CHAPTER NINE

Nanomaterials to target immunity

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Abstract

Critical advances have recently been made in the field of immunotherapy, contributing to an improved understanding of how to harness and balance the power of immune responses in the treatment of diseases such as cancer, cardiovascular disease, infectious diseases, and autoimmune diseases. Combining nanomedicine with immunotherapy provides the opportunity for customization, rational design, and targeting to minimize side effects and maximize efficacy. This review highlights current developments in the design and utilization of nano-based immunotherapy systems, including how rationally-designed nanosystems can target and modify immune cells to modulate

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immune responses in a therapeutic manner. We discuss the following topics: targeted immuno-engineered nanoformulations, commercial formulations, clinical applicability, challenges associated with current approaches, and future directions.

Abbreviations

CAR	chimeric antigen receptor
IFNγ	interferon-gamma
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage CSF
MALT	mucosa-associated lymphoid tissues
PLGA	poly(lactic-*co*-glycolic acid)
PEG	polyethylene glycol
APC	antigen-presenting cells
DC	dendritic cell
TGFβ	transforming growth factor beta
ITE	2-(1H-indole-3-carbonyl)-thiazole-4-carboxylic acid methyl ester
Foxp3	forkhead box protein P3
Treg	regulatory T cells
siRNA	small interfering ribonucleic acid
MHC	major histocompatibility complex
CTLA	cytotoxic T-lymphocyte-associated protein
MDSC	myeloid-derived suppressor cells
IL-10	interleukin-10
PD-1/PD-L1	programmed cell death/corresponding ligand PD- L1
NIR	near-infrared
RBC	red blood cells
WBC	white blood cells

1. Introduction

The present review article aims to provide a comprehensive update on recent developments for in vivo applications of nanoimmunotherapy in the last decade, including clinical translation strategies. We describe how a rational approach to nanodrug design facilitates customized management of a variety of diseases through nanoimmunotherapy by leveraging specific targeting moieties to optimize delivery, maximize efficacy, and provide specific customization of designed nanodrugs to disease processes. In this review, we focus primarily on the following topics: targeted immuno-engineered nanoformulations, clinical translation, challenges associated with current approaches, and future directions.

2. Nanoimmuno-engineering principles

2.1 Immunotherapy

The diverse groups of cells, molecules, and organs comprising the immune system perform the remarkable task of safeguarding individuals from foreign pathogens and infections. The immune system is generally divided into the innate and adaptive systems. The innate immune system provides immediate and generalized defense against foreign pathogens, whereas the adaptive immune response is slow but specific, and offers dynamic protection. Both immunities complement each other in a complex, multifaceted manner to recognize and eradicate any diseased cell (Walsh & Mills, 2013). Although this framework ensures an overarching effective immune response, such complexity also presents challenges because it creates a wealth of possibilities for dysfunction or destabilization within critical pathways. Thus, there is the potential for a variety of immune-mediated diseases such as asthma, allergy, malnutrition, and autoimmunity. Furthermore, certain types of infections cannot be destroyed consistently by the immune system. This ability to bypass the immune system is not limited to foreign pathogens, as some cells with mutations and/or dysregulation of growth and proliferation pathways (such as tumor cells) are known to escape immune surveillance, resulting in abnormal growth and metastasis. These abnormal responses impact treatment outcomes and are responsible for disease development and relapse.

Immunotherapy is a form of therapeutic management that manipulates the immune system with respect to its connection to disease processes. This therapeutic approach involves modulation of cells (e.g., lymphocytes, granulocytes, and myeloid cells), organs (e.g., lymphoid), and molecules of the immune system in order to reprogram the immune response, train/educate the immune system to identify and fight specific targets such as defective cells or foreign pathogens, and/or diminish the response to innocuous stimuli. Immunotherapies can include different approaches such as antibody therapy (Sliwkowski & Mellman, 2013), immunomodulation, and engineered T cells, among others (Khalil, Smith, Brentjens, & Wolchok, 2016). Immunotherapies can be classified based on the desired treatment outcome: *activating immunotherapies* involve an enhancement of the immune response, whereas *suppressive immunotherapies* are designed to dampen the immune response (Fig. 1A and B), and examples of both will be provided in this review. Another way to classify immunotherapies is to divide them into passive and active approaches. *Passive (adaptive) immunotherapy* includes

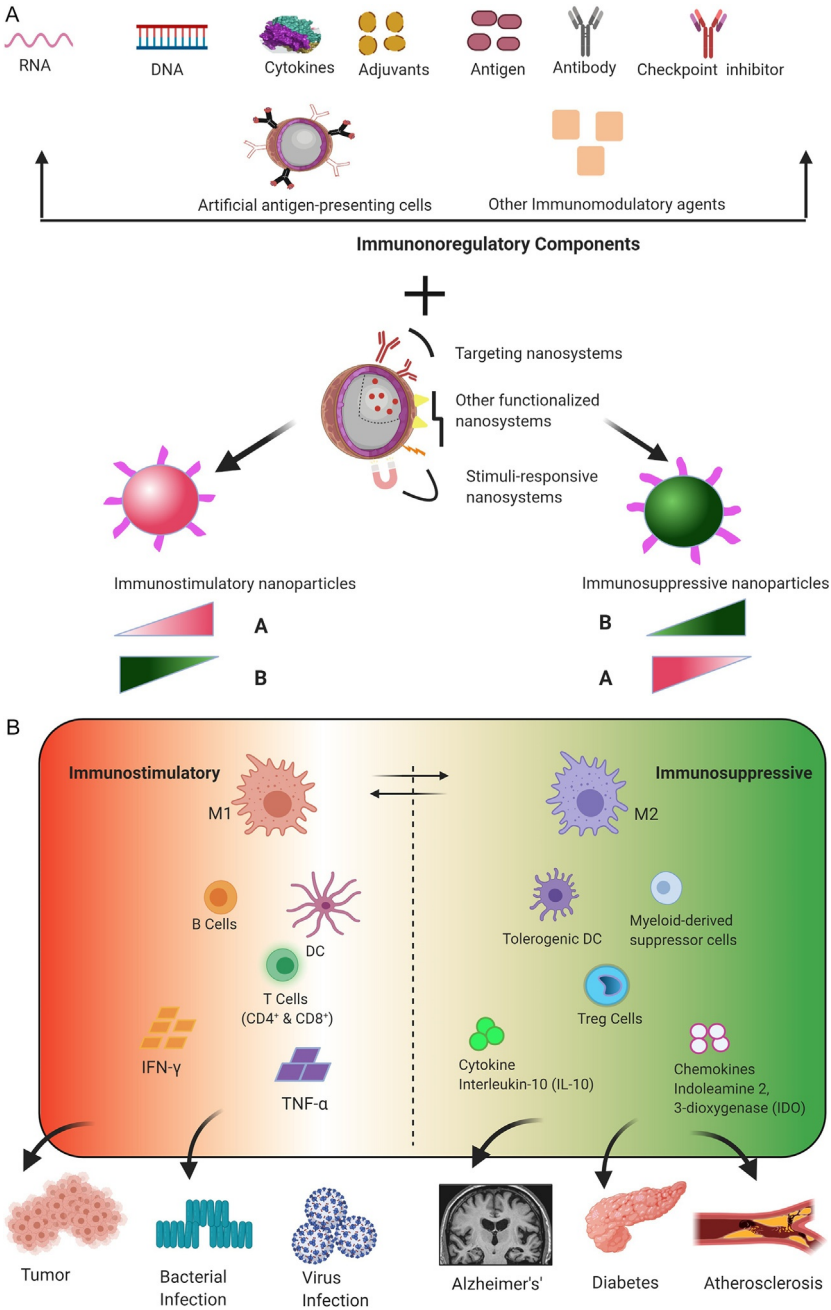

Fig. 1 (A and B) Fabrication of immunoactive and immunosuppressive nanodrug-based systems in disease treatments by modulating immune cells. *Figure created with Biorender.com*.

monoclonal antibody (mAb) and checkpoint inhibitors and does not stimulate the host immune response, so it generally does not result in long-lasting immunity. On the other hand, *active immunotherapy* (vaccine and allergen-specific) induces a specific immune response (antibodies and T cells) and imparts immunological memory. Active immunotherapy includes CAR therapy and cancer vaccines.

Immunotherapy has resulted in promising approaches to cancer treatment such as cancer vaccines (HPV vaccine), cytokine therapies (e.g. interferon, interleukin), adoptive T-cell transfer or CAR-T (e.g., axicabtagene ciloleucel), donor lymphocyte therapy, radioimmunotherapy (e.g., ibritumomab tiuxetan (Zevalin®)), and immune and checkpoint inhibitors (e.g., ipilimumab (Yervoy®), nivolumab (Opdivo®)). The use of immunotherapy in the treatment of various diseases has also expanded beyond these initial approaches. Many adjuvant methods use mAb and cytokines such as G-CS, GM-CSF, and IFNγ, which are employed as nonspecific immunomodulating agents to modulate the role of macrophages, neutrophils, and monocytes in various infectious diseases. IFNγ is used in chronic granulomatous disease, an immunodeficiency in phagocytic cells due to mutation in genes associated with the NADPH oxidase system (Vélez, Rocha, Arias, & López, 2015). Another example of the expansion of immunotherapy applications involves reengineering T cells to treat autoimmune-triggered skin disease (pemphigus) for human trials (Ellebrecht et al., 2016).

