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REVIEW

Immunosuppressive and anti-inflammatory properties of engineered nanomaterials

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Nanoparticle interactions with various components of the immune system are determined by their physicochemical properties such as size, charge, hydrophobicity and shape. Nanoparticles can be engineered to either specifically target the immune system or to avoid immune recognition. Nevertheless, identifying their unintended impacts on the immune system and understanding the mechanisms of such accidental effects are essential for establishing a nanoparticle's safety profile. While immunostimulatory properties have been reviewed before, little attention in the literature has been given to immunosuppressive and anti-inflammatory properties. The purpose of this review is to fill this gap. We will discuss intended immunosuppression achieved by either nanoparticle engineering, or the use of nanoparticles to carry immunosuppressive or anti-inflammatory drugs. We will also review unintended immunosuppressive properties of nanoparticles *per se* and consider how such properties could be either beneficial or adverse.

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Abbreviations
APC, antigen-presenting cell; DC, dendritic cells; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; FOXP3, forkhead box P3; IL-1RA, IL-1 receptor antagonist; iNOS, inducible NOS; IONPs, iron oxide nanoparticles; ITE, 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester; MDDC, monocyte-derived dendritic cells; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; MWCNT, multi-walled carbon nanotubes; NP, nanoparticles; ODNs, oligodeoxynucleotides; OVA, ovalbumin; PAMAM, polyamidoamine; PBMC, peripheral blood mononuclear cells; PEG, polyethylene glycol; PIBCA, polyisobutylcyanoacrylate; PIHCA, polyisohexylcyanoacrylate; PLGA, polylactic-co-glycolic acid; PVA-SPION, polyvinylalcohol coated super-paramagnetic iron oxide nanoparticles; RGD, Arg-Gly-Asp moiety; T-reg, regulatory T-cell; Th, T helper; TLR, toll like receptor; SAR, structure–activity relationship

Introduction

There is a growing body of evidence suggesting that immunotoxicity, defined as deregulated function of the immune system, contributes to the onset and development of various disorders including cancer and autoimmune diseases (Merk *et al.*, 2001; Descotes, 2004; 2012; Dobrovolskaia and Kozlov, 2005; Dietert, 2011). Nevertheless, it was not until recently

that this relatively new field of toxicology became an important interface of novel drug design and pharmacology. For the purpose of this introduction, immunotoxic effects will be separated into two categories: immunosuppression and immunostimulation. Each of these categories has been implicated in distinct adverse effects reported in human pathologies. Historically, the main concerns have been primarily directed towards immunosuppression, while immunostimulation has gained more attention only recently, as new biotechnology-derived pharmaceuticals have reached the clinical phase. Nanotechnology-derived products are complex, as they often combine small molecules, macromolecules and nanoparticles. For this reason, monitoring both immunosuppression and immunostimulation of nanomaterials is recognized as an important step in their safety assessment (Dobrovolskaia *et al.*, 2009). However, unlike biotechnology-derived pharmaceuticals, in nanotechnology more attention has been given to immunostimulation than to immunosuppression. Nanoparticles can be engineered to either specifically target the immune system or to avoid such interactions. They may change immune responses to small and macromolecular drugs, as well as be immunoreactive themselves (Alving *et al.*, 1996; Perkins *et al.*, 1997; Watanabe *et al.*, 2008; Libutti *et al.*, 2010; Van Beers *et al.*, 2012). Interactions between nanoparticles and the immune system can be beneficial or adverse. While immunostimulatory properties of nanoparticles have been reviewed before (Dobrovolskaia and McNeil, 2007; Pantic, 2011; Boraschi *et al.*, 2012; Elsabahy and Wooley, 2013), little attention has been given to their immunosuppressive and anti-inflammatory properties. Herein, we will review available data demonstrating both intended and unintended immunosuppressive and anti-inflammatory properties of nanoparticles. Intended immunosuppression is when inhibition of immune responses is expected to relieve immune-mediated pathologies (e.g. to treat inflammatory and autoimmune disorders or to prevent transplant rejections and allergic reactions). Unintended immunosuppression is when a decrease in immune function is unplanned, and can be either beneficial or adverse. Unintended immunosuppression can be beneficial when it aids in inhibiting inflammatory and autoimmune conditions, or adverse if it results in conditions such as myelosuppression, thymic suppression and lowered body responses to infections and cancer. In this review, we will consider the mechanism of action and discuss the immunosuppressive/anti-inflammatory properties attributed to nanoparticles *per se* and to those belonging to the drugs conjugated to or encapsulated into nanoparticles (Figure 1).

