carbon nanotubes.[171,172]

Cytokines play an important role in orchestrating and controlling the inflammatory process and their continual presence or excessive production can result in serious adverse reactions that can be life-threatening. They are also involved in important processes, such as fetal recognition, placental development and regulation of gene expression during organogenesis.[173] They are directly or indirectly involved in pathogenesis of several immune-mediated disorders and their levels are usually altered in patients with cancers, cardiovascular diseases, inflammatory diseases (*e.g.* rheumatoid arthritis) and infectious diseases (*e.g.* hepatitis).[24,26,174–177] For instance, it was found that the levels of several cytokines (IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, TNF, IFN-β, GM-CSF, G-CSF, MCP-1, MIP-1α, Eotaxin) were significantly higher in individuals before disease onset of rheumatoid arthritis than in control subjects and further increased after the disease.[24]

Cytokines can mediate both local and systemic inflammatory responses, and they can also be distributed throughout the circulation to various sites of activity. Resulting immune system derangement can lead to increased incidence of autoimmune, allergic and even neoplastic diseases. Some cytokines, such as IL-1, IL-2, IL-6, TNF, transforming growth factor (TGF)-β and IFNs are also involved in modulating the expression of several P450 isoforms (*i.e.* modification of hepatic metabolism).[173] Some of these circulating cytokines can enter some organs through fenestrated capillaries where they induce the production of prostaglandins (PG), such as PGE2, a centrally controlled mediator of fever.[173] The role of cytokines in the immunotoxicity of small and macromolecules has been established and molecular mechanisms are well understood. Studies connecting cytokines to specific toxicities associated with the use of engineered nanomaterials have been reported, but the molecular mechanisms have yet to be defined. For example, an interesting study was performed to confirm the role of cytokines in inducing toxicities. Systemic administration of siRNA-encapsulated in lipid nanoparticles induced the release of several cytokines such as TNF-α, IL-6, IFN-γ and MCP-1.[101] These cytokines were correlated with toxicity. Mild alleviation of the toxic responses was observed when the toxicity of the nanoparticles was re-evaluated in mouse lines deficient in one of these cytokines. Hence, it can be concluded that knocking down one or some of these cytokines is not enough to prevent the toxicity associated with the administration of the siRNA-loaded nanoparticles. Another recent study linked induction of IL-8 to dermal toxicity induced by iron oxide nanoparticles[178] and to pulmonary toxicity associated with the *in vivo* use of various metal oxide nanomaterials.[179] Similarly, induction of acute inflammatory cytokines (IL-1, TNF-α, IL-6, IL-8, *etc.*) was linked to nephrotoxicity associated with the *in vivo* use of TiO_2 nanoparticles.[180] Since the analysis of cytokine levels in blood is more accessible than analysis of specific pulmonary, dermal and nephrotoxicity markers, *ex vivo* analysis of cytokines may contribute to identification and understanding of the mechanisms of these toxicities.

7. Evaluation of nanoparticle immunotoxicity

There are a variety of methods for evaluating the immunotoxicity of nanomaterials both *in vitro* and *in vivo*, with each technique subject to specific limitations.[13] According to the practical strategies suggested during the National Cancer Institute's Nanoparticle Immunotoxicity Workshop, it is essential to test for endotoxin contamination before studying the immunotoxicity of nanomaterials *in vitro* and/or *in vivo*.[13] Recently, a book became available which outlines various approaches that can be pursued for testing the safety of nanomaterials including sterility (endotoxin and microbial contamination) and immunological assays (hemolytic and thrombogenic properties, complement activation, uptake by macrophages and cellular chemotaxis).[181] The various assays available to test the toxicity of nanomaterials have also been reviewed elsewhere.[182–185]

Most cytokines have a broad spectrum of biological activity on a wide variety of cells and are involved in hematopoiesis and recruitment of cells for host defense. Quantifying the levels of specific cytokines in cells, animals or patients treated with nanoparticles is of particular importance because variation in the levels may provide an insight to the mechanisms of immunomodulation. Understanding the underlying mechanisms that induce the secretion of cytokines and the associated adverse reactions is essential in the design of safe nanoparticulates. In addition, cytokines act as mediators of various adverse reactions and provide insight into the prognosis of several diseases. They are also commonly used as therapeutics, for instance, for cancer immunotherapy.[186,187] The use of dynamic assays that include the assessment of the immune response to nanoparticles *via* simultaneous measurements of various cytokines can offer significant advantages, although it may be difficult to predict a clinically relevant response. These levels are often compared to the levels in untreated subjects and can also be compared to the normal physiological range for the cytokine of interest, provided such data is available.[188]

Multiplexing principles and complications

Principles—Even in the absence of an acute cellular or *in vivo* toxicity, elevated cytokines may induce an immune response and result in long-term undesirable immunotoxicity by affecting immune cell function. Hence, it is important to measure cytokines while establishing the safety profiles of engineered nanomaterials. There are various techniques for quantitative measurements of cytokines, proteins, and various inflammatory mediators, with varying degrees of specificity and accuracy. One of the most common and earliest approaches is the enzyme-linked immunosorbent assay (ELISA). More recently, multiplex arrays gained popularity due to the reduced cost per single cytokine and often reduced sample volume.[174,189] There are several available technologies that utilize capture antibodies immobilized on microspheres, the most common being the cytometric bead assay, coupled particle light scattering and multi-analyte profiling (xMAP) technology.[190,191] This review will not detail the comprehensive procedures or types of these immunoassays, but will highlight the key features of the multiplex arrays as a new strategy for evaluating a large number of biomolecules (up to 100) in a few microliters (*e.g.* as low as 12 μL of serum or plasma samples) in a single sample with high sensitivity and accuracy.