However, immunotherapy is not without limitations. Notably, when the immune system is modulated or activated, it is highly likely that patients may develop undesirable side effects such as fatigue, flu-like symptoms, fever, swelling, diarrhea, and autoimmunity. Moreover, immunotherapy faces a challenge when attempting to expand the principles of immune modulation to a larger spectrum of patients and to different cancer types and disease processes. Innovation is necessary in order to overcome some of these hurdles and develop improved treatment approaches. Therefore, current research is focusing on exploring the possibilities of nanodrug design and novel delivery platforms for immunotherapy (Wang, Ye, Hu, Bellotti, & Gu, 2017) (Fig. 1A).

Nanomedicine can assist immunotherapy and augment and refine its effects by providing codelivery of therapeutics, site-specific targeting, and stimulation of key immune cells and organs for sustained response. Nanomedicine, in contrast to conventional delivery of cytotoxic agents,

can be used to activate key immune cells in order to deploy rapid proliferation and effective response against disease. A more sophisticated understanding of such modulation has changed the initial narrow focus of the nanomedicine field, which was centered on escaping from the immune system (e.g., sequestration of nanomaterials), to a broader view of how nanomaterials can work together with biological systems in order to obtain desired responses. Today, nanoimmunotherapy aims to facilitate and modulate beneficial interactions between nanoparticles and immune cells, and this mutual interface has become a crucial goal in the validation and design of nanosystems for nanoimmunotherapy.

The intrinsic properties of nanomaterials can be leveraged to promote targeted and purposeful interactions with host components, such as phagocytic cells and the reticuloendothelial system. Thus, there exists a collaborative approach to fight disease, rather than the initial narrow focus on engineering nanomaterials that have to bypass immune surveillance to exert their action. For instance, polymeric hydrogels and other nanomaterials have been shown to accumulate in lymph nodes and interact with immune components such as T cells and B cells in order to modulate the immune system (De Koker et al., 2016). A crucial tool in nanomaterial engineering is surface functionalization, which allows the design and preparation of antibody-based nanoparticles that demonstrate increased therapeutic efficacy over the administration of free soluble antibodies alone. Moreover, some nanosystems such as dextran- or dextran-sulfate-based systems show intrinsic targeting capability to macrophages (Heo et al., 2017; Ma et al., 2016). Therefore, nanoparticles can be used as much more than a delivery vehicle, and can serve multifunctional purposes when designed in a way that incorporates the potential for immune system interaction. Moreover, because the immune system maintains a challenging equilibrium between immunosuppressive and immunoactive components and pathways, a nano-mediated approach can assist in directing this equilibrium toward targeted and appropriate responses to potential threats, while maintaining tolerance toward healthy tissues.

2.2 Nano-focused immunotherapy

The intersection between immunotherapy and nanotechnology has remarkable potential to address major public health problems such as heart disease, autoimmune disorders, and cancer. Nanoscale-engineering can be used to enable nanoimmunotherapeutics to avoid immune system detection, or as

a means to introduce antigen inhibitors to improve immune system response, among other strategies. Nano-based approaches are often advantageous in the areas of cancer management, vaccines, and therapeutics for inflammatory and autoimmune disorders. Nanosystems are on an appropriate size scale to modulate immune system molecular and cellular constituents, or to induce immunotolerance against a specific antigen. Engineered immune-focused nanodevices should be equipped with desirable physicochemical characteristics. Some of these include hydrophilic/hydrophobic balance, surface charge, minimal toxicity, biocompatibility, colloidal stability, and disease-modifying properties. Indeed, nanomaterial size is a crucial factor that can be advantageous in reaching certain target tissues; but it can also constitute a challenge, because phagocytic cells (e.g., macrophages) are usually able to easily pick up nanoparticles identified as foreign materials. When nanoformulations interact with immune system components in undesirable ways, this can result in an inflammatory response, trigger autoimmune disorders, induce antibody production, and increase host susceptibility toward infections and cancer by giving rise to a multilevel immune response against the nanosystem (Poland et al., 2008). Thus, it is often critical for nanoimmunotherapeutic applications that nanoparticles be recognized as self, or that they evade immune recognition completely, in order to optimize biodistribution, treatment efficacy, and effectiveness of an imaging agent (Wang et al., 2020).

Nanosystems must have access to target tissues. Typically, this is achieved by overcoming physiological or physical barriers in order to effectively reach target tissue cells and exert the intended function. Nanoparticles can be administered by several routes (Nikolić, Ilić-Stojanović, Petrović, Tačić, & Nikolić, 2019), and the desired outcome should be matched by the administration route. Such routes include oral, nasal, parenteral, topical, transdermal, ocular, and pulmonary, among others. The route of nanoparticle administration greatly impacts both tissue distribution and the immune effects.

Mucosal administration, such as that associated with oral, nasal, or rectal delivery, is generally linked to M cells that connect the MALT with the mucosal microenvironment, and play a specific role in transcytosis of microbes and particles. Nanocarriers administered through mucosal routes have shown great potential for efficient mucosal vaccination (Cerutti, Chen, & Chorny, 2011). A cationic nanogel composed of cholesterol-modified pullulan was administered intra-nasally and showed the capacity to enhance murine survival rates in response to tetanus and botulin

neurotoxin tests (Nochi et al., 2010). Nanocarriers with mucodiffusive properties have also been engineered to cross the mucus layer to reach the epithelium. A defined equilibrium among mucoadhesive and mucodiffusive properties is important for the efficacy of nanocarriers delivered using mucosal pathways. Mucodiffusion properties largely depend on particle size, where particle size should be smaller than mucus mesh size. Thus, the composition and physicochemical properties of the core material, size, and surface structure of nanoparticles are key determining factors for enhanced transport mechanisms across the mucosal surface (Zhang, Gao, & Bao, 2015).

Parenteral administration includes intravenous, intramuscular, subcutaneous, intradermal, and intraperitoneal modes, which are used to administer nanoparticles to enter the systemic circulation, stay at the injection site, or travel to the nearest lymph node. Negatively-charged nanoparticles such as liposomes, dendrimers, and polymeric nanoparticles, e.g., PLGA are more easily drained into the lymphatic system when compared to positively-charged or neutral nanoparticles (Kaminskas & Porter, 2011). Increased uptake into the lymphatics from the interstitium is due to electrostatic repulsion between negatively-charged particles and the negative extracellular matrix (Rao, Forrest, Alani, Kwon, & Robinson, 2010). Conversely, drainage of cationic nanoparticles from the injection site to lymph nodes is slow due to attractive forces among the particles and the extracellular matrix. Cationic nanoparticles are likely to form a repository after injection and to be taken up by outer and migratory APC or slowly drain to lymphatics (Vicente, Goins, Sanchez, Alonso, & Phillips, 2014). Coating of nanocarriers with polyethylene glycol (PEG) can increase drainage into lymph nodes (De Koker et al., 2016), and PEG or other hydrophilic polymers are often used to shield engineered nanoparticles from immune surveillance in in vivo applications However, it has also been reported that an immunocompetent host's immune system may be capable of producing PEG-specific antibodies after administration of PEG-coated liposomes (Ishida, Wang, Shimizu, Nawata, & Kiwada, 2007), so this approach may not necessarily be effective in subsequent administrations.

Among parenteral routes, *intravenous (IV)* nanoparticle administration is the most commonly used because it provides a portal by which to access the entire body and nanoparticles are directly exposed to circulating immune cell components, and to the entire body's rich environment of proteins, cells, and tissues. Particle transport into tissues is dependent in part on the anatomy of blood vessel endothelium, as well as nanovehicle physical properties (Shah et al., 2018; Smith et al., 2013; Zhu, Vo, Taylor, & Smith,

2019). Lung and muscle capillaries display a continuous morphology and allow only small particles (i.e., typically less than ~3 nm) to filter across the capillary wall. On the other hand, kidney (with blood vessel fenestrated endothelia), and liver tissues (with discontinuous endothelia having larger pores) can allow for transport of particles up to ~60 nm (Almeida, Chen, Foster, & Drezek, 2011), but nanoparticles larger than 100–200 nm are more likely to accumulate in the spleen due to particle removal by splenic filtration (Cataldi, Vigliotti, Mosca, Cammarota, & Capone, 2017). Notably, the filtration values are likely not fixed and are reported heterogeneously across the literature, perhaps in part because they such values are affected by other nanoparticle parameters than size such as surface coating, shape, and charge.

Nanoparticle surface modification is an important factor when using the IV administration route. IV nanoparticle administration can stimulate immune tolerance and offers potential ways to modulate immune cells and molecular components in the blood. An important additional consideration, however, is that the surface and size of the nanoparticles are often influenced by biological blood components. In blood, nanoparticles tend to adsorb a protein layer (termed the protein corona, composed of proteins like albumin, immunoglobulin, etc.) that can change their overall hydrodynamic size and surface charge, both of which are critical factors in determining in vivo pharmacokinetics. The addition of the protein corona can also enhance agglomeration of nanoparticles and further lead to changes in hemodynamics and transport behaviors throughout the body. In addition, the presence of a protein corona can modify uptake mechanisms compared to those of bare nanoparticles, with nanoparticle-protein corona complexes more readily undergoing phagocytosis in macrophages (Corbo et al., 2016). This phenomenon occurs for example in cationic gold nanoparticles with a hydrophobic surface, which bind to serum proteins forming a protein corona and are then easily taken up by macrophages (Saha et al., 2016). Various modification methods such as the use of PEG, polysaccharide moieties, and zwitter ions have been used to regulate protein corona formation (Oh et al., 2018), but there is still much work that needs to be done in this field.

