*Minireview*

# Emerging nanotechnologies for cancer immunotherapy

**Sourabh Shukla[1,2] and Nicole F Steinmetz[1,2,3,4,5]**

[1]Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106, USA; [2]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH 44106, USA; [3]Department of Radiology, Case Western Reserve University, Cleveland, OH 44106, USA; [4]Department of Materials Science and Engineering, Case Western Reserve University, Cleveland, OH 44106, USA; [5]Department of Macromolecular Science and Engineering, Case Western Reserve University, Cleveland, OH 44106
Corresponding authors: Sourabh Shukla. Email: sourabh.shukla@case.edu; Nicole F Steinmetz. Email: nicole.steinmetz@case.edu

## Abstract

Founded on the growing insight into the complex cancer-immune system interactions, adjuvant immunotherapies are rapidly emerging and being adapted for the treatment of various human malignancies. Immune checkpoint inhibitors, for example, have already shown clinical success. Nevertheless, many approaches are not optimized, require frequent administration, are associated with systemic toxicities and only show modest efficacy as monotherapies. Nanotechnology can potentially enhance the efficacy of such immunotherapies by improving the delivery, retention and release of immunostimulatory agents and biologicals in targeted cell populations and tissues. This review presents the current status and emerging trends in such nanotechnology-based cancer immunotherapies including the role of nanoparticles as carriers of immunomodulators, nanoparticles-based cancer vaccines, and depots for sustained immunostimulation. Also highlighted are key translational challenges and opportunities in this rapidly growing field.

**Keywords:** Cancer immunotherapy, nanotechnology, vaccines, immunomodulation

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## Introduction

### Cancer immunotherapies – revitalizing the immune system against cancer

One of the major developments in the fight against cancer is the emergence of immunotherapies that are aimed at harnessing the exquisite and specific power of the immune system against malignancies.[1,2] The renewed excitement and push for novel immunotherapies stems from the success of two different strategies – adaptive T-cell therapy based on chimeric antigen receptors (CARs) and checkpoint blockade. The former, CAR T cells therapy, still under clinical evaluation, is based on genetically engineering patient's own T-cells with CARs that recognizes tumor antigens. These CAR T cells are then expanded *in vitro* and infused back in patients, where they are likely to recognize and kill cancer cells.[3] Checkpoint blockade therapies, on the other hand work by inhibiting pathways that keep the duration and strength of immune system in check.[4] The recent approval of two checkpoint blockade therapies targeting the receptors CTLA-4 and PD-1 have come on the back of several successful clinical trials where treatment with checkpoint blockade inhibitors has resulted in striking T cell function restoration in melanoma, renal cell carcinoma and lung cancer.[4,5]

These developments and other similar efforts are clearly fueled by growing insights into the nature and consequences of interactions between tumors and the immune system, which frequently impede the development and function of anti-tumor immune response.[6–8] The challenge for immunotherapies is to modulate such interactions towards successful recognition and elimination of cancer cells. The durability and specificity of such strategies has the potential to generate long-lived therapeutic effects with limited systemic toxicities.[9,10] However, growing evidences suggest that immunotherapies could be most beneficial as an adjuvant therapy to conventional chemo- and radiation therapies.[11]

The premise of an immune system-mediated intervention in cancer progression lies in the capacity of the immune system to distinguish between self and non-self.[12,13] While highly equipped and effective in the eradication of pathogens, the ability of the immune system to effectively deal with transforming cancer cells is hampered by the fact that the cancer cell's origin is self, and because of depletion of self-antigen reactive T lymphocytes during development.[14] Nevertheless, cancer is characterized by the accumulation of a variable number of genetic alterations and the loss of normal cellular regulatory processes, resulting in expression of neo-antigens arising from mutated

genes, chromosomal aberrations or overexpression of embryonic antigens.[15,16] These neo-antigens differentiate cancer from normal cells and enable their immune-recognition, as indicated by the presence of basal levels of tumor antigen-specific cellular and humoral responses in subsets of cancer patients.[13,17] However, a highly immunosuppressive microenvironment under the influence of cytokines such as TGF-$\beta$ and IL-2,[18] along with immunosuppressive cells such as Foxp3$^+$ $T_{regs}$ (regulatory T cells),[19] myeloid-derived suppressor cells (MDSCs)[20] and M2-type macrophages,[21] or combinations thereof, keeps such autologous immune response in check. Moreover, through intricate immunoediting mechanisms, including antigen shedding, negative selection of antigenic cancer cells, down-regulation of MHC-I molecules and turning off activated T cells via negative regulators such as PD-1,[4] cancer cells evade immunosurveillance and the tumor prevails.[7]