The multiplex assay is a modified ELISA assay that has been adapted to measure multiple cytokines in the same sample simultaneously by utilizing several techniques, most commonly flow cytometric technology. It is less time-consuming and labor intensive, and requires lower sample volume and provides higher throughput analysis than traditional ELISA assays. The assay is based on incubation of samples from various sources (plasma, serum, tissue culture supernatant, cell or tissue lysates, lavage samples, or other matrices) that contain cytokines or other inflammatory mediators with a mixture of beads (Figure 5). Each bead is coated with specific detection antibodies for every cytokine. The incubation is followed by a series of washes to remove the unbound cytokines. Biotinylated-antibodies are then added to bind to the cytokine-detection antibody complex (on a secondary site of the cytokine), which is conjugated onto the bead to form a sandwich-like complex. The detection products are then formed by the addition of a reporter molecule (*e.g.* streptavidin-phycoerythrin conjugate) that has a high binding affinity to the biotin (in the biotinylated antibody) and serves at the same time as a reporter or fluorescent indicator (correlated to cytokine concentration). The microscopic beads themselves are then recognized based on their chromogenic or florescence pattern (having unique color codes or spectral properties that correlate with the cytokine type) during the analysis (Figure 5). Although methods of cell stimulation are more or less optimized and harmonized, evaluation of cytokine levels is still subjective and usually conducted based on convenience, affordability and instrument availability in each individual laboratory. This emphasizes the need for greater harmonization and standardization of cytokine evaluation platforms.

Complications—The common limitations of the multiplex arrays include signal “leach-over” between various analytes and the necessity of testing the same sample at multiple dilutions to “concentrate” some low abundance cytokines (*e.g.* IL4, IL-5) and dilute higher abundance cytokines (*e.g.* IL-8, TNF-α, IL1) to the assay range. Information on the expected serum levels of various cytokines is required for the proper dilution of samples. For instance, among 48 different cytokines analyzed in human serum, the mean values for most of the cytokines were <100 pg/mL.[188] However, some cytokines (*e.g.* IL-1β, G-CSF, and β-nerve growth factor (NGF)) had levels lower than the detection limit of the instruments (*e.g.* ~ 1.5 pg/mL for Bio-plex®, Bio-Rad Laboratories, Inc., Hercules, CA), whereas platelet-derived growth factor (PDGF)-BB, regulated upon activation normal T-cell expressed and presumably secreted (RANTES) and stem cell growth factor (SCGF)-β concentrations were too high and required further dilutions.[188] Another hypothesis is that the antibody pair and the detection reagents need to be tuned to detect the cytokines that are either below or above the detection limits (*i.e.* these might be technique-related limitations). The multiplex arrays-based analyses are also extremely sensitive to the handling procedures and good laboratory practices (*e.g.* pipetting, consistency, use of standard operating procedures, validation, calibration, *etc.*).

Cytokines have short half-lives, which must be taken into consideration during analysis. Several cytokines have plasma half-lives ranging from minutes to a few hours (*e.g.* around 6–7 minutes for TNF-α.[192,193]). Hence, cytokines that can be detected in plasma over a long period of time suggests an intensive production. The timing is of particular importance for evaluating the levels of released cytokines. For instance, the release of 27 cytokines from

corneal bilayers treated with nanoparticles was measured 1 hour and 24 hours after exposure to cobalt-chromium nanoparticles. Among the 27 cytokines, IL6, GM-CSF, growth regulated oncogene (GRO), MCP-1 and IL8 were significantly induced, but this was only observed at the 24 hour time point.[194] A general trend throughout the literature is that the levels of cytokines change significantly during the time course of the treatment with nanomaterials, both *in vitro* and *in vivo*. Hence, it is critical to compare samples that were collected at the same time, and stored, transported and analyzed under identical conditions. In addition to the effect of timing, the possibility of interactions between nanoparticles and the secreted cytokines should also be considered. Adsorption of cytokines (*e.g.* GM-CSF, IL-6 and TNF-α) on various types of inorganic nanoparticles (*e.g.* carbon black and TiO_2 nanoparticles) has been observed *in vitro*, which might result in artefacts and misinterpretation of the data.[112,170,195,196] Other problems that could further complicate the assay and negatively affect the accuracy of the collected data include the presence of certain proteins in the analyzed samples, particularly the plentiful endogenous proteins in the blood, or the iron produced from blood hemolysis, which may interfere with the formation of cytokine-antibody complexes.

8. Controlling the immunotoxicity of nanomaterials

Proper design of nanomaterials has been always a subject of interest to improve their *in vitro* and *in vivo* characteristics, mainly to impart greater stability to their cargoes and to prolong blood circulation times to allow the accumulation at target sites, as well as greater safety. However, little impetus has been directed towards designing nanomaterials of low immunogenicity. When designing nanoparticles of low toxicity and immunogenicity, every component in the nanomaterial should be considered and systematic studies should be carried out to evaluate the effect of structural modifications on immunotoxicities. It is equally important to consider both the nanoparticle-forming material and the drug/payload (Figure 6).

8.1 Nanoparticle-related factors

The first parameter to be considered in the design of nanoparticles is the shell thickness, density and type, and accessibility of any molecules used for surface decoration (*e.g.* targeting ligands, contrast agents). Functionalization of PLGA nanoparticles with thiol groups was tested to decrease interactions with opsonins and phagocytic cells, and found to reduce protein adsorption, complement activation, and platelet activation of PLGA nanoparticles.[197] Cationic nanoparticles are believed to be more toxic, rapidly-cleared and induce higher inflammatory reactions than their anionic or neutral counterparts. Encapsulation of therapeutics inside the nanocarrier is expected to reduce the immune response induced by the drug. Considering the approximate size of plasma proteins and constituents (~1–10 nm), it is desirable to keep the spacing between PEG chains as small as possible to minimize the interactions between the plasma components and the core material.[198–200] Crosslinking the corona by biodegradable crosslinkers is also important to retain the spacing and avoid the dissociation and segregation of the PEG chains. Shell decoration with different moieties is necessary for the preparation of multifunctional nanocarriers. However, it is a prerequisite to keep the functionalization ratio as low as

possible. In addition, the use of moieties of low immunogenicity (*e.g.* galactose instead of antibody for targeting) is recommended. Comprehensive studies to identify the critical parameters (*e.g.* PEG length and density[198–200]) that influence the toxicity and immunotoxicity of nanoparticles are required. *In vitro* studies to determine which blood components are involved in the destabilization/opsonization of nanoparticles is equally important for the rational design of nanoparticles. Polymer biodegradability and biocompatibility are essential for patient safety.