Various nanodelivery systems have been formulated for the intra/subcellular localization of therapeutic agents. *Internalization of nanoparticles* typically involves endocytic mechanisms. Endocytosis encompasses at least two different cellular uptake routes: pinocytosis (uptake of fluids and small vesicles) and phagocytosis (engulfment of particles larger than 300 nm along with debris). Pinocytosis includes macropinocytosis, as well as clathrin-,

caveolae-, and scavenger receptor-mediated endocytic pathways (Kuhn et al., 2014). Phagocytosis and macropinocytosis are both dependent on actin and form protrusions at the cell membrane (Kumari, Mg, & Mayor, 2010). Phagocytosis is carried out by professional phagocytes viz. macrophages, neutrophils, dendritic cells, and monocytes. Gold (Frana et al., 2011), silica, TiO_2, and Fe_2O_3 nanomaterials, among others, have been employed to understand diffusion pathways across the cell membrane. Particle size, shape, charge, composition of nanoparticles, and hydrophobicity/hydrophilicity are some of the processing parameters governing the internalization of nanoparticles. Modular multifunctional nanoparticles can be purposefully designed to enable stimulation of different responses, by carefully choosing the appropriate molecular components to obtain desirable physiochemical properties for specific applications (Smith & Gambhir, 2017).

Nanoparticle size is a critical parameter in modulating the innate immune response. Several groups have studied the immunomodulatory properties of polystyrene nanoparticles with diameters ranging from 20 to 1000 nm. There were significant differences in uptake between the smallest and the largest sizes. Lung DCs captured 20 nm polystyrene particles more effectively compared to 1000 nm particles (Seydoux et al., 2014), whereas larger sized particles tended to be taken up by migratory DCs to the lung-draining lymph nodes (Blank et al., 2013). The uptake of 20-nm particles enhanced antigen presentation to $CD4^+$ T cells. *The shape of nanoparticles* also has a significant role in interactions with the innate immune system (Getts, Shea, Miller, & King, 2015). Bartneck and coworkers found that macrophages took up nanorods (15×50 nm) more efficiently than nanospheres (either 15 nm or 50 nm diameter) through macropinocytosis (Bartneck et al., 2010). Talamini et al. prepared spherical (10 and 50 nm), rod (60×30 nm), and star-shaped gold nanoparticles (55 nm). They found that spherical- and star-shaped gold nanoparticles accumulated in the same ratio in the liver, but star-shaped gold nanoparticles tended to accumulate more in the lungs (Talamini et al., 2017). The enhanced surface *hydrophobicity* of polymeric particles improves internalization into DCs and increases the expression of CD86 in DC populations (Liu et al., 2013).

Surface charge also plays a critical role in the interactions between innate immune cell components and nanoparticles. Cationic gold nanoparticles are more easily internalized by macrophages and DCs compared to negatively-charged particles due to enhanced electrostatic attraction between

positively-charged nanoparticles and the negatively-charged cell membrane (Fytianos et al., 2017). However, a protein corona on the nanoparticles can mask surface charges and influence targeting ability, thus hindering the biological efficacy of targeted nanoparticles.

2.3 Nanoimmunotargeting

One of the critical advantages of nanosystems is the ability to actively target them to increase therapeutic efficacy and reduce off-target toxicity. Actively-targeted nanosystems can be developed by surface functionalization of nanocarriers with bioactive ligands. These nanosystems can identify tissues or cells that have molecular patterns/receptors at their surface, accelerating subsequent receptor-mediated endocytosis of the nanoformulation. This nanoparticle surface modification is an effective tool that can enhance interactions of the nanosystem with a specific population(s) of immune cells. Surface modification of nanoparticles with ligands may be attained via Michael addition, a biotin–streptavidin method, or a carbodiimide chemistry approach (Yüce & Kurt, 2017). Antibody-targeted nanoparticles are an example of how these modifications are capable of improving antitumor immunity (Schmid et al., 2017). TGFβ is a major mediator of immunosuppression and T cell signaling, and is dysregulated in cancer. PLGA nanoparticles loaded with the inhibitor TGFβ receptor 1 were specifically designed to bind to $CD8^+$ T cells by conjugating anti-CD8a F(ab')2 fragments to the particle surface. The accumulation of TGFβ inhibitor at the target site resulted in delayed tumor growth and improved survival (Schmid et al., 2017).

Macrophages are involved in a variety of diseases and disorders including inflammation, metabolic disorders, rheumatoid arthritis, atherosclerosis, and cancer, making them an attractive therapeutic target. Many other cellular and molecular immune system components have also been targeted. In the case of autoimmune disorders, gold nanoparticles loaded with autoantigens such as ITE have been used to target antigen-presenting cells such as DCs, and to induce the differentiation of Foxp3 Tregs, leading to decreased autoimmunity (Serra & Santamaria, 2018). Gold nanoparticles loaded with ITE and myelin oligodendrocyte glycoprotein are taken up by DCs and promote $FoxP3^+$ Treg differentiation. This approach has been shown to be effective against mouse models of autoimmune encephalomyelitis and type 1 diabetes (Neef & Miller, 2017; Yeste, Nadeau, Burns, Weiner, & Quintana, 2012). Lipid nanoparticles have also been used for targeted delivery of siRNA to inflammatory monocytes in order to

downregulate chemokine receptor (CCR2) expression that decreases monocytic tissue localization (Leuschner et al., 2011).

2.4 Rational nanodrug design

Rational design of nanomedicines can endow advantageous features, such as improved efficacy and safety compared to conventional therapeutics, creation of versatile agents with multifunctional applications (such as simultaneous real-time imaging, targeting, and treatment), and utilization as molecular biomarkers. Although solid tumors and antimicrobials remain a critical target for nanodrugs, research is also rapidly gearing toward applications in metabolic, neurological and autoimmune disorders (Margulis et al., 2020; Ventola, 2017). Nanoscale platforms can help overcome some of the physicochemical and biological barriers for conventional pharmaceuticals, such as poor aqueous solubility (e.g., cyclosporin), low bioavailability, short half-lives, off-target toxicity, or immunogenicity (Patra et al., 2018). Formulating a drug in a properly-designed nanoscale cargo can increase its circulation half-life and enhance target delivery. Additionally, controlled drug release can reduce the required dosage and minimize the need for repeated administrations, which is especially advantageous in inflammatory conditions. Lowering the dose while maintaining efficacy results in an increased therapeutic index for nanoscale drugs. Another area where nanodrug design can prove useful is for low molecular weight drugs (<55 kDa), including vasoactive intestinal peptide, which are otherwise rapidly cleared in blood circulation via renal clearance. Nanoformulations of these drugs have enhanced bioavailability and pharmacological activity, and slowed clearance kinetics (Klippstein & Pozo, 2015; Rubinstein, Soos, & Onyuksel, 2008; Ventola, 2017). Many nanodrugs have the added advantage of bypassing membrane drug efflux pumps such as *P*-glycoprotein, which are upregulated in cancer cells and lead to multidrug resistance.

Nevertheless, there are still challenges in nanodrug design that need to be overcome in order to facilitate clinical translation. It is crucial to study how the physiological and bioactive properties of nanodrugs differ from parent drug-only forms, and the subsequent impact and potential clinical significance of those differences. In addition, nanodrugs must overcome biological barriers such as opsonization followed by sequestration by mononuclear phagocytes, distribution in *endo*-lysosomes, nonspecific distribution in the vascular system, pressure gradients, and drug efflux pumps among others (Blanco, Shen, & Ferrari, 2015).

3. Nanoimmunotherapy applications

Given the extensive and complex role the immune system plays in different disease states, there are several potential applications for nanoimmunotherapy. These applications span diverse areas such as cancer, cardiovascular disease, central nervous system disorders, diabetes, infection, and autoimmune disease, among others.

3.1 Nanoimmunotherapy applications: Cancer

Cancer is a multifaceted disease of the genome that occurs in part due to the failure of the immune response to recognize cancer cells as a threat and eliminate them. These malignant cells are able to escape immune surveillance by stimulating various tumor-associated mechanisms, including reduction of MHC I fragments, reduction in tumor antigen production, defective death receptor signaling, reduction of co-stimulation, and production of immunosuppressive cytokines and suppressive cells (Pandya, Murray, Pollok, & Renbarger, 2016). Conventional cancer treatment regimens including surgery, chemotherapy, and radiotherapy have well-characterized intrinsic limitations. Prognosis and treatment selection are mainly determined by diagnosis, and these treatment approaches are often not sufficiently specific to cancer tissue. Additionally, these conventional therapies can affect the body's ability to fight the tumor. For instance, most chemotherapeutic agents can cause depression of the immune system, which leads to a decrease in available antitumor immune components. The goal of immunotherapy, on the other hand, is to promote an approach in which the body's own immune system is an active participant in cancer management. The goal is to unleash the inherent power of the immune system to attack cancer cells continuously and dynamically, while sparing healthy cells. The immune system has the potential to detect, attack, and continuously monitor abnormal growth, resulting in a prolonged and reliable mechanism of action. Moreover, immune system memory can offer long-term protection in case of re-occurrence.