To overcome these hurdles and tip the scale in favor of anti-tumor immune response, a diverse set of immunotherapeutic approaches have been explored. While adaptive T cell transfer (ACT)[22] and cancer vaccines[23] are aimed at boosting tumor reactive immune cell populations, cytokines, immunomodulatory antibodies and small molecule drugs have been employed to overcome the immunosuppressive tumor microenvironment.[24] The goal of cancer immunotherapy is to initiate or reinitiate a self-sustaining cycle of cancer immunity, enabling it to amplify and propagate.[2] Although numerous such strategies have been explored, only a handful of them have been approved and adapted for clinical use with only a modest rate of success.[1] Thus, there is a huge scope for further development in terms of specificity, enhanced effectiveness and reduced toxicities.

Many immunotherapeutic strategies currently in preclinical or clinical evaluation are based on traditional drug development approaches where nanomedicine has already made significant contributions by improving stability, biodistribution through targeted delivery, bioavailability and efficacy of cytotoxic drugs or imaging contrast agents.[25,26] Ongoing clinical evaluations of several candidate nanoformulations in conjugation with a wide range of chemotherapeutic or immunotherapeutic payloads are a testament of the efficacy of nanoparticle-based drug delivery approaches.[27,28] For example, Nanoplatin NC-6004, a cisplatin containing polymeric nanocarrier is under Phase I/II clinical trial,[29] whereas cyclodextrin-based nanoparticles CALAA-01 that delivers small-interfering RNA (siRNA) agent to shut down a key enzyme (ribonucleotide reductase) in cancer cells is under Phase I evaluation.[30] Similarly, an engineered adenovirus nanoparticle-based drug delivery platform is under Phase-1 dose escalation study for delivery of cancer immunotherapy to patients with chronic lymphocytic leukemia (CLL)[31] while the pH sensitive polymeric nanoparticle CRLX101 loaded with camptothecin is undergoing Phase II clinical trials.[32]

Based on those very possibilities, an excellent opportunity for nanotechnology-mediated refinement exists in the field of immunotherapy.[33–35] This review details some of the key immunotherapeutic strategies and highlights nanotechnology-based interventions that are being pursued to improve the overall efficacy of such approaches, as summarized in Figure 1. Additionally, critical barriers to the successful translation of these emerging technologies are also discussed.

## Nanocarriers to deliver tumor microenvironment immunomodulators

A number of immunomodulatory and immunostimulatory molecules such as cytokines, chemokines and targeted antibodies have been identified for their important roles in countering the highly immunosuppressive tumor microenvironment. Cytokine IL-2 promotes proliferation of effector functions of cytotoxic T lymphocytes (CTLs) and has shown clinical efficacy in malignant melanoma and renal carcinoma.[36] IL-2 has also resulted in enhanced efficiency of other immunotherapies.[37] Other cytokines such as IL-21 and IL-18 modulate both innate and adaptive immune responses through activation of CD4$^+$/CD8$^+$ T cells, natural killer (NK) cells, and B cells while suppressing $T_{reg}$ cells.[38,39] Systemic administration of IL-21 and IL-18 also leads to enhanced production of IFN-$\gamma$, IL-2, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1$\beta$ and IL-6 by activating T cells. Similarly, type I interferons (IFN-$\alpha$ and $\beta$) demonstrate anti-tumor activities through stimulation of NK cell activity and suppression of allospecific suppressor T cells. Indeed, the administration of type I interferons has shown promise and efficacy in clinical trials in the setting of leukemia, melanoma and renal cell carcinoma.[40] Type II interferons (IFN-$\gamma$) induce apoptosis, upregulate HLA-I and HLA-II and therefore promote antigen presentation in cancer cells, which in turn mediates tumor rejection and has shown efficacy in the setting of ACT therapies.[41] Other non-specific immunomodulators such as Toll-like receptor (TLR7/9) agonists (e.g., synthetic oligonucleotides CpG) promote Th1 polarization, trigger activation of innate and adaptive immune responses, lead to dendritic cell (DC) activation and proliferation of CD4$^+$/CD8$^+$ T cells and modulate suppressive functions of $T_{reg}$ cells.[42,43]