Careful selection of the ingredients of nanomaterials is critical in predicting the immune response upon *in vivo* administration. Indeed, Huang and coworker have been systemically investigating the effect of the nanoparticle design on the immunotoxicity of nanoparticles designed for siRNA delivery. Targeted nanoparticles-based on anisamide (ligand for sigma receptors overexpressed in some cancer cell lines)-PEGylated liposomes (DOTAP and cholesterol)-protamine-hyaluronic acid complexes (diameter of 115 nm and zeta-potential of 25 mV) were developed for the systemic delivery of siRNA.[201] These nanoparticles showed low immunotoxicity in a dose range of 0.15–1.2 mg siRNA/kg in mice. On the contrary, another formulation that has the same composition but uses pDNA or calf thymus DNA instead of the hyaluronic acid had a narrower therapeutic index (*e.g.* a dose range of 0.15–0.45 mg/kg for the calf thymus DNA).[201,202] The differences between the two formulations were the type of the condensing materials that employed to enhance the condensation of siRNA by cationic liposomes (DNA instead of hyaluronic acid). Intravenous injection of the siRNA formulated in both formulations in mice was tested and proinflammatory cytokines (IL-6 and IL-12) levels in serum of treated mice were measured 2-h after injection. The hyaluronic acid-based formulation induced lower immunotoxicity than the calf thymus or the pDNA-based nanoparticles, although both formulations showed similar characteristics and gene silencing activity. The lower immunotoxicity might be due to the removal of the foreign DNA and the CpG immunostimulatory sequences and also because hyaluronic acid is a biogenic compound that widely distributed in the human body fluids. Continuing on their work, nanoparticles built with a degradable calcium phosphate core were utilized instead of the non-degradable ones for the systemic delivery of siRNA and resulted in higher silencing activity *in vitro* and similar *in vivo* gene silencing. Importantly, they induced lower immunogenicity in mice as compared to the DNA/protamine-based nanoparticles (lower induction of IL-6 and IL-12 at higher doses).[203]

8.2 Drug-related factors

Careful design of the drug itself can modulate the immunotoxicity of nanoparticles. For instance, while the injection of pDNA usually induces strong immune responses, designing plasmid structures that do not contain unmethylated CpG motifs could reduce the immunological reactions and enhance the transgene expression efficiency.[43] Surprisingly, even a single CpG motif in the DNA structure could elicit an inflammatory response. Methylation of pDNA has also been confirmed to significantly reduce the levels of inflammatory cytokines.[44] Similarly, chemical modifications and sequence selection of other small nucleic acids (*e.g.* siRNA) can greatly modify/amend their immunotoxicity.[204] It has been shown in one study that the immunostimulatory response of siRNA could be completely abolished, with no induction of cytokines (*e.g.* IFN-α and TNF), by designing

chemically modified siRNA contains 2'-*O*-methyl modified nucleosides (<20%) into one of the siRNA strands without affecting their gene-silencing activity, both *in vitro* and *in vivo*, whereas the unmodified siRNA induced the induction of cytokines and toxicity in the treated mice.[140]

9. Conclusions

With the rapid and extensive research now underway into the design of novel nanomedicines, it is critical that attention be directed to their potential immunotoxicity. Evaluation of the immunotoxicity of nanomaterials, for example, by measuring the levels of cytokines or other immune-indicators, is of particular importance for their clinical safety and for maximizing therapeutic benefits. Measuring the levels of proinflammatory cytokines and other inflammatory mediators and monitoring the balance of T_H1/T_H2 cytokines can be useful tools in evaluating nanoparticle immunotoxicity. Moreover, screening of a single reporter is inadequate; combinations of cytokine levels should be measured, due to the cytokine network influence on the immune system. Monitoring the levels of cytokines, in particular the proinflammatory ones, following administration of nanomaterials, appears to be an important tool for partially screening their immunomodulatory effects. High levels of cytokines, as compared to the untreated controls, are considered as biomarkers of nanoparticle immunotoxicity. The latter is usually associated with toxicity, adverse reactions and lower therapeutic efficacy. Sometimes, treatment with anti-inflammatory drugs might be useful in reducing the inflammatory effects associated with the administration of nanoparticles. Designing nanocarriers with biodegradable cores and coatings that are capable of shielding the core ingredients during circulation can minimize the immune response. Equally important is the design of the drug itself, for example *via* chemical modifications of nucleic acid (DNA/RNA) backbones to mask or remove immunostimulatory sequences. Further understanding of the underlying mechanisms of nanomaterial-mediated toxicities and inflammatory reactions, and the role of the various chemical mediators and ligands will secure the development of biocompatible and non-immunogenic nanomaterials for a variety of biomedical delivery applications.

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Box 1 | The immune system

Organs of the immune system: *Bone marrow, thymus, spleen, lymph nodes and mucosa-associated lymphoid tissues*. During hematopoiesis, bone marrow-derived stem cells differentiate into either mature cells or into precursors of cells that migrate out of the bone marrow to continue their maturation elsewhere, for example, maturation of T lymphocytes occurs in the thymus. The spleen acts as an immunologic filter of the blood, whereas the nodes filter the tissue fluids (*i.e.* lymph). The mucosa-associated lymphoid tissues are aggregates of lymphoid tissues near the mucosal surfaces.

Cells of the immune system: Leukocytes (white blood cells) consist of: **(a)** Polymorphonuclear granulocytes (PMN): Neutrophils, eosinophils and basophils; **(b)** Monocytes: can be differentiated into macrophages; **(c)** Natural killer cells; **(d)** B lymphocytes: produce antibodies and **(e)** T lymphocytes: T helper cells, suppressor T cells and cytotoxic T cells. Lymphocytes and mononuclear phagocytes play a central role in the immune response.[37]

Immune response: Antigen presenting cells (APCs) are cells that capable of processing an antigen and presenting part of it onto the MHC II where it can interact with the appropriate immune cell receptors. Dendritic cells, macrophages and B cells are the main APCs for T cells, whereas follicular dendritic cells are the main APCs for B cells. APCs and B or T cells communicate together, either directly or *via* cytokines, to initiate an immune response. Antigen processing and presentation signal these cells to proliferate and secrete antibodies (B cells), cytokines ($CD4^+$), or become activated to kill cells expressing the antigen presented by the APC ($CD8^+$). The antibodies secreted by B cells can directly bind to that antigen, which accelerate the clearance by the PMN or macrophages. The antibodies may also initiate the complement destruction cascade by attracting the serum protein to bind to the immobilized antibodies that are bound to the antigen, and thereby aiding in the phagocytosis process and elimination of the immune complex.