In general, there are three prerequisites for cancer immunotherapeutic strategy success, not necessarily simultaneous. First, cancer antigens should be effectively escorted to immune cells such as DCs. Second, the anticancer immune response should be initiated after delivery of the therapeutic supplement (adjuvant) and cancer antigen. Lastly, it is important to consider that immunosuppressive T cells such as Tregs and myeloid cells such as

myeloid-derived suppressor cells can suppress the activity of anticancer T-effector cells and thereby create an immunosuppressive veil throughout the cancer; therefore, the immunosuppressive tumor microenvironment must often be modified to improve immunotherapeutic success. Treg-targeting nanoparticles can be engineered to circumvent this tumor immunosuppressive microenvironment; for instance, a checkpoint blockade such as CTLA-4, which mediates immunosuppression by decreasing immune signaling activity, can be utilized to control the activity of Tregs (Ou et al., 2018; Sacchetti et al., 2013). In the tumor microenvironment, MDSC generate various factors such as cytokines (interleukin-10 (IL-10)), which facilitates the production and activation of Tregs. Therapeutic efficacy can be improved by adding combinational treatments, such as chemotherapy, at appropriate times (Emens & Middleton, 2015).

Various types of cancer immunotherapies have shown promising clinical outcomes. These include checkpoint inhibitors (PD-1/PD-L1 blockade and CTLA4 inhibition), cytokine-based therapies (including interferons, interleukins, and granulocyte-macrophage colony stimulating factor), engineered T cells like chimeric antigen receptor T (CAR T) and T cell receptor (TCR) T cells, agonistic and antagonistic antibodies (which target inhibitory or stimulatory receptors), and cancer vaccines. Cytokine-based therapies such as IL-2 can impact both T cells and Tregs. These therapies can either cause death of stimulated T cells or induce immune tolerance and promote Treg growth, thus potentially leading to inhibition of unwanted autoimmune responses (Milling, Zhang, & Irvine, 2017). Approaches utilizing CAR T Cells and TCR T cells require only a single administration and some have resulted in extended patient survival. Nevertheless, there are ongoing efforts to design CAR T cells that can target diverse antigens despite concerns about cost and side effects (O'Rourke et al., 2017; Schuster et al., 2017). Another promising field is that of cancer vaccines. Some existing cancer vaccines have been formulated based on DCs, nucleic acids (DNA, RNA and such as mRNA), and neo-antigens, which are a type of tumor-specific antigens (TSA) that arise from altered DNA in cancerous cells. In the vaccine field, studies show that antigen valency (i.e., number of binding sites) impacts the types of B cell responses and the magnitude of the response, with high valency enhancing effector differentiation (Kato et al., 2020).

A variety of *nano-based methodologies* can be used to enhance the comprehensive application of cancer immunotherapy and refine its action. For example, nano-based cancer vaccines have been employed in order to present tumor-associated antigens (TAA) to APC (DC) and to facilitate specific

cytotoxic T cell and antibody responses. Diverse studies published over the past 10 years show that typically less than 1% of the administered nanoparticle dose actually reaches malignant tissue (Wilhelm et al., 2016), emphasizing the importance of rational design to maximize dose delivery and efficacy. Recently, simultaneous delivery of tumor-specific antigens (TSAs), adjuvant (3-O-desacyl-4′-monophosphoryl lipid A), and DDA (dimethyldioctadecylammonium, used as a cell invasion moiety) was achieved using nanoliposomes termed "tumosomes" (Noh et al., 2017). Tumor-bearing C57BL/6 mice treated with this formulation showed improved immune response and survival, with tumor growth reduction. Co-delivery of engineered cancer-specific antigen (OVA) and adjuvants in the context of CpG or toll-like receptors using micelles and liposomes has also shown better immunogenic activity compared to a conventional therapeutic approach (Wilson et al., 2013). Liposomal delivery platforms have commonly been used as nanoimmunotherapeutic modulators (CpG, anti-PD-1 and CpG, TGF-β, IL-2 and IL-12) in tumor preclinical studies (Fan et al., 2017; Lu et al., 2016; Xu, Wang, Zhang, & Huang, 2014), and the sheer number of available clinical liposomal formulations suggest nanoimmunotherapeutic liposomal formulations bear watching for regulatory trials and approvals in the near future.

Nanosystems can also be used in biomimetic approaches, in which the nanosystem is designed to elicit an immune response by mimicking a key aspect of immune biology. For instance, synthetic artificial antigen-presenting cells (sAPCs) are artificial systems that include T-cell stimulating molecules attached to their surface and thereby mimic APCs. The use of sAPCs is a possible alternative to conventional adoptive T cell therapy, since these synthetic particles have the capability to present multiple signals to activate T cells (Kosmides et al., 2017), potentially without some of the limitations. In the treatment of multiple melanoma (MM), limitations associated with clinically successful CAR- and bispecific-T cell engagers (BiTEs) include difficulty in targeting multiple cancer antigens, short half-lives (2 h), and high cost for clinical formulations. Alternatively, a nano-based liposomal system conjugated with two antibodies (nanoBiTE) was developed to identify the epitope on MM and to engage T cells. Multispecific T cell engager nanoliposomes (nanoMuTE) were also developed to target more than one epitope and engage T cells (Alhallak et al., 2020).

Nano-based vehicles can be employed to improve the delivery of mRNA and neoantigen vaccines to target sites, and nanoformulation versatility allows for surface modifications and *combinational approaches*, which can

help induce immunogenicity and enhance therapeutic efficacy in tumors treated by immunotherapy. For instance, combining nanoimmunotherapy with other therapies such as chemotherapy, radiotherapy or phototherapy can enhance treatment outcomes and reduce the toxicities involved in each therapy. Like other combination treatments, combinational nanoapproaches can take advantage of the beneficial effects of two or more treatment mechanisms; the difference is that by using nanoapproaches, the multiple treatment mechanisms can be packaged into one nanosystem made possible by the versatility and multifunctionality of nanodesign. For example, to leverage the effects of chemotherapy combined with immunotherapy, a PDL1 antibody and gemcitabine-loaded polyvinyl alcohol (PVA) hydrogel network was prepared to degrade in response to radical oxygen species within the tumor microenvironment. Murine models of B16F10 melanoma and 4T1 breast carcinoma were treated with the hydrogel formulations. Results showed that the hydrogel degraded and released the chemotherapeutic drug, as well as the anti-PDL1 antibody, resulting in a synergistic effect and increased anticancer immunity compared to nonencapsulated drugs, as indicated by bioluminescence imaging (Wang et al., 2018). Another example of nano-based cancer immunotherapy is the combination of the chemotherapy agent oxaliplatin with tumor-targeted lipid-protamine-DNA (LPD) nanoparticles and liposomal nanoparticles coupled with PD-L1 entrapped with plasmid. In a colorectal cancer murine model, LPD nanoparticles and oxaliplatin showed a synergistic effect in reducing tumor growth compared to free nanoparticles or oxaliplatin alone. The authors showed that CD8+ T cells, CD4+ T cells and activated DCs were significantly increased inside the tumor, and suggest that oxaliplatin may support an immunologically "cold" tumor becoming "hot" and thus susceptible to LDP therapy (Song et al., 2018).

Besides chemotherapy, another example of a combinational approach is nanoimmunotherapy agents plus phototherapy. Phototherapy can be used in cancer treatment by two different strategies: photothermal therapy (PTT) and photodynamic therapy (PDT). These methods use a specific wavelength of light to stimulate light-sensitive molecules and initiate a specific therapeutic effect. PTT uses a light-absorbing material whose light-induced temperature increase results in tissue ablation. PDT requires light sources, a photosensitizer and oxygen. The photosensitizer may be in active form or in prodrug form when administered. When the active photosensitizer is irradiated with light of a specific wavelength, it creates reactive oxygen species

(ROS) that are cytotoxic to the surrounding environment. Combination platforms (nanoimmunotherapy + PTT) were developed using PLGA nanoparticles encapsulated with anti-PD-1 peptide (APP) and hollow gold nanoshells (HAuNS). After NIR irradiation, HAuNS generated a PTT effect in the tumor and simultaneously released APP (Luo et al., 2018). These combinations of phototherapy and nano-based delivery systems are particularly beneficial for inhibition of metastatic tumors that are close to the skin surface or near the surface of other fiber-accessible sites, since light penetration in deeper areas is limited even at longer wavelengths.

3.2 Nanoimmunotherapy applications: Cardiovascular disease

Despite extensive developments and progress in cure and prevention, heart disease continues to be the highest collective cause of mortality in the United States in the developed world (WHO, 2017). Heart disease includes a spectrum of disorders with various etiologies and presentations including congenital heart disease, ischemic disease, valvular disorders, inflammatory disease, hypertension, and cardiomyopathy, which may collectively result in congestive heart failure. The main cause of cardiovascular disease (CVD) is atherosclerosis, which is a multifaceted inflammatory disorder with thickening and plaque formation within the intimal layer of arterial vessels, potentially associated with low-density lipoprotein accumulation (Ference et al., 2017). Abnormal thickening and plaque accumulation can lead to vessel occlusion and irregular flow patterns. Thrombosis, the formation of a clot within a blood vessel, is a potentially fatal consequence of atherosclerosis because plaques that have built up over time can rupture and elicit a clotting response, leading to vessel occlusion and tissue injury or necrosis. Given the potentially severe consequences of atherosclerosis, plaque development has been extensively studied. Growth of atherosclerotic lesions can lead to the development of new blood vessels inside the arterial walls (Camaré, Pucelle, Nègre-Salvayre, & Salvayre, 2017), involving neoangiogenesis in a manner similar to the progression of cancerous tumors. Monocyte recruitment, macrophage accumulation, and B cells in the vessel wall play key roles in plaque development, so immunotherapy is likely able to impact these processes and provide an approach to prevention and/or treatment. Importantly, however, the toxicity of immunomodulatory drugs should be considered, which could limit therapeutic applications in heart disease treatments (Bringhen et al., 2018) due to side effects such as arrhythmias. Nanoimmunotherapeutic

approaches can provide an avenue to target therapy site- and cell-specifically and thereby diminish these concerns (Dutta et al., 2012).