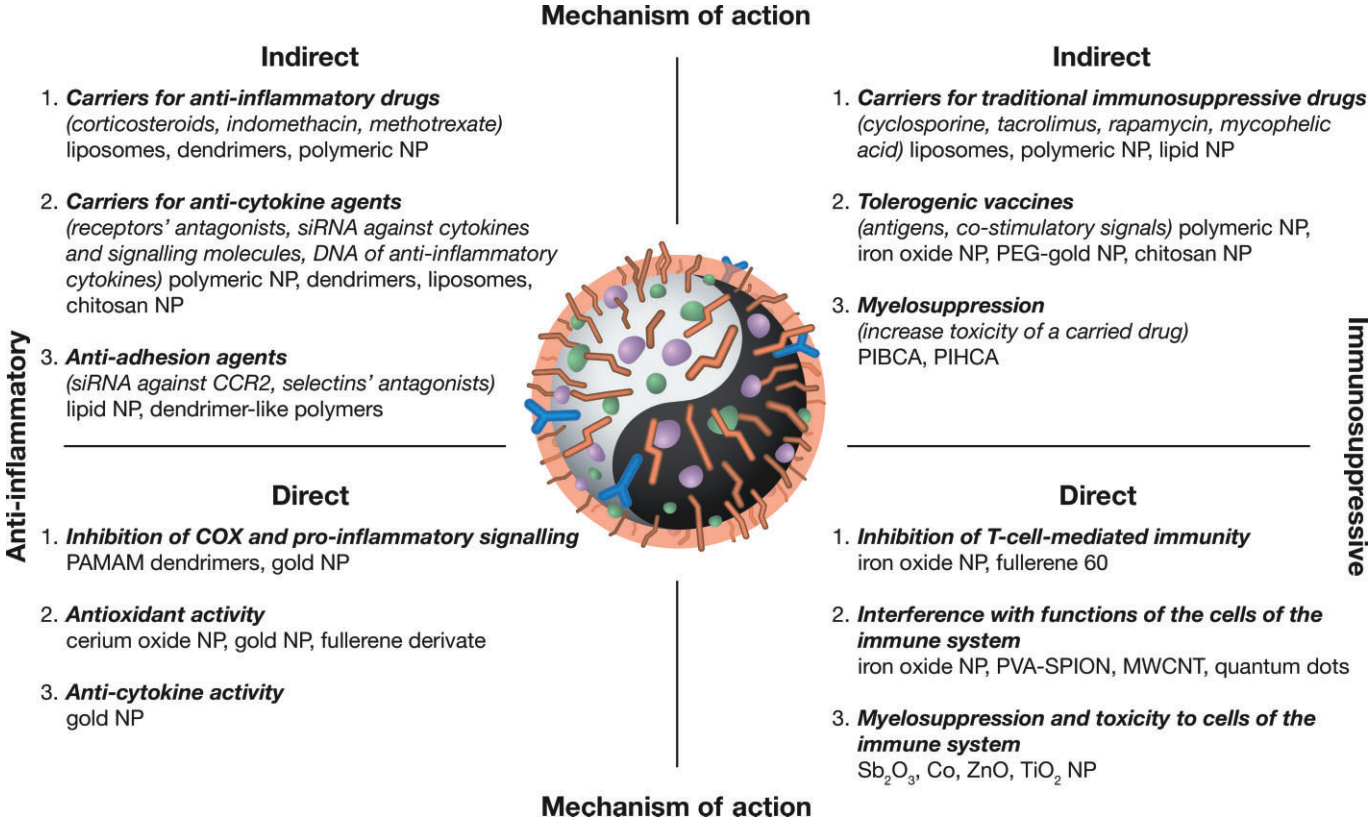

Figure 1

The Yin and Yang of nanoparticle immunosuppressive and anti-inflammatory properties. Immunosuppressive and anti-inflammatory properties can be achieved by either using nanoparticles as carriers for immunosuppressive and anti-inflammatory drugs (indirect mechanism of action), or by optimizing nanoparticle properties to allow particles to suppress the immune system (direct mechanism of action). Immunosuppression and anti-inflammatory properties of nanomaterials can be either therapeutically beneficial or detrimental. Shown are examples of immunosuppressive and anti-inflammatory nanoparticles and their mechanisms of action.

Intended immunosuppression

Suppressing immune system function can be desirable under certain circumstances: (i) when it reacts to allogenic (foreign) antigens after organ and tissue transplantation (graft rejection); (ii) when leukocytes in transplanted tissue attack host cells as foreign antigens (graft vs. host disease); (iii) when the immune system loses tolerance to self-antigens (autoimmunity); and (iv) when it overtly reacts to environmental and dietary factors (allergies and atopic disorders). However, the preparation and use of conventional immunosuppressive agents can cause a lot of complications. Most of these agents, for example, tacrolimus, cyclosporine and rapamycin, are hydrophobic and as such have poor bioavailability. They also require solvents that themselves may cause toxic side effects such as nephro-, neuronal and immunotoxicities (Dye and Watkins, 1980; Varma *et al.*, 1985; van Zuylen *et al.*, 2001). For example, Cremophor-EL®, composed of polyethoxylated castor oil and ethanol, is known to cause complement activation-related pseudoallergy in sensitive individuals (Szebeni, 2005) and contributes to neuronal toxicity of certain drugs (Windebank *et al.*, 1994; Scripture *et al.*, 2006). In addition, it is believed that Cremophor-EL forms large micelles, which can entrap small molecules co-administered with Cremophor-formulated drugs and lead to alterations in biodistribution, interfere with efficacy, and contribute to off-target toxicities of the entrapped drugs (Hawkins *et al.*, 2008).

Reformulation of immunosuppressive agents using nanotechnology platforms is intended to alleviate these problems by improving solubility, providing fine targeting, allowing a lower dosage, reducing side effects and offering alternative less-invasive delivery routes. In this section, we will review examples demonstrating challenges with traditional immunosuppressive drugs and advantages imparted by their reformulation using nanotechnology. Available data will be discussed in four sections based on the mechanism of action of the immunosuppressive drugs. Examples of traditional drugs and nanotechnology platforms used for their reformulation are summarized in Table 1.

Inhibition of T-cells

T-lymphocytes represent one of the most common targets in immunosupressive intervention. Activation of these cells is initiated by interaction with antigen-presenting cells [APC; e.g. dendritic cells (DC)] displaying antigen-major histocompatibility complexes (MHC) and co-stimulatory molecules on their surface. One of the consequences of T-cell activation is the initiation of transcription and synthesis of IL-2 (see Alexander *et al.*, 2013b), a cytokine essential for T-cell proliferation. As such, the activation of T-cells can be interrupted during both antigen recognition and signal transduction (Getts *et al.*, 2011).

The fungal peptide cyclosporine A and the bacterial macrolide lactone tacrolimus, approved by the US FDA in 1983 and 1994, respectively, are widely used in transplant medicine and for treatment of autoimmune disorders (Liu *et al.*, 2007). Cyclosporine A and tacrolimus both interfere with activity of calcineurin, a factor critical for activation of the transcription factor, nuclear factor of activated T-cells, thus preventing transcription of genes encoding cytokines and decreasing the rate of graft rejection (Abbas *et al.*, 2012). For traditional medical use, these agents are typically formulated in vegetable oils (e.g. Sandimmune® or Cipol®) or in gelatin capsules (e.g. Neoral®). Common side effects from these agents include kidney damage, cardiotoxicity and high BP (Bottiger *et al.*, 1999; Liu *et al.*, 2007). Their poor solubility in water, low bioavailability and high inter-patient variability in

Table 1

Reformulation of traditional immunosuppressive and anti-inflammatory drugs into nanotechnology platforms