Mononuclear phagocytic system (MPS): The original term used to refer to the phagocytic system is reticulo-endothelial system (RES). It refers to the mononuclear cells of mysenchymal origin that reside in the reticular organs including the liver, spleen and lymph nodes and other organs, and possess the collective property of rapidly engulfing colloids and particulate materials. Since not all endothelial and reticular cells are phagocytic, the term RES was recently replaced with MPS, however in the literature, RES and MPS are often used interchangeably. MPS is distributed throughout the body and are mainly responsible for the phagocytosis, clearance and initiation of the immune response due to the introduction of the nanomaterials, foreign particles or antigens in the body, and it also engulfs and clears aged cells in the blood and tissues. Phagocytic cells include PMN, blood monocytes and tissue macrophages (Kupffer cells in the liver, alveolar macrophages in the lung, splenic macrophages, peritoneal cells in the peritoneal fluids, microglial cells in the central nervous tissues, histiocytes of the connective tissues, and dendritic cells). These cells have a number of surface receptors that allow them to bind carbohydrates, complement protein and immunoglobulins. If the MHC II is expressed on the surface of the phagocytes, it enables them to function as APCs.

Opsonins (“prepare food for”) are antibodies, complement proteins and other serum components that upon binding to foreign particles make them easier targets for phagocytes.

Complement system: It is a part of the immune system comprised of a biochemical proteolytic cascade that aids (complement) the antibodies and phagocytic cells to eliminate pathogens from the body.

Box 2 | Nanomaterials in biomedicine

Nanomedicine: "The design of diagnostics and/or therapeutics on the nanoscale, which provides opportunities of coincident transport and delivery of the active species with mediation of their navigation within the biological systems for the treatment, prevention and diagnosis of diseases".[4]

Types:

A. Organic nanoparticles:

1. **Macromolecular conjugates (*e.g.* Xyotax®, Oncaspar®):** Conjugating drug to a polymeric or lipidic segment to impart specific properties to the drug to facilitate or direct its delivery.[45,46]
2. **Nanoemulsions (*e.g.* Diprivan®):** Heterogeneous mixture of two immiscible liquids with emulsifier that stabilizes the dispersed droplets. They can be utilized as carriers for hydrophilic or hydrophobic drugs for various therapeutic applications.[47,48]
3. **Polymeric micelles (*e.g.* NK012, NK105, SP1049C, NC-6004, Genexol):** Self-assembly of amphiphilic copolymer chains in aqueous milieu presenting a core/shell architecture with a hydrophobic core and hydrophilic corona. Depending on the structure of the core forming polymer and the forces driving the assembly, they may be classified also as polyion complex micelles or polymer-metal complex micelles.[49–51]
4. **Shell crosslinked knedel-like nanoparticles:** Shell crosslinked polymeric nanoparticles to enhance the kinetic stability and prevent the dissociation upon *in vivo* administration. Degradation of the core is also possible to form nanocages.[1,52,53]
5. **Protein-based and polymeric nanoparticles (*e.g.* Abraxane®, BIND-014):** Colloidal particles with a rigid core that are either made from a polymeric or lipidic matrix in which a drug is dissolved or dispersed or from drug nanocrystals stabilized by a polymer.[41,53,54] Polymer brushes, unimolecular (*e.g.* dendrimers) and hyperbranched structures utilized for drug delivery may also fall into this category.[55,56]
6. **Liposomes (*e.g.* Doxil®, DaunoXome, Ambisome):** spherical vesicles composed of lipid bilayers and a hydrophilic core with capability of solubilizing both hydrophilic and lipophilic drugs into the core and lipid bilayers, respectively.[57–59]
7. **Lipoplexes and polyplexes (*e.g.* ALN-VSP):** Complexes between nucleic acids (DNA/RNA) and cationic lipids or polymers, and when PEGylated are categorized as PEGylated lipoplexes and polyion

complex micelles, respectively.[9,60–62] Stable nucleic acid lipid particles (SNALP) may also fall into this category.[63,64]

B. Inorganic nanoparticles:

1. **Metal and metal-oxide nanoparticles:**
    a. **Gold nanoparticles (*e.g.* Aurimune®):** Nanoparticles that display interesting optical and electrical properties.[65]
    b. **Magnetic nanoparticles (Ferridex, Ferumoxytol, Resovist):** Nanoparticles with magnetic properties (*e.g.* paramagnetic iron oxide nanoparticles).[66,67]
    c. **Other nanoparticles:** Other kinds of nanoparticles do also exist, such as, titanium oxide, platinum- and diamond-based nanoparticles, *etc.*
2. **Carbon nanotubes:** single wall or multi-wall cylindrical graphene sheets.[67–69]
3. **Quantum dots:** Inorganic semiconductor nanoparticles that are widely used as fluorophores for biological imaging.[67]