Therapeutic approaches such as reducing low-density lipoproteins (LDL), using antithrombotic drugs, and aggressive revascularization methods have been employed against atherosclerosis (Arnett et al., 2019). Two statin clinical trials (JUPITER and ASTEROID) showed remarkable control over CVD, but controlling lipid levels to achieve a sustained treatment effect has proven difficult. Novel nanomaterials that can be combined with immunotherapy are being designed to optimize targeting and develop site-specific interventions in cardiac disease. A multifaceted approach, made possible by combining nanotechnology with immunotherapy, may best address the variety of mechanisms that contribute to plaque development and resulting morbidity/mortality. Nanomaterial design and selection must consider several factors including the type, function, and role of immune cells in the pathological microenvironment, and how each cell contributes to the progression of disease. Monocyte and macrophage targeting offer a distinctive opportunity to modulate inflammatory disease. Nanodrugs that specifically target macrophages attenuate pro-inflammatory activities and have an athero-protective effect (Cervadoro et al., 2018). One example involves lowering serum levels of LDL with statins in combination with nanosystems to immunomodulate plaque-resistant cells. For example, high-density lipoprotein nanoparticles loaded with simvastatin (S-HDL) reduced plaque inflammation by inhibition of macrophages (Mφ) in apolipoprotein *E*-deficient ($Apoe^{-/-}$) mice. Inflammatory gene expression (including TNF-α, CCL2, VCAM-1, and CCL3) in plaque macrophages decreased remarkably (Tang et al., 2015). A novel macrophage-specific nano-immunotherapy was recently developed to clear atherosclerosis, and efficacy was demonstrated in several murine atherosclerosis models (Flores et al., 2020). This "Trojan horse system" (SWNT-SHP1i), which very selectively localized in inflammatory monocytes, consisted of PEG-coated single walled carbon nanotubes (SWNT) co-loaded with fluorescent probe Cy5.5 and an inhibitor of Src homology 2 domain-containing phosphatase-1 (SHP-1i). The nanosystem effectively cleared atherosclerotic plaque by reactivating lesional phagocytosis (MΦ) in murine models, decreased inflammation based on scRNA-seq studies, and showed no off-target toxicity based on a variety of evaluations including histology, clinical blood panels, and phenotypic observations.

Numerous studies have also investigated anti-inflammatory therapeutic approaches to reduce macrophage growth in plaques, such as a targeted

approach using statin-loaded reconstituted high-density lipoprotein nanoparticles to inhibit plaque macrophage propagation in $APOE^{-/-}$ mice (Duivenvoorden et al., 2014). Integrating radiolabeling with targeted nanoimmunotherapy of atherosclerosis can help reveal mechanisms of plaque formation and the role of immune cells therein. Real-time imaging can report thickening of the vessel wall, inflammation, and macrophage propagation in vivo (Calcagno, Mulder, Nahrendorf, & Fayad, 2016; Herbin et al., 2016).

In the last few decades, as our understanding of the pathogenic mechanisms has improved, efforts to design advanced cardiovascular disease treatments have shifted to include not only lipid management strategies, but particularly the inflammatory underpinnings of disease, and to better understand the role of immune cells and inflammatory modulators in disease progression, particularly in atherosclerosis. The use of nanoimmunotherapy thus represents a more holistic, overarching strategy compared with the increasingly antiquated notion of lipid management as the sole treatment approach. Nanosystems provide opportunities for theranostic approaches to thrombosis management, as they can potentially be used for thrombus imaging as well as for delivery of therapeutics or even prevention of thrombus formation. For example, a lipopeptide nanosystem for combined antithrombotic therapy and fibrin-targeted imaging (using an NIR fluorescent dye coupled with boronate antioxidant polymer) was developed to inhibit thrombus formation and visualize atherosclerotic plaque. Thrombus formation is related to the presence of hydrogen peroxide which promotes platelet aggregation (Ambrosio, Tritto, & Golino, 1997) and inflammation. The nanosystem derived its efficacy through inhibition of hydrogen peroxide generation, serving as an anti-inflammatory and anti-platelet aggregation system (Kang et al., 2017).

In summary, CVD therapeutic strategies increasingly lean on our improved understanding of the underlying inflammatory processes including the broad role of the immune system; because of these close links, there is tremendous potential for nanoimmunotherapeutic treatment of atherosclerosis and thrombosis. Further development of nano-based systems combined with immunotherapy will likely help create effective site-specific therapeutic modalities that can also reduce off-target effects.

3.3 Nanoimmunotherapy applications: Central nervous system

The central nervous system (CNS) comprises the brain and the spinal cord. CNS diseases include a broad spectrum of disorders in which brain or spinal

cord function is diminished or impaired, resulting in diminished motor, sensory, or cognitive performance. The etiologies vary, including but not limited to genetic disorders, infection, stroke, neurodegenerative conditions, brain tumors, trauma, and many other potential sources of damage to CNS structures. One of the most common issues with pharmacological management of CNS disorders is the existence of natural barriers to molecular transport into the CNS. The three major barriers include the arachnoid epithelium (AE), the choroid plexus epithelium (CPE), and the blood-brain barrier (BBB). Among them, the BBB is the major concern for conventional IV-injected treatments due to the restrictions presented by tight junctions between endothelial cells, permitting very limited penetration to even small molecules. In particular, the BBB limits transport of peripheral circulating immune components (Takeshita & Ransohoff, 2012). Special challenges may exist for immunotherapy in CNS treatment because of the scarcity of lymphatic structures in the CNS, and because of what is traditionally known as "immune privilege," i.e., a protective mechanism that minimizes the chance of CNS inflammation through the ability to tolerate the presence of antigens without eliciting an immune response (Louveau, Harris, & Kipnis, 2015). However, some of these concepts are currently being reevaluated based on the presence of resident antigen-presenting cells in the CNS including astrocytes, microglia, macrophages, and DCs (Louveau et al., 2015; Perng & Lim, 2015; Schetters, Gomez-Nicola, Garcia-Vallejo, & Van Kooyk, 2018). Recent evidence also suggests the existence of meningeal lymphatic vessels, which permit communication between the CNS and the immune system (Schetters et al., 2018). Although the brain has limited lymphatic vessels in the meninges, these can allow antigens to sink into the cervical lymph nodes, and T cells can be activated there and reach the brain parenchyma through the cerebrospinal fluid (McGranahan, Li, & Nagpal, 2017).

Treatment approaches targeting the CNS must consider both transport through the BBB and the unique characteristics of local immune responses. Various delivery strategies have been employed to bypass the BBB and reach the CNS, including transnasal, transcranial, transvascular, carrier-, and receptor-mediated transcytosis (Saraiva et al., 2016); all display inherent limitations. Nanomedicine has the potential to improve therapeutic agent delivery via rational design to tailor size and surface characteristics, so that effective transport across the BBB is achieved. Nanoparticles that have been proven to cross the BBB include lipid nanoparticles, polymeric nanoparticles, polymeric micelles, carbon nanotubes, and dendrimers, where they were used to treat CNS disorders (Saeedi, Eslamifar,

Khezri, & Dizaj, 2019). In this review, we focus on immunotherapy-based interventions to particularly address CNS cancers, Alzheimer's disease, and Parkinson's disease.

Gliomas comprise a main cancer of the CNS, with origins in the glial cells that account for 1%–2% of all types of cancers. The most common type of glioma is glioblastoma multiforme (GBM). Glioblastomas are highly aggressive and spread fast due to their rapid cell replication and ability to induce immunosuppression by, for example, tumor cell-secreted immunosuppressive factors. One potential approach to treat glioblastomas, among many other cancers, is by reversing immunosuppression. As previously discussed, an immunosuppressive environment is characteristic of tumors, but the immunosuppressive capabilities of glioblastomas (Perng & Lim, 2015) are enhanced by CNS barriers to transport as well as vigorous cytokine production. Various nano-based immunotherapies including CAR-T cells, and checkpoint inhibitors have been employed. One example of glioma-focused nanoimmunotherapy comprises targeted PD-1 and CTLA-4 protein antibodies and antibodies specific to the transferrin receptor to facilitate BBB transport. This nanosystem applied checkpoint inhibitors to shut down macrophages and Treg cells, which had acted as a tumor-protective "shield" against other immune cells. The resulting activation of cancer-destroying cells (e.g., lymphocytes and microglia) yielded enhanced survival in GL261 tumor-bearing mice (Galstyan et al., 2019).