Drug	Nanocarrier	Status
Cyclosporine	Liposomes (Freise *et al.*, 1994; Shah *et al.*, 2006); polymeric NP (Gref *et al.*, 2001; Italia *et al.*, 2007; Azzi *et al.*, 2010; Tang *et al.*, 2012); lipid NP (Muller *et al.*, 2008)	Preclinical
Tacrolimus	Lipid NP (Pople and Singh, 2012); polymeric NP (Tammam *et al.*, 2012); liposomes (Erdogan *et al.*, 2002; Zhang *et al.*, 2010)	Preclinical
Rapamycin/sirolimus	Polymeric NP (Yuan *et al.*, 2008; Woo *et al.*, 2012; Shah *et al.*, 2013); micelles (Yanez *et al.*, 2008; Chen *et al.*, 2013); liposomes (Rouf *et al.*, 2009; Ghanbarzadeh *et al.*, 2013)	Preclinical
Mycophenolic acid	Polymeric NP (Shirali *et al.*, 2011); nanogels (Look *et al.*, 2013); dendrimers (Hu *et al.*, 2009)	Preclinical
Corticosteroids	Liposomes (Metselaar *et al.*, 2003; Linker *et al.*, 2008; Schweingruber *et al.*, 2011; Ulmansky *et al.*, 2012); polymeric NP (Ishihara *et al.*, 2005; Matsuo *et al.*, 2009); solid lipid NP (Jensen *et al.*, 2010; Zhang and Smith, 2011); dendrimers (Khandare *et al.*, 2005)	Preclinical
Non-steroidal anti-inflammatory	Dendrimers (Chauhan *et al.*, 2004; Na *et al.*, 2006; Chandrasekar *et al.*, 2007; Cheng *et al.*, 2007); nanocolloid (Milkova *et al.*, 2013); lipid NP (Castelli *et al.*, 2005); liposomes (Paavola *et al.*, 2000; Srinath *et al.*, 2000; Turker *et al.*, 2008); polymeric NP (Agnihotri and Vavia, 2009; Cooper and Harirforoosh, 2014)	Preclinical

Many traditional immunosuppressive and anti-inflammatory drugs were attempted for reformulation using a variety of nanotechnology carriers. Examples of such studies and nanoparticle carriers are summarized in this table. All of these novel formulations are in preclinical phase of drug development.

metabolism and excretion render dose monitoring of these drugs difficult (Bottiger *et al.*, 1999; Liu *et al.*, 2007).

Some of these challenges may be circumvented by formulating the immunosuppressants into nanoparticles (Canadas *et al.*, 2004; Liu *et al.*, 2007; Czogalla, 2009; Shin *et al.*, 2010; Pople and Singh, 2012; Tammam *et al.*, 2012). For example, liposomal and polymeric nanoparticle reformulation of cyclosporine significantly reduced nephrotoxicity of the drug in rats and in a rat ischaemic kidney model (Freise *et al.*, 1994; Italia *et al.*, 2007). Incorporation of tacrolimus into 75 nm lipid nanoparticles resulted in improved skin penetration and deposition, and reduced side effects in comparison to the traditional formulation Protopic® (Pople and Singh, 2012). Another example of using nanotechnology to improve delivery of T-cell-specific immunosuppressive drugs is the study by J. Azzi *et al.*, in which polylactide nanoparticles were used for e*x vivo* delivery of cyclosporine A into DC (Azzi *et al.*, 2010). Re-injection of these drug-loaded DC into footpads of BALB/c mice facilitated delivery of the drug to the lymph nodes *in vivo* (Azzi *et al.*, 2010). Interestingly, nanoparticles protected DC from the toxic effects of cyclosporine A, ensuring its delivery to lymph nodes where released cyclosporine A suppressed T-cell proliferation.

Rapamycin is another traditional immunosuppressive agent used to inhibit T-cells through a mechanism distinct from that of cyclosporine A and tacrolimus (Kahan, 2011). Rapamycin inhibits mammalian target of rapamycin (mTOR) complex 1, a protein complex that includes serine/threonine protein kinase mTOR (Thomson *et al.*, 2009). mTOR provides an important link in many signalling pathways and in processes such as protein synthesis, intracellular trafficking, mRNA turnover and autophagy. Inhibition of mTOR suppresses T-cell activation, proliferation, and development of forkhead box P3 (FOXP3) positive cells. However, it is not specific to T-cells, and as such, mTOR blockade has an effect on a variety of cells including other immune cells. Inhibition of mTOR leads to the suppression of DC maturation, B-cell activation, neutrophil chemotaxis and uptake of antigen by APC (Thomson *et al.*, 2009). The adverse effects observed in patients treated with mTOR inhibitors include, but are not limited to, dose-dependent hyperlipidaemias, kidney toxicity, dermatological complications and bone marrow suppression (Campistol *et al.*, 2010; Kahan, 2011). Formulation of rapamycin using nanotechnology has helped to overcome its poor solubility, improve its safety profile and increase its therapeutic efficacy (Woo *et al.*, 2012; Chen *et al.*, 2013; Shah *et al.*, 2013). Delivery of rapamycin by elastin-like polymeric nanoparticles has been associated with reduced kidney toxicity and injection site reactions, yet demonstrated therapeutic efficacy comparable to, or in some parameters even exceeding, that of the free drug in a mouse model of Sjogren syndrome, a systemic autoimmune disorder destroying exocrine glands, which produce tears and saliva (Shah *et al.*, 2013). Rapamycin delivered on chitosan/polylactic acid nanoparticles prolonged the presence of drug at the procorneal area of rabbits' eyes after corneal transplantation and increased the median allograft survival time (Yuan *et al.*, 2008).

Mycophenolic acid (MPA) is a conventional immunosuppressive drug inhibiting T- and B-cells. Common side effects observed clinically include nausea, vomiting, diarrhoea, leucopaenia and anaemia. MPA reformulation using polylactic-co-glycolic acid (PLGA) nanoparticles or nanogel platforms have achieved extended skin graft survival using lower doses, which eventually resulted in decreased systemic toxicity (Shirali *et al.*, 2011; Look *et al.*, 2013; 2014). It has also been demonstrated that internalization of MPA-loaded nanoparticles by DC results in stronger suppression of IL-12 and IFN-γ levels as compared with conventional MPA. In addition, nanoparticle-formulated MPA resulted in up-regulation of surface expression of programmed death ligand-1, a negative regulator of T-cells, which did not occur with conventional MPA (Shirali *et al.*, 2011; Look *et al.*, 2013).