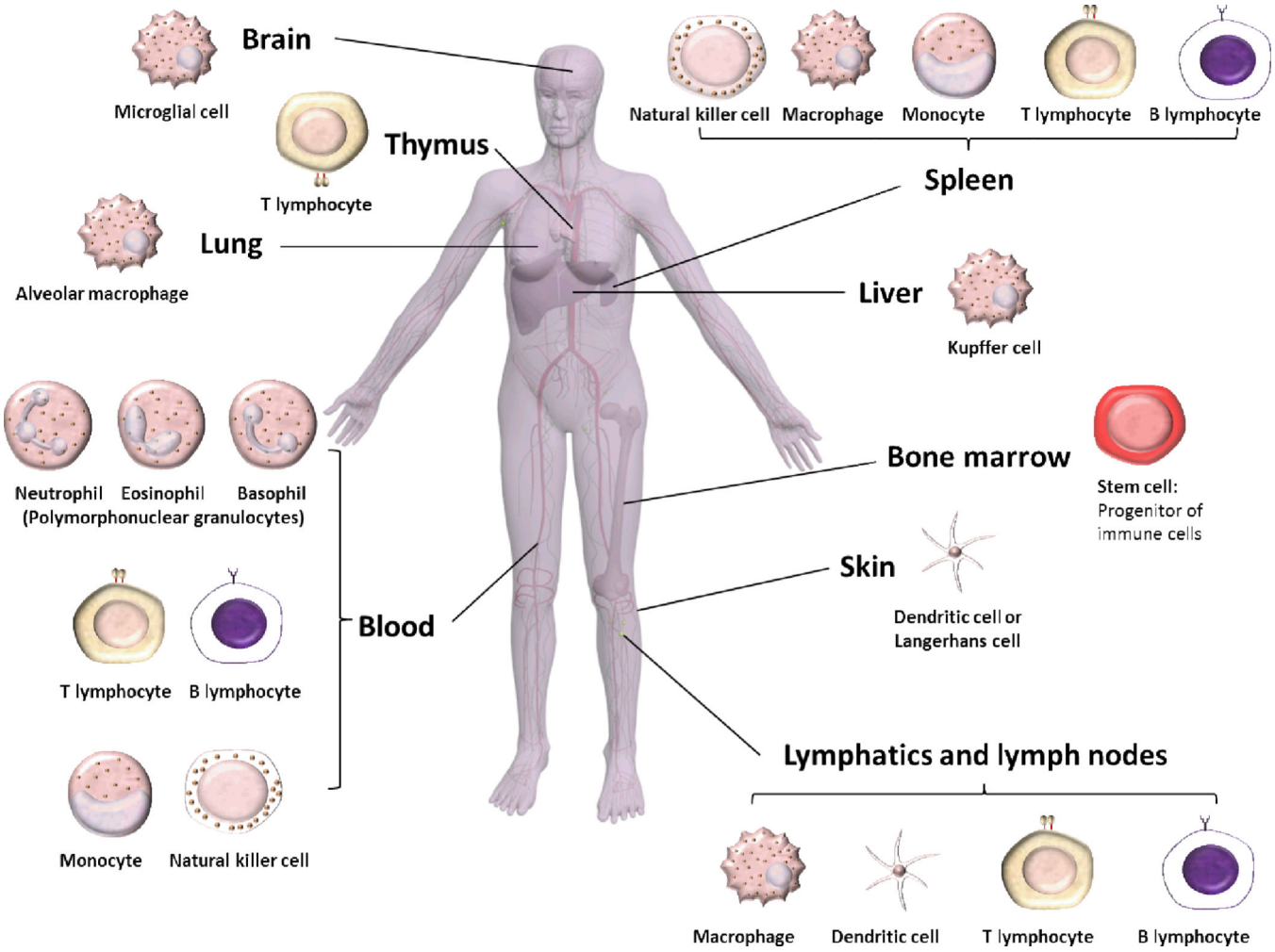

Figure 1.

Human body showing the various organs of the immune system and the distribution of the various immune cells into the immune organs and other organs that are rich in macrophages.