Alzheimer's disease (AD) is a permanent form of dementia that results in a typically gradual decrease in an individual's cognitive abilities. It is especially prevalent with aging, and remains a major global health concern. Multiple hypotheses regarding its pathogenesis exist, but we still do not fully understand the molecular processes that trigger and perpetuate the disease. Two key known processes in AD include the formation of amyloid plaques as a result of the β-amyloid (Aβ) cascade, and the formation of neurofibrillary tangles (NFTs) composed of abnormal tau (τ) protein; however, neither process fully describes the AD mechanism (Bloom, 2014). Initially, immunotherapy research in AD focused on targeting and clearing Aβ using nano-based and other strategies, but attention has more recently shifted to the τ protein, as the formation of τ neurofibrillary tangles seems to correlate with cognitive deterioration (DeTure & Dickson, 2019). Nevertheless, causation remains unclear. Active and passive immunotherapies have eliminated the deposition of Aβ plaques and target tau proteins, with very limited clinical success thus far (Bittar, Sengupta, & Kayed, 2018). While the greatest advances in AD will undoubtedly emerge as mechanistic understanding improves, new

developments in nanoimmunotherapy may provide an opportunity to overcome some of the aforementioned challenges of traditional immunotherapy, by exploiting features such as controlled antigen release, lack of need for other adjuvants, and reduction in immunization numbers. Liposomes {(1,2-distearoyl-sn-glycerol-3-phosphatidylcholine (DSPC) and choline (Chol)} coated with antibody (Aβ-monoclonal antibody) showed high stability and targeting to $A\beta_{42}$ and were developed as a potential proof of concept for AD treatment. However, the study was done ex vivo in post-mortem tissue. Aβ-monoclonal antibody (MAb) liposomes were found to bind amyloid deposits and enhance amyloid clearance (Canovi et al., 2011). Immune-modulating nano-strategies to deliver anti-inflammatory drugs to inflammatory sites may also have therapeutic potential in AD. Since BBB permeability can be improved using cationic nanocarriers, peptide K16ApoE was physically adsorbed to curcumin-loaded PLGA-chitosan nanoparticles, thereby maintaining the integrity of IgG4. Physical adsorption of K16ApoE onto PLGA-chitosan nanoparticles improved the permeability of nanoparticles across the BBB, and the system showed high affinity to bind amyloid plaques (Ahlschwede et al., 2019). These studies are preclinical and measured no cognitive outcomes, so they provide only a proof of concept of the strategy, but not an indication of therapeutic significance.

Parkinson's disease is a degenerative disease stemming from the death of dopaminergic neurons in the substantia nigra of the brain. While the causes are not well-understood, recent findings suggest roles of certain proteins (such as α-synuclein) and immune cells (such as DCs, microglia, T cells, and B cells) in the pathogenesis or progression of the disorder (Jiang, Li, Xu, Gao, & Chen, 2018). To date, the available treatments, such as dopamine replacement strategies, only seek to ameliorate the symptoms of Parkinson's. This approach is effective in the early stages, but loses efficacy over time and has many undesirable long-term side effects. Current PD immunotherapy approaches are based on two strategies: (i) targeting α-synuclein and (ii) employing the neuroprotective properties of regulatory cells. Multifunctional magnetic nanoparticles carrying alpha-synuclein RNAi plasmid can inhibit α-synuclein synthesis and reduce neuron apoptosis, with some functional improvement of locomotion in a mouse model (Niu et al., 2017). Because overexpression of α-synuclein results in significant microglial activation, and suppressing microglial activation can reduce neural degeneration (Allen Reish & Standaert, 2015), there is a clear connection of this approach to modulation of the immune response that characterizes pathological inflammation in PD. More broadly, granulocyte-macrophage

colony stimulating factor (GM-CSF; sargramostim) can modulate microglial neurotoxicity and may be effective in treating many neurological disorders (Gendelman et al., 2017). The potential for combination of Parkinson-specific approaches with a nanosystem in order to bypass the BBB and target microglia is an interesting direction to explore, as research shows that microglia uptake nanoparticles by clathrin-mediated endocytosis, and nanocarriers can mediate microglia/macrophage modulation (Zhang, Lin, Kannan, & Kannan, 2016).

Although nanoimmunotherapy for CNS disorders remains in the very early stages of development, there appears to be much promise in the potential for multifunctional nanoagents to enhance CNS delivery and address immune-related responses. Those responses that generate or perpetuate CNS damage (e.g., inflammatory cascades) could be inhibited, and responses that help detect or minimize damage (such as the immune's system ability to fight glioma cells) could be enhanced.

3.4 Nanoimmunotherapy applications: Autoimmune disorders and diabetes

In autoimmune disorders, the immune system generally identifies autologous proteins/molecules as "non-self" antigens, and starts producing autoreactive T cell and B cell clones (Rosenblum, Gratz, Paw, & Abbas, 2012). Since co-stimulation is essential for T cell activation, interrupting co-stimulatory pathways is one potential therapeutic route (Barranco, 2016; Maxwell & Singh, 2010). Treg therapy is another approach to suppress pathological immune responses directed at self-antigens which characterize autoimmune diseases. Allergen-specific immunotherapy and manipulation of the IL-2 pathway have also been employed. A limitation of these treatments is that they carry a high risk of systematic immune suppression, and there is still a lack of biological knowledge of molecular pathways for activation of Tregs. Another important consideration in the treatment of autoimmune disorders is that patients are usually prescribed nonsteroidal anti-inflammatory drugs and immunosuppressive drugs. There are major biological limitations associated with these medications, including the fact that their transport in physiological fluids is negatively impacted by their hydrophobicity, their cell uptake is nonspecific, and they also have intrinsic side effects with long-term treatments. Considering both the role of the immune system and the required adjuvant medication, nano-mediated immunotherapy could provide a way to support immunosuppression against

overactive autoimmune diseases, while also protecting encapsulated drugs from degradation with increased half-life and enhanced delivery to target cells. Various nanobiomaterials have been used to overcome immune dysregulation by immunosuppressing autoreactive T cells through modulation of the cytokine microenvironment (Feng et al., 2019).

Diabetes mellitus is a disease in which the body's ability to process blood sugar is impaired. In healthy individuals, insulin secreted by pancreatic β cells regulates blood sugar levels. Individuals with diabetes are either unable to produce insulin due to β cell damage (type 1 diabetes, T1D), or have acquired insulin resistance (type 2 diabetes, T2D). The immune system plays an important role in the pathogenesis of diabetes. T1D is an autoimmune disease that is both T-cell based (autoreactive CD4 + and CD8 + T cells specific to β cells) and B cell based (loss of tolerance by autoreactive B cells), and causes continuous damage to β cells (recognized as autoantigens) in pancreatic islets (Smith, Simmons, & Cambier, 2017). Traditional diabetes treatments include islet transplantations and glucose level maintenance by insulin administration, which are limited by either being invasive or requiring patience compliance and continuous monitoring. Although historically it was believed that only T1D was autoimmune-related, new evidence indicates that T2D also displays autoimmune features with chronic, low-grade inflammation (LGI) and involvement of T-cell populations including Tregs (de Candia et al., 2019). Management of T2D also requires patient compliance and continued monitoring for successful outcomes.

Innovative approaches to diabetes treatment have recently been developed by leveraging emerging knowledge of the immunological mechanisms associated with autoimmunity. For instance, Treg induction or DC modulation can be used to suppress inflammation and reverse autoimmunity (Mukherjee & Dilorenzo, 2010). The main focus is on reducing the damaging autoimmune reaction against β cells, thereby arresting T1D development. A variety of antigens for autoimmune reversal have been studied, including glutamate decarboxylase 65 (GAD65), ZnT8, chromogranin A (ChgA), glucose-6-phosphatase catalytic subunit-related protein (IGRP), insulin peptide chains A and B, and proinsulin C-peptide (Han, Donelan, Wang, Reeves, & Yang, 2013; Yang et al., 2014). However, these methods are limited by poor targeting and imprecise intracellular delivery to specific cells. Nano-mediated immunotherapy approaches to T1D management primarily focus on controlling and modulating immune cells and suppressing autoreactive cells by modulating the cytokine microenvironment or by

controlling tolerogenic characteristics of APCs. Nano-based systems have the capacity for utilizing multiple loaded pharmacological molecules to modulate APCs; surface modifications can also be introduced to customize in vivo properties and behavior. For instance, non-viral siRNA based PEGylated liposomes were targeted to pancreatic β cells and APCs (CD11b + macrophages and DCs), yielding improved Treg activation in the pancreas of lipid/Alox15 siRNA-treated NOD mice (Leconet, Petit, Peraldi-Roux, & Bresson, 2012). Polymeric nanoparticles have also shown potential in reversing autoimmune T1D. PLGA nanoparticles loaded with β-cell antigens were tested for reversing the destruction of β cells in diabetic mice. Tolerance was restored by inducing Tregs to suppress activated diabetogenic CD4 and CD8 T cells (Prasad et al., 2018). Nano-based codelivery of proinsulin and tolerogenic molecules (aryl hydrocarbon receptor (AhR) ligand ITE) was tested in a T1D mouse model, and showed decreased activation of inflammatory effector T cells and increased differentiation of FoxP3 + Treg cells (Yeste et al., 2016). Nano-mediated immunotherapy to treat T1D-associated (and now even T2D-associated) autoimmunity has opened new avenues for therapies in diabetes, but more research is needed to understand diabetes in terms of its pathogenesis, mechanisms, autoreactive T cell targets, and antigen identification.

Multiple sclerosis (MS) is an autoimmune neurodegenerative condition with no curative therapy. Destruction and demyelination of axons in the CNS lead to impaired nerve signaling and play a central role in MS progression. Although several FDA-approved drugs can help slow disease progression and relapse, myelin destruction can make even the diagnosis challenging (Ojha & Kumar, 2018). Lipid-based nanosystems such as liposomes and solid lipid nanoparticles have shown promising therapeutic results. PEGylated solid lipid nanoparticles were modified with axoglial glycoprotein antigens (either anti-contactin-2 or anti-neurofascin) and used to successfully deliver methylprednisolone to the brain in mice (Chountoulesi & Demetzos, 2020; Gandomi et al., 2017). These antigens are important targets of autoimmune reaction in MS. More recently, a phase 2 clinical study using gold nanocrystals (CNM Au8) from Clene Nanomedicine, Inc. showed signs of robust remyelination and an improvement in murine motor function (Robinson et al., 2020). The nanocatalytic activity of gold nanocrystals employed in this study facilitates nicotinamide adenine dinucleotide hydride (NADH) oxidation, which is an important step in energy-dependent processes such as myelination.