Although reformulation of immunosuppressive agents using nanotechnology platforms helps to reduce undesirable side effects, suppression of T-cells can still be non-specific, leaving these improved formulations prone to causing off-target toxicity. To further improve specificity of T-cell suppression, nanoparticulate vaccines inducing tolerance to a particular antigen have been developed. Improved specificity of these nanoformulations has been achieved by engineering nanoparticles to tune induction of the cytokine profile supporting particular T-cell subtypes, to inhibit self-reactive T-lymphocytes or exclusively increase regulatory T-cell (T-reg or $FOXP3^+$ T-cells) subpopulations, which are crucial in supporting self-tolerance and down-regulating the immune response (Beissert *et al.*, 2006), Examples of nanoparticle-based tolerogenic vaccines utilizing these mechanisms are described in more detail below. In the model of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, administration of polystyrene nanoparticles coupled with the myelin antigen were proven effective in suppressing both acute and relapse phases of multiple sclerosis (Getts *et al.*, 2012). Activation of $CD4^+CD25^+$ T-cells, T-cell anergy and abortive activations were proposed as potential mechanisms contributing to the observed tolerance. Interestingly, induction of tolerance by polystyrene-myelin nanoparticles was dependent on particle size: smaller nanoparticles were more immunosuppressive than their larger counterparts. However, this effect was limited to the relapse and was not observed in the acute phase. The study suggested that smaller nanoparticles are not recognized by the macrophage scavenger receptor macrophage receptor with collagenous domain involved in development of tolerance, and a clear understanding of the differences between the acute phase and relapse has yet to be attained (Getts *et al.*, 2012). PLGA nanoparticles loaded with leukaemia inhibitory factor and decorated with anti-CD4 antibody were used to augment generation of $FOXP3^+$ T-cells. These particles extended the survival time of the fully mismatched graft from 7 to 12 days in a mouse model of vascularized heart allografts (Park *et al.*, 2011). In another study, iron oxide nanoparticles (IONPs) coated with diabetes-specific peptides in the context of MHC were successful in suppressing the response to diabetic antigens in mice (Tsai *et al.*, 2010). Of interest, the disease-induced autoregulatory cells expanded using IONPs were $CD8^+$ and $FOXP3^-$, yet they expressed markers of memory cells CD44 and CD122. The significant finding of this study is that these regulatory $CD8^+$ cells specifically targeted autoantigen-loaded APC, suppressing the response to the pool of diabetic antigens.

Nanoparticles have also been used to co-deliver an antigen and a co-stimulatory moiety to DC. It is known from earlier studies that 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), a ligand for aryl hydrocarbon receptor, facilitates generation of T-reg through induction of the tolerogenic DC (Quintana *et al.*, 2010). Yeste *et al*. prepared gold nanoparticles covered with polyethylene glycol (PEG) carrying both ITE and myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$-specific T-cell epitope to induce generation of T-regs restricted to $MOG_{35–55}$ antigen (Yeste *et al.*, 2012).

Lastly, nanoparticles have been successfully employed for the delivery of vaccines inducing tolerance to food allergens. Oral administration of chitosan nanoparticles engineered to deliver DNA-encoding chicken ovalbumin (OVA) resulted in the development of tolerance to this protein antigen. This tolerance development was mediated by $CD4^+CD25^+$ T-cells and confirmed by adoptive transfer. The study also documented a favourable change in the cytokine profile for development of T-reg cells (Goldmann *et al.*, 2012).

Delivery of anti-inflammatory drugs

Corticosteroids have a long history of use for the treatment of chronic inflammatory disorders (Schweingruber *et al.*, 2011). Sustaining efficacious concentrations of glucocorticoids in the blood requires high doses and frequent injections due to their rapid clearance from circulation. Therefore, it is not surprising that chronic use of corticosteroids is associated with severe side effects. Conceivably resolving challenges with current anti-inflammatory standard-of-care could be achieved by extending drug circulation time, active targeting, controlled release and retention of the corticosteroids in the inflamed tissue through the use of engineered nanomaterials as delivery vehicles (Mitragotri and Yoo, 2011). Below we will provide several examples demonstrating the efficacy of nanoparticles as carriers for corticosteroids. For example, loading glucocorticoids into liposomes prolonged drug circulation time. As a consequence, it allowed for a reduced number of injections and dose while still achieving similar efficacy to that of the free drug in rat models of rheumatoid arthritis (Metselaar *et al.*, 2003; Anderson *et al.*, 2010; Ulmansky *et al.*, 2012) and EAE (Schmidt *et al.*, 2003; Linker *et al.*, 2008). Furthermore, incorporation of glucocorticoids into liposomes changed drug distribution: while free glucocorticoids acted mainly through T-lymphocytes, their liposomal counterparts targeted macrophages to induce M2 phenotype expressing anti-inflammatory cytokines (Schweingruber *et al.*, 2011). Nanoparticles have also been employed for co-delivery of anti-inflammatory agents. For example, dexamethasone-loaded PLGA nanoparticles were combined with siRNA targeting COX-2 to suppress inflammatory responses (Park *et al.*, 2012). PEGylation of the polymeric nanoparticles loaded with betamethasone phosphate prolonged particle circulation and increased drug accumulation in the inflamed tissues leading to a stronger anti-inflammatory effect (Ishihara *et al.*, 2009; Sakai *et al.*, 2011).