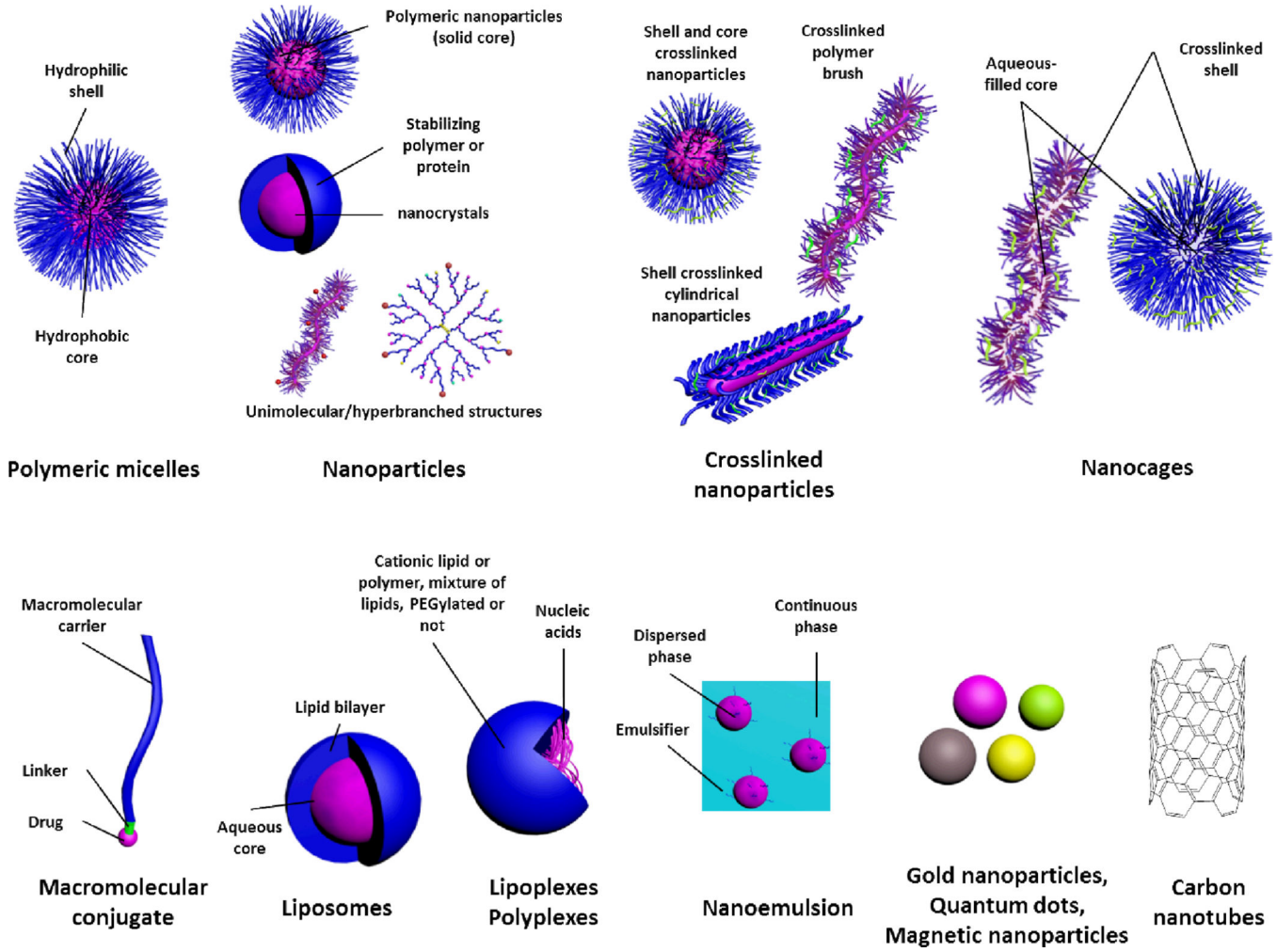

Figure 2.

Common nanoparticulates utilized for delivery of a wide range of therapeutics. Brief explanation of each category is indicated in Box 2.

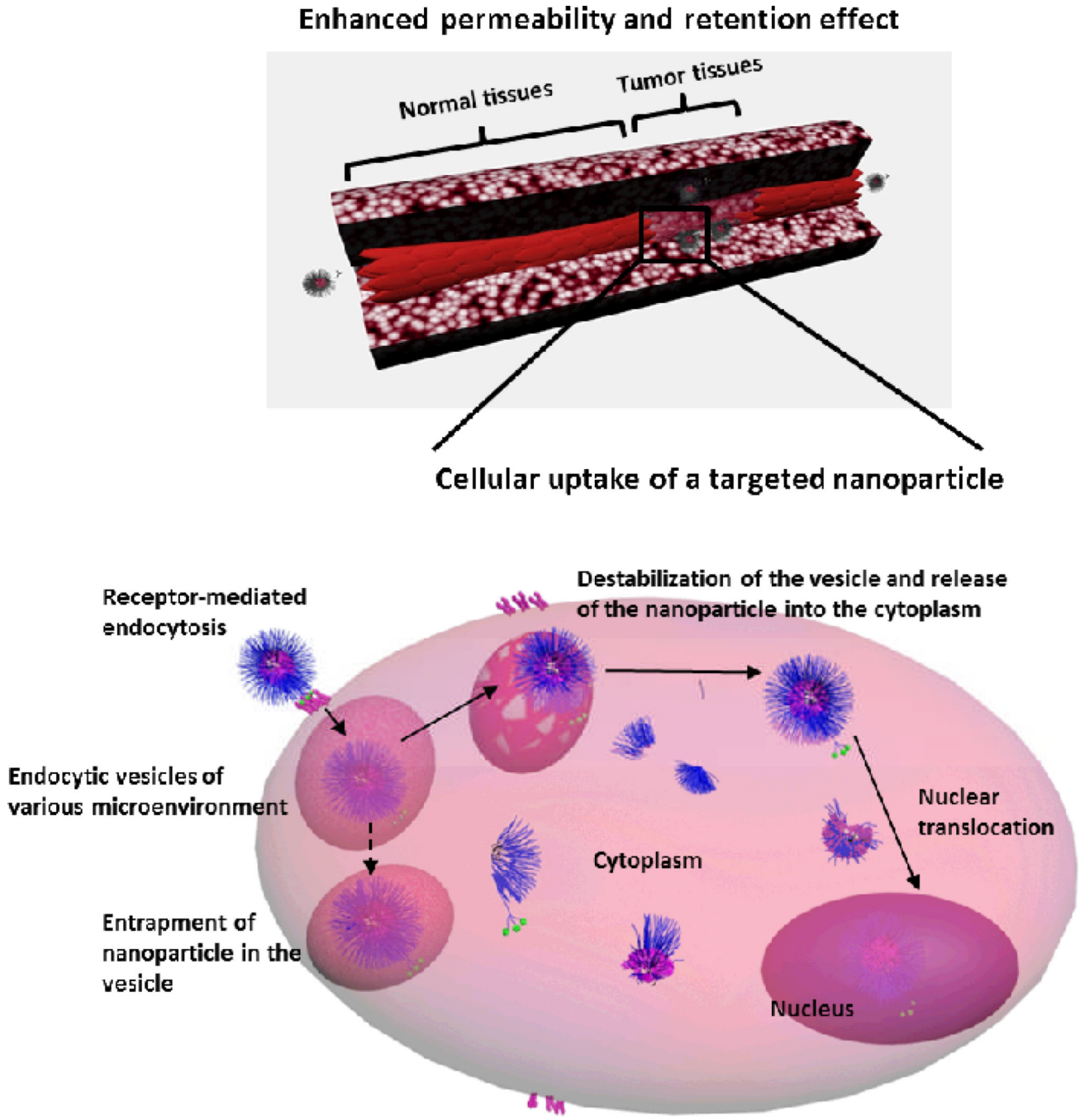

Figure 3.