3.5 Nanoimmunotherapy applications: Infectious diseases

Immunomodulatory nanoparticles have been used to inhibit or attack bacteria or viruses and treat their associated infections, including therapies against human immunodeficiency virus (HIV), influenza, encephalitis, hepatitis, Ebola, pneumonia, etc. (Deng et al., 2018; Draz, Wang, Chen, Xu, & Shafiee, 2017; Liu et al., 2016; Vetro et al., 2017; Yang et al., 2017). Vaccines specific to antigenic bacterial constituents are often used instead of entire microbes in order to enhance immune efficiency, such as protein-based vaccines against Haemophilus influenza type b, diphtheria, or tetanus; still, adjuvants are generally required in association with these antigens to activate the immune system. DNA vaccines against infectious diseases have shown limited promise for clinical application due to their low effectiveness as well as their safety concerns. In contrast, nano-based systems such as liposomal nanovaccines show strong efficacy in humans (Schütz, Juillerat-Jeanneret, Mueller, Lynch, & Riediker, 2013). Nanoparticles may co-deliver antigens and adjuvants to immune cells with improved delivery efficiency, and can be engineered for slow, controlled release of antigen, further increasing APC exposure time and requiring less frequent administration. Controlled delivery of vaccine/drug from nanoparticles is a desirable approach to design therapeutic strategies against a range of infectious diseases.

Nano-based systems can be used to regulate the immune system and may also have germicidal properties. For example, silver nanoparticles display immunostimulatory properties and intrinsic HIV inhibition. Modified silver nanorods have been prepared using PEG or polyvinylpyrrolidone to increase IgG response and T cell production against HIV (Liu, Balachandran, Li, Shao, & Jiang, 2016). In another study, peptide-based anti-HIV DNA-vaccine-loaded nanofibers were shown to be effectively internalized by APCs, because DNA condensed into nanovectors was protected from degradation, thus enhancing DNA entry into cells and vaccine activity (Tian et al., 2014). Nanoimmunotherapy developments present important opportunities to improve disease management in the fight against HIV. HIV destroys the host's immune cells (mainly $CD4^+$ and macrophages), and conventional therapies such as active antiretroviral therapy can control but typically not remove the virus, so treatment is often lifelong and may cause undesirable side effects, such as damage to the liver and immune system. The ability to modulate immune response while reducing off-target effects is an important characteristic of a nanoimmunotherapy approach.

Cell-mimicking nanoparticles have also been exploited to enhance performance in complex biological environments, for instance by cloaking nanomaterials with natural cell membranes such as that of RBCs, platelets, and WBCs, thereby minimizing undesirable interactions with immune system components by fooling them (Fang, Kroll, Gao, & Zhang, 2018). One such nanosystem endowed with anti-HIV properties consisted of PLGA nanoparticles coated with CD4+ T cell plasma membranes. This nanosystem specifically bound HIV envelop glycoprotein 120, inhibiting viral infection (Wei et al., 2018). Researchers have also utilized the intrinsic adjuvant properties of nanoparticles against other viruses, such as the pandemic H1N1 influenza virus. For instance, poly (γ-glutamic acid) (γPGA)-chitosan nanogel has been applied as an adjuvant against H1N1 influenza virus to induce virus-specific T cell memory. This adjuvant system was more effective than aluminum compound, a well-known human vaccine adjuvant (Yang et al., 2017).

Inorganic and polymeric nanovaccines for delivery of antigen and/or adjuvants have also been designed with improved anti-bacterial infection outcomes (Pati, Shevtsov, & Sonawane, 2018). Gold nanoparticles are ideal for nanovaccine formulation due to their biocompatibility, ease of preparation, and adjuvant activity. A glycoconjugate-based gold nanovaccine loaded with T-helper OVA stimulated the specific IgG antibody-dependent immune response in *S. pneumonia* in mice (Vetro et al., 2017). Polymeric and inorganic nanoparticles have produced fairly strong host immune responses against tuberculosis (Bekale et al., 2019). Stimulation of APCs using 1,3-β-glucan, i.e., curdlan has been explored using various nanoparticles. In one study, a 1,3-β-glucan adsorbed chitosan shell, a rifampicin-loaded PLGA core multifunctional immunomodulatory nanosystem was designed to stimulate ROS and cytokine production. Results showed that these multifunctional nanoparticles promoted ROS and pro-inflammatory cytokines in human macrophages, and simultaneously increased intracellular concentrations of the anti-mycobacterial drug rifampicin (Dube et al., 2014). Curdlan-conjugated PLGA nanoparticles stimulated THP-1 monocytes, yielding improved phosphorylated extracellular-signal-regulated kinase (ERK) production (Tukulula et al., 2015). These studies show that β-glucan coated nanoparticles are able to stimulate APCs, but further studies in cells infected with *M. tuberculosis* are required. Pore-forming toxins (PFT), a key virulent component of bacteria, can be eliminated using polymeric nanoparticles. In these studies, RBC membrane vesicles on PLGA nanoparticles were used to remove PFT (Hu, Fang, Copp, Luk, & Zhang, 2013), with the PLGA

nanoparticles providing mechanical stability to these membrane vesicles. A variety of PLGA-RBC membrane sponges have been employed for PFT neutralization, which has been effective against *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, and other pathogens (Escajadillo, Olson, Luk, Zhang, & Nizet, 2017).

4. Nanoimmunotherapy translation

4.1 Nanoimmunotherapy: From bench to clinic

The principle underlying the combination of nanomedicine and immunotherapy is the ability to leverage nanomaterial interactions with the immune system in a manner that maximizes therapeutic efficacy, functionality, and specificity of these combined approaches. Ultimately, the goals of a therapeutic strategy are regulatory approval, clinical translation, and bedside deployment with high efficacy and low side effects. While many entities show benefits preclinically, few formulations ultimately succeed in clinical trials. Currently, many ongoing and completed clinical trials are exploring nanoimmunotherapeutics such as metallic and polymeric nanoparticles, with varied degrees of success as we describe in this section. An important consideration when developing nanoimmunotherapeutics, as with any therapeutic agent, is that systems using FDA-approved materials are likely to more rapidly and successfully translate from the bench to the clinic, as their biodistributions and toxicity profiles have been extensively studied. Most formulations that have advanced into clinical trials employ FDA-approved materials such as lipids or polymers incorporated in various configurations of liposomal and polymeric nanoparticles. Examples of materials used in nano-based delivery systems for clinical translation include FDA-approved polymers such as PLGA, and lipids such as DOTMA (1,2-diO-octadecenyl-3-trimethylammonium-propane), DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane), or the zwitterionic lipid DOPE (1,2-dioleoylsn -glycero-3phosphoethanolamine) (Kranz et al., 2016).

Many recent translational efforts of nanoimmunotherapy focus on cancer treatment. Liposomal immunotherapeutics for cancer applications currently in clinical trials include Lipovaxin-MM vaccine for DC targeting in stage IV melanoma (Gargett et al., 2018), DepoVax (DPX) containing the tumor antigen survivin and focused on ovarian cancer (Berinstein et al., 2015), and OncoVAX (Id/IL-2) to induce T cell responses in follicular lymphoma (Grippin, Sayour, & Mitchell, 2017). Additionally, NBTXR3 hafnium

oxide nanoparticles are currently being investigated in combination with anti-PD-1 antibody nivolumab and radiotherapy for treating prostate cancer, squamous cell carcinoma, and rectal cancer (Bonvalot et al., 2017). Stimuvax®, another liposomal based system designed to stimulate the T-cell mediated immune response against cancer cells that overexpress Mucin 1 (MUC-1), underwent three worldwide phase III clinical trials: START (Stimulating Targeted Antigenic Responses to NSCLC), INSPIRE (Stimuvax® trial In Asian NSCLC Patients: Stimulating Immune Response), and STRIDE (Stimulating immune Response in advanced breast cancer). Unfortunately, in 2014, the company stopped Stimuvax® clinical trials due to failure to meet primary or secondary endpoints, including survival and time-to-symptom progression (Kroemer, Zitvogel, & Galluzzi, 2013). Gold nanoparticle-based therapies are also being explored clinically, including Cytimmune's CYT-6091 Aurimune® (NCT00356980, NCT00436410). Aurimune® is made of colloidal gold nanoparticles conjugated to thiolated PEG and recombinant tumor necrosis factor α (rhTNF-α) to treat patients with varied types of solid tumors (such as pancreatic, ovarian and breast cancers, melanoma and soft tissue sarcoma), causing vascular breakdown and stimulating an immune response. Another gold nanoparticle therapy that completed a phase I trial for safety in 2018 employs CpG oligonucleotides around a gold core, acting as a toll-like receptor 9 (TLR9) agonist to stimulate innate and adaptive immune responses against advanced or metastatic solid tumors (https://clinicaltrials.gov/ct2/show/NCT03086278).