Dendrimers have also been successfully used for delivery of anti-inflammatory drugs (Kolhe *et al.*, 2003; Chauhan *et al.*, 2004; Na *et al.*, 2006; Chandrasekar *et al.*, 2007; Cheng *et al.*, 2007). In addition to improving drug solubility, incorporation of celastrol into G4-OH polyamidoamine (PAMAM) dendrimers allowed for reduced drug toxicity (Boridy *et al.*, 2012). However, it is important to note that drug loading into nanocarriers has the potential to change the drug's original properties. The celastrol-conjugated dendrimer, for example, retained its capacity to suppress LPS-induced NO release; however, it lost its ability to inhibit production of pro-inflammatory cytokines (Boridy *et al.*, 2012). PAMAM dendrimers have also been used to deliver methotrexate and indomethacin to reduce inflammation in the rat model of arthritis (Chandrasekar *et al.*, 2007; Thomas *et al.*, 2011). Since folate receptor β is expressed on activated but not on resting macrophages, functionalization of dendrimers with folate as a targeting ligand allowed for delivery of anti-inflammatory drugs to activated macrophages (Chandrasekar *et al.*, 2007; Thomas *et al.*, 2011).

Anti-cytokine activity

Overwhelming expression of pro-inflammatory cytokines can damage healthy tissues. As such, anti-cytokine approaches have been developed for the therapeutic intervention of cytokine mediated toxicities. Two main approaches were considered: (i) preventing interaction between cytokine and its receptor, and (ii) reducing cytokine gene expression. The former was achieved through neutralization of either the cytokine itself or its respective receptor. The main challenge with this was to deliver the cytokine or receptor antagonist to, and retain within, the inflamed tissue. Functionalization of the nanoparticle surface with targeting moieties has allowed for some of these challenges to be addressed. For example, coating the nanoparticle surface with an Arg-Gly-Asp moiety (RGD) peptide specific to αVβ3 integrin ensured delivery of nanoparticles to the sites of active angiogenesis, which often accompanies inflammation (Scheinman *et al.*, 2011).

Traditional therapy for rheumatoid arthritis involves injection of anti-inflammatory agents directly into joints. However, good retention inside the joint is achieved only for molecules larger than 100 kDa, while smaller drugs rapidly leave the joint (Whitmire *et al.*, 2012). Nanoparticles have been engineered to address this challenge. Furthermore, nanoparticles have been shown to better penetrate the synovium than microparticles (Horisawa *et al.*, 2002). Copolymeric particles loaded with a IL-1 receptor antagonist (IL-1RA) have been shown to reside in joints longer than IL-1RA itself; however, the efficacy of this construct was not studied in a disease model *in vivo*. (Whitmire *et al.*, 2012). Glucosamine attached to anionic generation 3.5 PAMAM dendrimers suppressed LPS-triggered secretion of pro-inflammatory cytokines through a mechanism involving competition for the LPS-binding pocket of the MD2 component of the LPS receptor complex (Shaunak *et al.*, 2004; Barata *et al.*, 2011).

Cytokine production can also be significantly reduced by siRNA silencing expression of either the cytokine gene itself, or of components of the signalling pathways leading to activation of cytokine gene expression (Scheinman *et al.*, 2011). Challenges common to therapeutic delivery of siRNA include lack of targeting, low potency, off-target toxicities and poor stability in biological matrices, all of which conceivably can be addressed through the use of nanocarriers. For example, chitosan nanoparticles and cationic liposomes were used for delivery of siRNA inhibiting TNF-α. Both formulations significantly decreased TNF-α secretion and

improved the disease score in the mouse model of collagen-induced arthritis (Khoury *et al.*, 2006; Howard *et al.*, 2009). RGD-coated PLGA nanoparticles were used to protect STAT1 siRNA from degradation by serum nucleases. RGD targeting improved siRNA uptake in the paw tissue of arthritic mice and increased delivery of nanoparticles into lungs. Animals treated with RGD-PLGA-STAT1-siRNA nanoparticles recovered while disease progressed in all control groups (Scheinman *et al.*, 2011). Another approach to decrease the level of pro-inflammatory cytokines is to induce synthesis of anti-inflammatory cytokines. For example, cationic polymeric nanoparticles loaded with DNA encoding the anti-inflammatory cytokine IL-10 prevented severe autoimmune damage of the pancreas in a mouse model of autoimmune diabetes (Basarkar and Singh, 2009). In some cases, nanoparticles may switch on anti-inflammatory signalling in cells. For instance, dendrimers consisting of a cyclotriphosphazene core, phenoxymethyl-methylhydrazone branches and capped with azabisphosphonate decreased levels of pro-inflammatory cytokines and induced production of anti-inflammatory cytokines in mice with experimental arthritis (Hayder *et al.*, 2011).

Anti-adhesive and anti-cell recruitment activity

Mitigation of inflammation and the associated tissue damage can be achieved by harnessing the cell types recruited to the inflamed tissue. Nanoparticles may be used to inhibit recruitment of inflammatory monocytes to the compromised tissue. For example, lipid nanoparticles encapsulating the chemokine receptor CCR2-specific siRNA (for receptor nomenclature see Alexander *et al.*, 2013a) delayed graft rejection of pancreatic islet allografts in mice with streptozotocin-induced diabetes (Leuschner *et al.*, 2011). Adhesion of leukocytes to endothelial cells forming the blood vessel walls is an initial step facilitating leukocyte migration to the sites of inflammation. The initial interaction between leukocytes and endothelial cells occurs through adhesion molecules and carbohydrates ligands on the surface of these cells. Breaking this interaction will inhibit leukocyte recruitment to the site of inflammation. Dendrimer-like nanoparticles were used in several studies for exactly this purpose (Rele *et al.*, 2005; Dernedde *et al.*, 2010). For instance, β-lactose functionalized poly(ethylene oxide) dendrimer-like polymers inhibited leukocyte adhesion through L-selectin (Rele *et al.*, 2005). Moreover, dendritic polyglycerol sulfates not only prevented leukocyte interaction with both L- and P-selectins, but they also reduced levels of pro-inflammatory anaphylatoxins (Dernedde *et al.*, 2010). Polymerized lipid nanoparticles bearing P-selectin inhibitors on the surface demonstrated anti-inflammatory activities in a murine model of asthma (John *et al.*, 2003). As in earlier examples with anti-inflammatory agents, reformulation of conventional anti-adhesive agents onto nanotechnology platforms could improve efficacy of these inhibitors. For instance, the cholesteryl butyrate solid lipid nanoparticles were more effective inhibitors of neutrophil adhesion to endothelial cells than free sodium butyrate; this greater efficacy was hypothesized to result from rapid internalization of solid lipid nanoparticles into cells (Dianzani *et al.*, 2006).