Passive and active targeting features of multifunctional nanomaterials. In passive targeting, nanoparticles accumulate into pathological sites with leaky vasculature (*e.g.* tumor) due to the enhanced permeability and retention effect. In active targeting, the targeting ligands on the surface of nanoparticles enhance cellular uptake by binding to specific receptors overexpressed on the diseased cells, and can also be achieved *via* facilitating the escape from endosomes and/or enhancing nuclear translocation. Multifunctional nanoparticles have additional functionalities to deliver more than one cargo (*e.g.* more than one type of

therapeutic and/or diagnostic), or combine more than one targeting mechanism (*i.e.* passive and active targeting). Reproduced by permission of the Royal Society of Chemistry.[4]

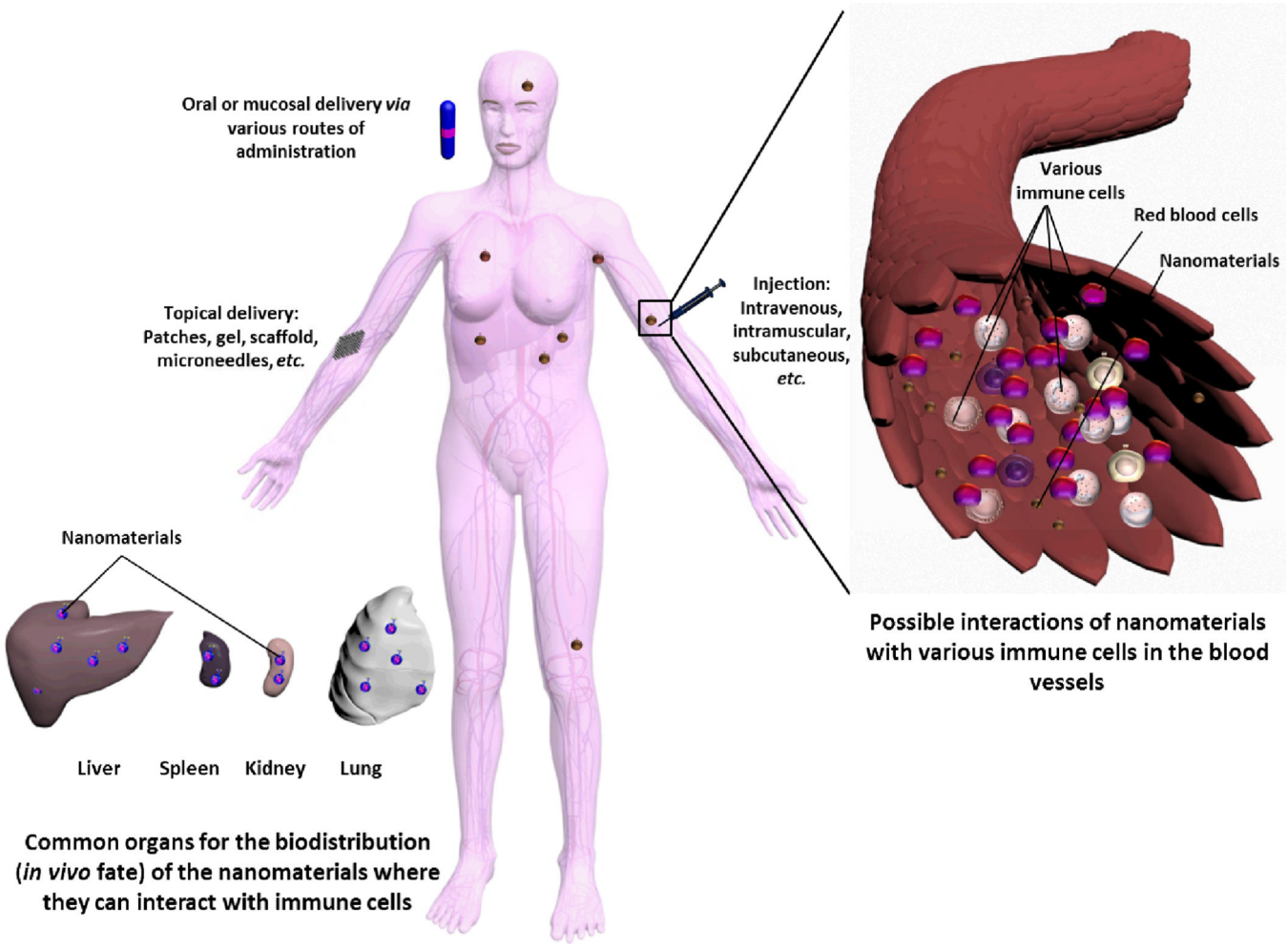

Figure 4.

The possible interactions of nanoparticles with the various components of the immune system after entering the body *via* various routes of administration (oral, mucosal, systemic or topical).