There are other nanoimmunotherapeutic approaches to cancer treatment beyond liposomes and gold nanoparticles that are being clinically tested: one is a protein nanogel-based "backpack" strategy, with ex vivo modification of T cells prior to adoptive cell transfer. The nanogel is in phase I trials (https://clinicaltrials.gov/ct2/show/NCT03815682) for patients with advanced solid tumors and lymphomas (Tang et al., 2018). Another is a cancer vaccine (WDVAX) against stage IV melanoma, consisting of the patient's own tumor lysate combined with other proteins (GM-CSF and CpG) to elicit DC activation, and PLGA as base carrier material, for which Novartis has received a license for phase I clinical trials (https://clinicaltrials.gov/ct2/show/NCT01753089). A different vaccine approach involves the "Lipo-MERIT" vaccine, which is currently being evaluated for safety and tolerability and consists of RNA-lipoplexes created by combining liposomes with various RNA-drug products that are specifically designed to elicit T-cell responses to four cancer antigens

(NY-ESO-1, MAGE-A3, tyrosinase and TPTE). The lipoplexes are designed to enhance plasma half-life after IV administration and target systemic antigen-presenting cells (APC), and are being assessed in clinical trials to treat advanced malignant melanoma (https://clinicaltrials.gov/ct2/show/NCT02410733).

The use of nanoparticles to promote immune tolerance and regulate immune response is being explored clinically for diseases other than cancer by several companies including Courpharma, Selecta Biosciences, and others for applications such as pemphigus vulgaris, T1D, multiple sclerosis, celiac disease, and other disorders. Selecta's Selective Immune Tolerance Technology (ImmTOR) is being used to generate DCs and $Foxp3^+$ $CD4^+$ T cells in vivo and inhibit $CD4^+$ and $CD8^+$ T cell effector cell activation, and a Phase II clinical trial using this approach is underway to explore the use of rapamycin and antigen co-loaded PLA and PLGA nanoparticles in treating chronic refractory gout, and is currently in Phase II clinical trials (Kishimoto & Maldonado, 2018).

Clinical nanomedicine use remains sparse in cardiovascular therapy, but human trials are beginning to emerge. For instance, second-generation lipid nanoparticles loaded with a siRNA targeted to Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) were studied in patients with high plasma LDL cholesterol with promising results, including a 70% reduction in circulating PCSK9 plasma protein and a 40% reduction in LDL cholesterol from baseline relative to placebo. Additionally, long-circulating prednisone-containing liposomes for atherosclerosis accumulated in plaque macrophages in patients, providing proof-of-concept for the potential development of macrophage-focused therapies (Fitzgerald et al., 2014; van der Valk et al., 2015, 2016; Zhang et al., 2021).

Two key barriers to successful translation from bench to clinic are stratification of patients who will benefit most from specific therapies, and controlling immunotoxicity effects. Deployment in clinical practice remains elusive due to the lack of precise strategies to effectively manage potential toxicity, even in cases where the formulation has obtained FDA approval. For instance, checkpoint inhibitor therapies can cause adverse effects including skin reactions, such as rash and psoriasis, and gastrointestinal tract problems such as inflammatory colitis (Postow, Sidlow, & Hellmann, 2018). These known shortcomings of immunotherapies must be considered in developing and translating future nanoimmunotherapy formulations for clinical use. However, some of the desirable properties of nanosystems, including versatility of their design, targeting ability, and specificity of

delivery, could help minimize some of these issues. A thorough understanding of nanomaterials in terms of the safety profiles of nanomaterials, as well as their interactions with immune cells, mechanisms of action, and standardized protocols for treatment and detection and management of side effects, are all critical to engender successful deployment of nanoimmunotherapies.

5. Toxicology of nanoimmunotherapies

In the last 10 years, there have been great advances in our understanding of the immunocompatibility of nanoparticles, and how the characteristics of nanoparticles change their interaction with plasma proteins and immune cells to safeguard drug delivery (Kadam, Bourne, & Kompella, 2012; Petschauer, Madden, Kirschbrown, Song, & Zamboni, 2015). This knowledge has greatly enhanced the development of safe and efficacious nanoimmunotherapeutic platforms. Still, critical concerns remain regarding the safety of these engineered nanosystems, including their interactions with innate immune subcomponents, which may result in immunological reactions and lead to immunotoxicity. Also, repeated stimulation of the immune system can intensify the development of allergic and autoimmune diseases. Conversely, uncontrolled tolerance or suppression of the immune system may result in increased risk of infectious diseases and tumor growth. Furthermore, physicochemical characteristics of nanoparticles such as their size, shape, chemical composition, surface chemistry in relation to charge, area, porosity, etc., are crucial in determining not only efficacy, but also safety and compatibility with the immune system. The size of nanoparticles influences overall biodistribution, and nanoparticle size and shape affects their uptake by immune system cells and the subsequent immune response (Liu, Hardie, Zhang, & Rotello, 2017), with larger and non-spherically shaped nanoparticles being more easily internalized. Similarly, increasing nanoparticle hydrophobicity results in enhanced immune cell internalization, whereas hydrophilic formulations seem to be less immunogenic. Surface charge is another important nanoparticle consideration (Liu et al., 2017); for instance, cationic nanoparticles interact more readily with negatively-charged cell membranes. As our understanding increases in terms of how tailored design of nanomaterials can impact interactions with the immune system, this can lead to improved safety and compatibility profiles.

The most common safety concerns associated with nanosystems include erythrocyte damage, thrombogenicity, cytokine-mediated inflammation, and other complement activation-induced responses (Dobrovolskaia,

Shurin, & Shvedova, 2016). Some nanoparticles may induce generation of more ROS during the pro-inflammatory stage in cells, which may in turn damage cellular components including proteins, lipids, and the cell membrane. Although there is data on the immunomodulatory effects of nanoparticles, it is not sufficient to fully understand all the mechanisms in play, and the immunological effects of a given formulation do not necessarily reflect their immunotoxicities (Engin & Hayes, 2018). The lack of standards or specific safety protocols and toxicity guidelines for nanoimmunotherapy also makes it difficult to interpret the relevance of a given study's observations regarding immune toxicity. It also creates issues in understanding which observations should constitute a concern or trigger further review, or even how to properly and accurately measure/evaluate toxicity. In the last decade, to improve this situation ASTM and ISO launched the E56 and TC229 committees to develop reference test methods for nanomaterials (ASTM E56–2524-08(2013), ASTM E56–2525-08(2013), ASTM E2526–08(2013), and ISO 29701:210). These methods provide guidelines for testing hemolytic properties of nanoparticles, the effect of nanoparticles on the formation of mouse granulocyte-macrophage colonies, evaluation of cytotoxicity of nanoparticles on different types of cells, and testing of contamination of nanomaterials by endotoxins. Although these are helpful guidelines, there remains much work to be done in developing a sufficiently broad set of tests and guidelines that is applicable to overall global immunotoxicology, and which can properly assess the interactions of nanoimmunotherapy formulations with living systems, and the potential consequences of this interplay.

6. Conclusion

Nano-mediated immunotherapy faces the complex task of tailoring a specific, desirable immune response within the human body, bringing these types of therapies into the realm of personalized medicine. However, many associated challenges remain. Although a variety of nanoimmunotherapies have reached clinical trials, particularly in the area of cancer management, clinical translation remains elusive for most nanoimmunotherapeutic agents. The delicate equilibrium between immune protection and immune tolerance, along with the intricate array of factors that affect nanosystem delivery, safety, and efficacy, create many challenges for successful translation. Of these factors, nanosystem immunotoxicity and safety are key areas of concern, since nanoparticles typically interact closely with immune cells. Environmentally-conscious or "green" nanomaterials typically have

excellent biocompatibility and sometimes better safety profiles, although more research is required to ensure their utility.

A detailed understanding of how a nanosystem interacts with immune components is required in order to select the appropriate platform for precision treatment. Some nanoparticles are intrinsic adjuvants, so immunogenicity should be controlled to circumvent excessive immune response that may decrease the efficacy of therapy or become maladaptive. Another challenge is successful delivery of a nanodrug to specific immune cell populations. Targeted delivery and controlled release of antigen and adjuvants must often be achieved, and a variety of ligands or intrinsic proclivities can be employed to functionalize nanoparticles for precise transportation to a target (Flores et al., 2020; Kanthi, de la Zerda, & Smith, 2020; Smith et al., 2014; Zhang et al., 2021). The intrinsic features of nanoparticles such as their size, shape, surface charge, and hydrophobic/hydrophilic balance are also crucial factors that influence local delivery and accumulation within a target. Nanoparticle physicochemical characteristics influence their pharmacokinetics and may promote or prevent interaction with biological components (e.g., protein corona formation on the surface of the nanoparticle), which can impact how much of the agent reaches the target site. Systematic studies are needed to enrich our understanding of how nanoimmunotherapeutic agents behave in biological environments, and how we can modulate or tailor that behavior to our advantage. The eventual success of translational nanoimmunotherapy also depends on testing in advanced preclinical animal models that accurately simulate human illness progression. Various established models are effectively used to study disease mechanisms, but a major remaining challenge is how effectively these models can address therapeutic efficacy of a given formulation, so that the potential for clinical translation can be more accurately evaluated.

Nano-mediated immunotherapy is a growing area of research for generating novel strategies to treat a variety of diseases, given the immune system's central role in human health. Although many obstacles lie ahead, exciting clinical developments are also likely approaching as we better learn how to harness the power of the immune system for therapeutic modulation using nanotechnology.

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Conflict of interest

Authors declare no conflict of interest.

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