Unintended immunosuppression

Since inhibition of the immune system may decrease host resistance to infections and cancer, as well as lead to thymic suppression and myelosuppression, identification of undesirable immunosuppressive properties of engineered nanomaterials is an important component of establishing their safety profile. It is generally recognized that a nanoparticle's physicochemical properties determine their interactions with the immune system. Such structure activity relationships have been described for a variety of components of the immune system in the context of adverse immunostimulation, including, but not limited to, complement activation, platelet activation and induction of leukocyte procoagulant activity (Dobrovolskaia *et al.*, 2012; Ilinskaya *et al.*, 2013). However, studies investigating immunosuppressive properties of nanoparticles *per se* are scarce (Chen *et al.*, 2004; Mitchell *et al.*, 2009; Yamashita *et al.*, 2009; Shen *et al.*, 2011; 2012). In part, this may be explained by methodological challenges and the lack of a systematic approach. Immunosuppression is not an acute toxicity, which can be easily monitored *in vitro*; it affects function of the immune system, and assessing functional changes involves long-term, systematic, multi-parameter *in vivo* studies evaluating various aspects of immunity. Many *in vitro* studies demonstrating the immunosuppressive properties of tested nanomaterials are focused on a limited number of cellular processes – most commonly cytokine production and surface marker expression – and do not provide sufficient details for gaining insight into the immunosuppressive potential of nanoparticles. For example, nanoparticles inducing production of the anti-inflammatory cytokine TGF-β are not necessarily immunosuppressive. Although TGF-β suppresses the proliferation of lymphocytes, in the presence of certain cytokines (e.g. IL-6 and IL-1) it also induces development of T helper 17 (Th17) cells, which induce inflammation in a variety of autoimmune disorders (Abbas *et al.*, 2012). It is also important to note what cell type is producing TGF-β, as this cytokine can be expressed by M2 macrophages, Th3 cells and T-regs, each of which perform distinct functions. Unfortunately, none of the studies describing induction of TGF-β by engineered nanomaterials attempted to identify the cell type producing this cytokine. To further complicate this subject, some nanoparticles can suppress one immune function while stimulating another one. For example, silica oxide nanoparticles decreased expression of the innate immune receptor toll like receptor 9 (TLR9; for nomenclature see Alexander *et al.*, 2013b) thus halting immune responses to CpG oligonucleotides, but enhanced TLR4-mediated LPS-induced production of the pro-inflammatory cytokines IL-1β and TNF-α (Lucarelli *et al.*, 2004). We speculate that such diverse reactions occurred because nanoparticles can enter cells through different pathways and interfere with immune cell function through a variety of mechanisms. While several mechanisms attributing certain structural properties of nanoparticles to their pro-inflammatory effects have been described, it is largely a grey area for immunosuppressive properties (Nel *et al.*, 2006; Dobrovolskaia and McNeil, 2007).

Below, we will review studies describing unintended immunosuppression and anti-inflammatory properties of nanoparticles. By 'unintended' we refer to suppression of

immune responses or inhibition of inflammatory reactions by nanoparticles designed for applications other than suppressing immunity. Among these unintended properties, some can be considered as beneficial while others to be adverse. Delineation between beneficial and adverse unintended immunosuppression is not always straightforward. In many cases, this depends on the model studied and end points (e.g. cytokine secretion, cell adhesion, cell viability) evaluated. The same nanoparticles may be beneficial in one model and/or using one end point, and adverse when using another model or end point. Until more data allowing for a foundation of clear criteria for separation between beneficial and adverse unintended immunosuppression becomes available, we will refer to these 'dual' properties as modulatory. We will categorize myelosuppression and toxicity to the cells of the immune system as adverse due to the clear detrimental consequences to the function of the immune system. We further stress the need for a systematic approach in the evaluation of immunosuppressive properties of engineered nanomaterials.

Modulatory effects

Anti-inflammatory nanoparticles. The anti-inflammatory properties of PAMAM dendrimers were discovered spontaneously while exploring the dendrimers as carriers for indomethacin (Chauhan *et al.*, 2009). These properties were confirmed by *in vitro* studies measuring NO production and inhibition of COX, as well as *in vivo* in three different models: (i) carrageenan-induced oedema, (ii) cotton pellet test and (iii) adjuvant-induced arthritis in rats. Interestingly, the anti-inflammatory properties of PAMAM dendrimers depended on the surface functionalization and generation (i.e. particle size), but not on the core. Only large amine- and hydroxyl-terminated dendrimers were able to inhibit inflammation, while there was no difference between 1,2-diaminoethane and 1,12-diaminododecane core dendrimers of the same generation and the same surface functionality (Chauhan *et al.*, 2009). Furthermore, the anti-inflammatory activity of generation 4 amine terminated dendrimers was concentration-dependent (Chauhan *et al.*, 2009). Through *in vitro* mechanistic experiments, this study suggested that the observed anti-inflammatory activity of amine- and aminoethylethanolamine-terminated PAMAM dendrimers was due to the inhibition of COX-1 (for nomenclature see Alexander *et al.*, 2013c) and COX-2 (Chauhan *et al.*, 2009). Another study demonstrated that hydroxyl-terminated G4 PAMAM dendrimers reduced synthesis of LPS-triggered IL-6 through the mechanism involving interference with the LPS signalling pathway and p38 phosphorylation in N9 microglia cells (Boridy *et al.*, 2012).

Antioxidants. Despite the fact that toxicity of certain nanomaterials is attributed to their ability to induce oxidative stress and free radical formation, there are some nanoparticles with intrinsic antioxidant properties (Nel *et al.*, 2006). For example, cerium oxide nanoparticles, due to their ability to switch between a 3^+ and 4^+ oxidative state, possess 'reactive sites' quenching free radicals. In addition to direct quenching of radicals, cerium oxide nanoparticles could reduce inducible NOS (iNOS) amounts at both mRNA and protein levels (Hirst *et al.*, 2009). Reduction in iNOS mRNA and NO levels triggered by LPS was also observed in macrophages treated with gold nanoparticles (Ma *et al.*, 2010). Further mechanistic studies revealed that gold nanoparticles can interfere with NF-κB and STAT1 signalling pathways in that pretreatment of macrophages with gold nanoparticles decreased degradation of IκB-α and amounts of p-Akt in response to LPS stimulation (Ma *et al.*, 2010).