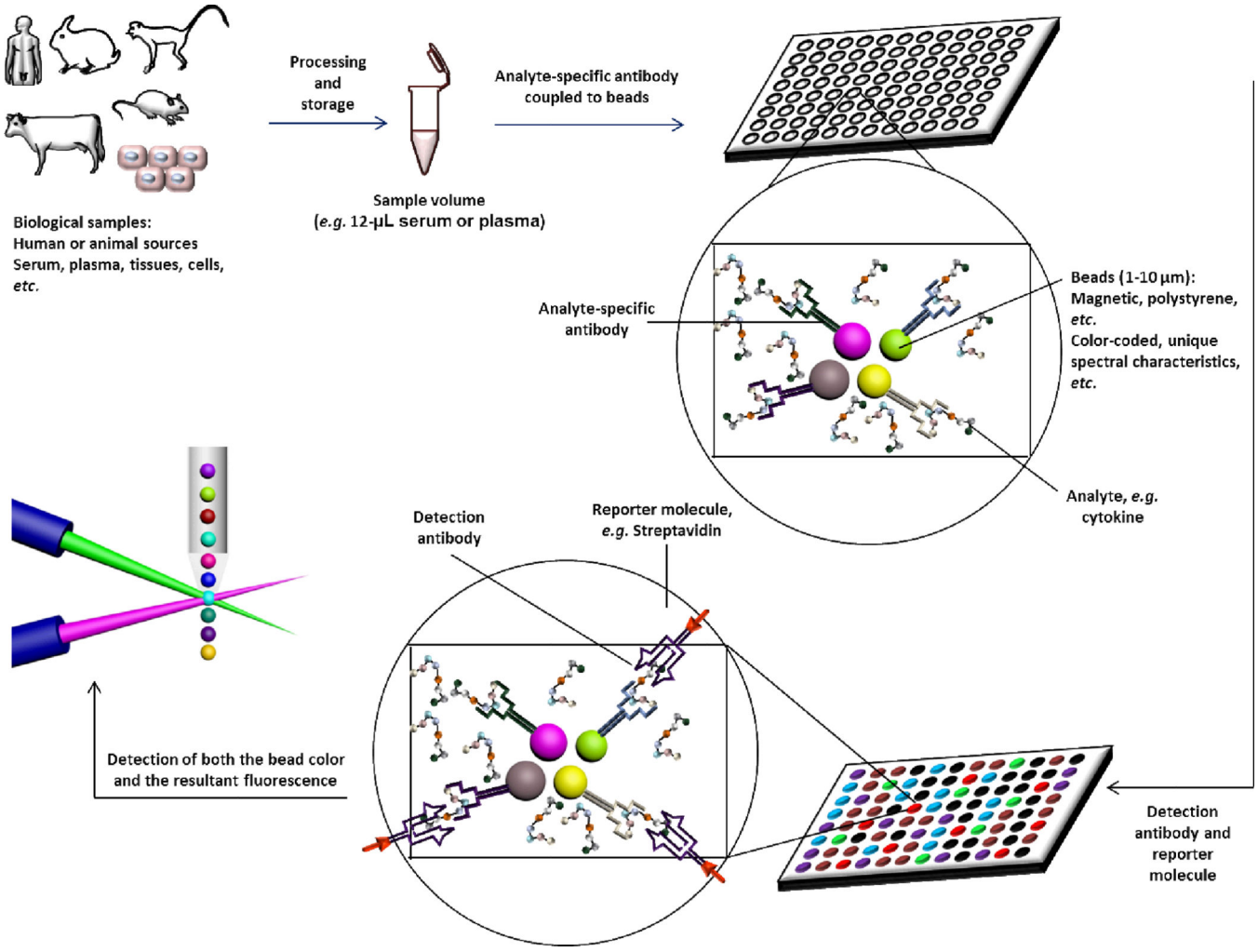

Figure 5.

Schematic representation of the steps of multiplex assay for the simultaneous detection and measurements of cytokines in biological samples of various sources. Detailed procedures are not included in this schematic diagram and the detection methods (*e.g.* color code *vs.* fluorescence, or the use of various reporters, or the flow cytometric detection) vary depending on the specific instrument and kit utilized for the assay.

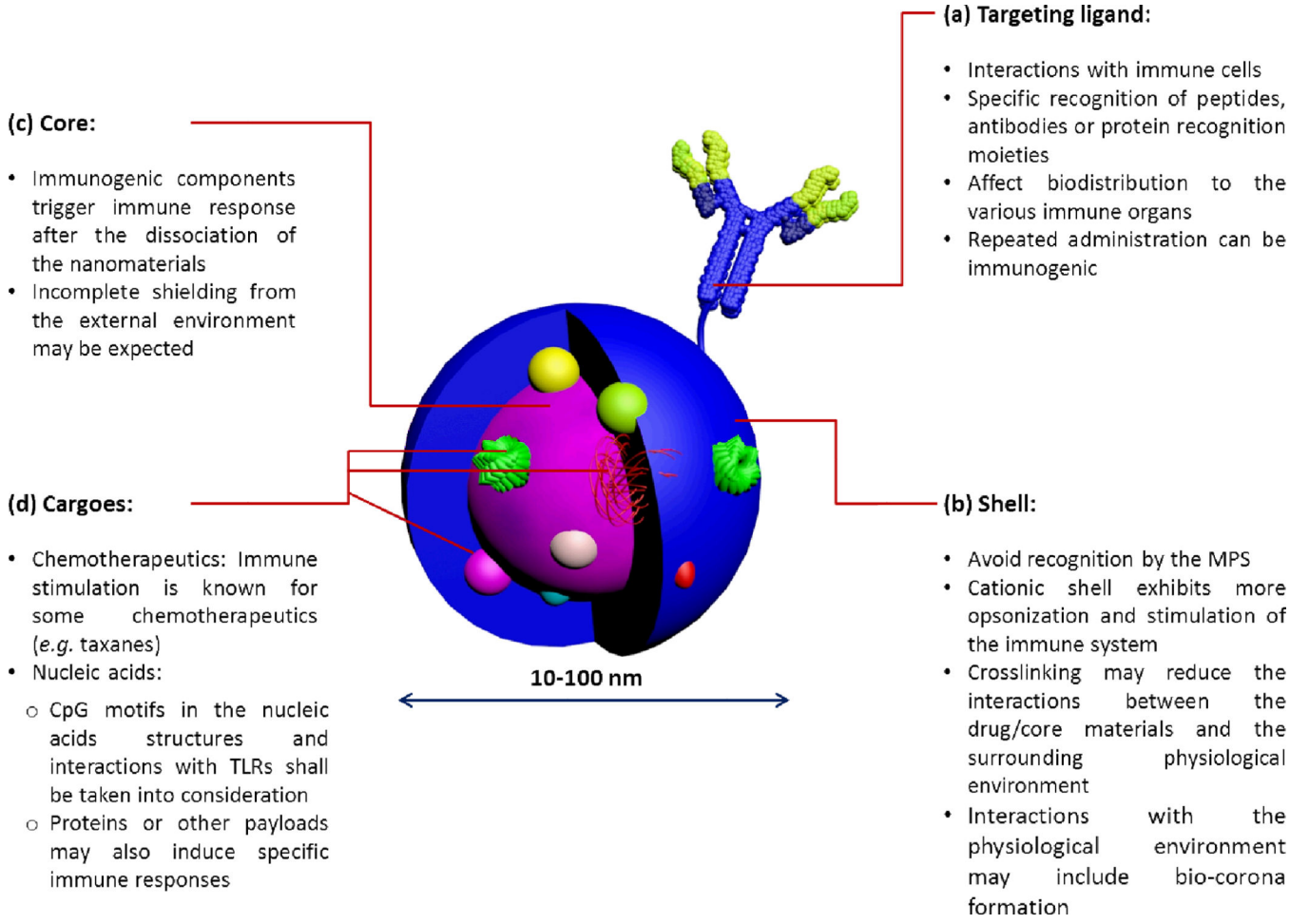

Figure 6.

The general composition of a multifunctional nanoparticle for biomedical delivery applications is illustrated with highlighting some important considerations for the design of nanoparticles of low immunogenicity. The size of nanomaterials usually ranges from 10–100 nm.