Certain fullerene derivatives can effectively lower levels of reactive oxygen species and prevent oxidative stress-mediated cell death *in vitro* (Chen *et al.*, 2004). Moreover, some of these fullerene derivatives are effective *in vivo* and can mitigate ischaemia-reperfusion-induced oxidative stress in rats (Chen *et al.*, 2004). The main problem limiting application of fullerenes as antioxidants is dose-dependence of the oxidation protective effect and toxicity of fullerenes at high doses.

Anti-cytokine activity. Several examples of anti-cytokine activity have been described for gold colloids. Particularly, citrate stabilized gold nanoparticles prevented the development of pro-inflammatory responses initiated by IL-1β in THP-1 cells (Sumbayev *et al.*, 2012). Of interest is the selectivity of this effect: gold nanoparticles inhibited only an IL-1β-induced response but not that triggered by other factors such as TLR7/8 ligand R848 and stem cell factor. Anti-inflammatory properties of gold nanoparticles were size-dependent in that smaller particles were more effective than their larger counterparts (Sumbayev *et al.*, 2012). Another recent study reported that citrate stabilized gold nanoparticles attenuate TNF-α induction triggered by CpG-oligodeoxynucleotides (ODNs; Tsai *et al.*, 2012). Ligands to other TLRs (imiquimod to TLR7, LPS to TLR4, Poly I:C to TLR3 and lipoteichoic acid to TLR2) were also included in this study; however, consistent inhibition of TNF secretion was observed only in response to TLR9 ligand CpG ODN (Tsai *et al.*, 2012). Interestingly, imiquimod-induced TNF-α was inhibited by gold nanoparticles only at one concentration 1 $\mu g \cdot mL^{-1}$, but not at 10 $\mu g \cdot mL^{-1}$. Consistent with IL-1 studies discussed above, the inhibition of TNF-α production in this case was particle size-dependent, with smaller particles being more potent than their larger counterparts (Tsai *et al.*, 2012). Colloidal gold is known to readily bind proteins. This property has been widely applied for years in immunoelectron microscopy in which gold nanoparticle-tagged antibodies are used for detection of cellular antigens (Baschong and Wrigley, 1990). Intuitively, the mechanism of cytokine inhibition may be due to gold nanoparticle binding to the cytokine or its receptor and/or any other element critical in signal transduction leading to cytokine protein expression. Ivanov *et al*. suggested that gold nanoparticles interfere with TLR9 trafficking and accumulate in the lysosomes where they bind to high-mobility group box-1 protein essential for TLR9 function (Ivanov *et al.*, 2007). The reason for the observed size-dependence is probably due to the difference in particle number and surface area. At equivalent concentrations of gold, there are a greater number of smaller sized particles than larger sized particles. Consequently, the total surface area is greater in smaller nanoparticles. What is harder to explain is the specificity and concentration-dependence of the observed inhibition. One also has to be careful in interpreting cytokine inhibition studies, because nanoparticles

present in supernatants used for cytokine analysis may interfere with ELISA and other immunoassays used for cytokine detection and lead to erroneous data (Dobrovolskaia *et al.*, 2008; Kroll *et al.*, 2012; Guadagnini *et al.*, 2013).

Inhibition of cell-mediated immunity. Type IV (cell-mediated) hypersensitivity reactions include delayed-type hypersensitivity (DTH) triggered by Th1 and Th17 cells. Th1 lymphocyte cytokines, particularly IFN-γ, dominate in the development of the DTH (Kobayashi *et al.*, 2001). Different types of nanoparticles have been shown to reduce DTH through different mechanisms. For example, both a colloidal suspension of crystalline fullerene C60 and IONPs (Resovist®) reduced footpad swelling caused by methyl BSA and OVA, respectively, in the murine model of DTH (Yamashita *et al.*, 2009; Shen *et al.*, 2012). In the latter case, it was suggested that the IONPs inhibited DTH by shifting the cytokine balance from Th1 to Th2 because a decrease in IFN-γ and an increase in IL-4 production was detected in splenocytes treated with these nanoparticles (Shen *et al.*, 2012). In contrast to Resovist, fullerene suppressed IL-4 and enhanced TNF-α production, but did not affect IFN-γ secretion. Furthermore, fullerene suppressed the production of the pro-inflammatory cytokines IL-6 and IL-17 (Yamashita *et al.*, 2009). These data suggest that immunosuppression observed with the colloidal suspension of crystalline C60 is due to elevation of T-reg cell number and the inhibition of Th17 cells (Yamashita *et al.*, 2009). However, the exact mechanism(s) is unknown in both fullerene and iron oxide examples and clearly warrants further investigation.

Interference with normal response to antigens. Unintended immunosuppression may weaken host resistance to infections and cancer. A single i.p. dose of iron oxide nanoparticles (Resovist) administered to Balb/c mice 1 h prior to challenge with model antigen (OVA) attenuated production of OVA-specific antibodies. Furthermore, the production of IFN-γ and IL-4 was significantly decreased in splenocytes isolated from these mice (Shen *et al.*, 2011). Inhalation of multi-walled carbon nanotubes (MWCNT) resulted in suppression of antibody production and T-lymphocyte proliferation in response to the sheep red blood cell challenge (Mitchell *et al.*, 2009). Since inhaled MWCNT do not enter the systemic circulation, it was hypothesized that the observed immunosuppressive effect did not result from a direct interaction between the carbon nanotubes and spleen cells. Through the series of experiments, Mitchell *et al.* demonstrated that inhaled MWCNT induced production of TGF-β in alveolar macrophages. When TGF-β distributed systemically, it triggered activation of the COX pathway and IL-10 production in the spleen leading to suppression of antibody production (Mitchell *et al.*, 2009).

Some nanoparticles may affect the antigen-presenting capacity of DC. Using different types of fluorescent dyes conjugated to OVA, it was demonstrated that poly(vinylalcohol)-coated super-paramagnetic iron oxide nanoparticles (PVA-SPIONs) did not inhibit antigen uptake by monocyte-derived dendritic cells (MDDC), but rather interfered with antigen processing. In addition, PVA-SPION significantly reduced proliferation of T-cells promoted by the autologous MDDC, decreased production of pro-inflammatory cytokines (IL-1β, IL-5, IL-6, IL-12p70, IFN-γ, TNF-α) and enhanced LPS-induced production of the anti-inflammatory cytokine IL-10 (Blank *et al.*, 2011). Phagocytic cells are more prone to nanoparticle toxicity due to their greater likelihood of internalizing nanoparticles. For example, quantum dots, at non-cytotoxic concentrations, accumulated in J774A.1 macrophages, but not in Hepa-1 hepatocytes, *in vitro*. Such accumulation reduced functional activity of J774A.1 through interferences with normal cytoskeleton function (Qu *et al.*, 2012).

Adverse effects

Myelosuppression. Myelosuppression is a condition in which activity of the bone marrow is decreased so that fewer erythrocytes, lymphocytes and platelets are present in the blood. Suppression of fine activity of the bone marrow may lead to life-threatening conditions such as anaemia, thrombocytopenia and decreased resistance to pathogens and cancer. Some nanoparticles can be toxic to bone marrow cells. For example, toxicity to haematopoietic progenitors was reported for antimony oxide (Sb_2O_3) and cobalt nanoparticles (Bregoli *et al.*, 2009). Among seven tested nanoparticles (Fe_2O_3, Fe_3O_4, Sb_2O_3, Au, TiO_2, Co and Ag), only Co and Sb_2O_3 nanoparticles at concentrations of 100 and 25 ppm suppressed formation of colonies from both erythroid and granulocytic-monocytic precursors in primary cultures of human haematopoietic progenitor cells (Bregoli *et al.*, 2009).

Myelosuppression is also a common dose-limiting toxicity of cytotoxic oncology drugs. The main intention in using nanoparticles for delivery of cytotoxic drugs is to decrease toxicity of the latter through precise targeting, slow release and a decreased dose. However, not all nanoparticles can achieve this goal. If a nanoparticle carrier is toxic to bone marrow, it may exaggerate the toxicity of the drug. In the example discussed above, cobalt and antimony nanoparticles would not be suitable for oncology drug delivery (Bregoli *et al.*, 2009). One has to also keep in mind that nanoparticles *per se* may be harmless to bone marrow cells, but may enhance the myelosuppressive effects of drugs they carry due to a change in the biodistribution. For example, doxorubicin conjugated to polyisobutyl (PIBCA) and polyisohexylcyanoacrylate (PIHCA) nanoparticles was significantly more myelosuppressive than the free drug (Gibaud *et al.*, 1994). Moreover, the severity of the myelosuppression was carrier-dependent; it was greater in PIHCA than in PIBCA nanoparticles. This unfortunate effect was due to accumulation and retention of the conjugated doxorubicin in bone marrow and the spleen due to a greater particle uptake by the phagocytic cells. Passivation of nanoparticle surfaces with hydrophilic polymers such as PEG is generally recognized as a reliable means of increasing the 'stealthness' of nanoparticles, leading to lower accumulation in mononuclear phagocytic cells. Intuitively, nanoparticles that increase myelosuppression of cytotoxic drugs due to increased phagocytic uptake could be engineered to avoid myelosuppression through this mechanism.

Cytotoxicity to the immune cells. Different types of primary immune cells may exert different sensitivities to the same nanoparticle type. For example, widely used in cosmetic products and sunscreens, ZnO nanoparticles are toxic to

monocytes but do not affect the viability of lymphocytes (Hanley *et al.*, 2009). NK cells are more sensitive to ZnO than T and B lymphocytes, but less sensitive than monocytes exposed to the same dose of nanoparticles (Hanley *et al.*, 2009). These results were partially confirmed by another study demonstrating that ZnO nanoparticles at concentrations leading to low toxicity in human peripheral blood mononuclear cells (PBMC) were extremely cytotoxic to MDDC (Andersson-Willman *et al.*, 2012). This study did not evaluate the viability of various cell populations in PBMC; therefore, it is hard to say whether the toxicity observed in bulk PBMC was due to nanoparticle effects on monocytes only, or whether it affected lymphocytes as well (Andersson-Willman *et al.*, 2012). The mechanism of toxicity was attributed to dissolution of ZnO nanoparticles leading to an elevation of Zn ion concentration inside the cells and subsequent mitochondrial dysfunction-triggered apoptosis (Kao *et al.*, 2012). Another metal oxide nanoparticle, TiO_2, was not found to be cytotoxic *in vitro*, but caused significant immunosuppression *in vivo* (Moon *et al.*, 2011; Andersson-Willman *et al.*, 2012). Systemic administration of TiO_2 nanoparticles inhibited T-cells, B-cells, macrophages and NK cells, and was associated with greater susceptibility to a melanoma challenge (Moon *et al.*, 2011; Andersson-Willman *et al.*, 2012).

Conclusion and future directions

Immunosuppressive and anti-inflammatory effects of engineered nanomaterials can be intentionally achieved by engineering the nanoparticle physicochemical properties and by using nanoparticles as carriers for immunosuppressive and anti-inflammatory agents. Existing data suggest that similar to immunostimulation, nanoparticle-mediated suppression and inhibition of immune function is determined by the nanoparticle's physicochemical properties. However, systematic structure–activity relationship (SAR) studies are needed to further advance this area of nanoimmunology. In addition to SAR investigations, future studies should focus on the mechanisms of nanoparticle-mediated immunosuppression and on identifying key elements (dose, route of administration, physicochemical properties and composition) triggering immunomodulatory effects. Understanding what makes the same nanoparticle immunostimulatory in one model and immunosuppressive in another model is critical. This will aid drug delivery formulation scientists in choosing appropriate nanoparticle carriers and will clearly advance the rapidly growing field of nanoimmunotoxicology.

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Conflict of interest

The authors declare that they do not have conflicting interest with any drug or device mentioned in this manuscript.

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