Even though the immunomodulatory effects of these small molecules in overturning the suppressive tumor microenvironment are well documented, drawbacks are associated with such therapies: Besides the short half-life, stability and bioavailability challenges akin to many conventional therapeutic candidates, systemic toxicities of cytokines arising due to their broad spectrum of biological activity on a wide variety of cells are major safety concerns.[44–46] Cytokines could lead to non-specific lymphocyte activation in circulation and increased incidences of autoimmune and allergic responses. IL-1, IL-2, IL-6, TNF, and TGF-β could lead to modulation of hepatic metabolisms.[9] Similarly, systemic administration of CD-40 agonist used to trigger CD40 signaling for activation of antigen presenting cells can lead to widespread symptoms of cytokine release syndrome, ocular inflammation, elevated levels of hepatic enzymes, and hematologic toxicities including T-cell depletion. Besides, agonistic anti-CD40 therapy has also been linked to long-term immunosuppression mediated by activation-induced apoptosis of CD4$^+$ and CD8$^+$ T cells.[47,48] Similarly, overexposure to CpG could result in suppression

of adaptive T cell immunity.[49,50] Likewise, IL-2 administration at high doses causes vascular leak syndrome (VLS; also known as capillary leak syndrome), which is associated with increased vascular permeability, hypotension, pulmonary edema, liver cell damage and renal failure.[51]

Other side effects of IL-2 are hypothyroidism, thrombocytopenia, anemia, coagulopathy, or impairment of neutrophil chemotaxis, autoimmunity, neurotoxicity and myocarditis.[52,53] In addition, the ability of IL-2 to stimulate $T_{Reg}$ cells diminishes the beneficial effects of stimulating tumor-specific T cell response.[54] Systemic administration of IL-2 with adaptively transferred T cells could also cause multi organ failures in severe cases. At the same time, dose dependent toxicities including thrombocytopenia, fatigue, and pyrexia have been associated with checkpoint blockade inhibitors.[9]

To overcome these challenges and provide a pathway for safe clinical use of immunomodulatory cytokines/chemokines, nanotechnology approaches hold great promise for the path forward. Nanoformulations consisting of such immunomodulatory molecules have improved bioavailability due to significantly prolonged circulation times of the carrier particles and *in vivo* stability of payload against serum inactivation and enzyme degradation.[55,56] For example, intravenous administrations of liposomes containing cytokines such as IFN-γ, IFN-α, IL-2 or TNF-α enhance the plasma residence time.[55,57,58] On the other hand, intraperitoneal, intramuscular, subcutaneous or intranasal administration of cytokine carrying liposomes and polymeric particles creates local depots and increases residence times of the immunostimulatory payloads at the site (discussed in details later).[59–61] Additionally, requirement of external or physiological stimuli ensures the release of immunostimulatory cargo only at targeted sites and further improve its bioavailability and safety.[62–65]

Specifically, nanoparticle-based delivery promotes the preferential accumulation and retention of immunomodulators in tumors due to the enhanced permeability and retention (EPR) effect, while minimizing off-target systemic toxicities, thereby improving potential for clinical translation of such therapies.[66,67] Building on EPR effect-mediated nanoparticle homing, nanotechnologies are undergoing development targeting and modulating the immunosuppressive tumor microenvironment to attain efficacy of immunotherapies. For example, based on the passive tumor homing properties, lipid-coated calcium phosphate nanoparticles (LCP-NPs) have been used for tumor microenvironment immunomodulation by delivering TGF-$\beta$ siRNA and thereby down-regulating the levels immunosuppressive TGF-$\beta$ within the tumor. LCP-NPs have also been used to deliver a broad spectrum anti-inflammatory triterpenoid – methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) – that significantly reduced $T_{reg}$ and MDSC populations. The delivery of immunostimulatory molecules by LCP-NP has been combined with vaccination strategies using an LCP vaccine delivering a tumor antigen (Trp 2 peptide) and adjuvant (CpG oligonucleotide) to DCs – the combination therapy resulted in improved efficacy over vaccine only treatments.[68,69]

Similarly, EPR-mediated accumulation of liposome-encapsulated polymer nanogels has been utilized for intratumoral delivery of cytokine (IL-2) and TGF-β receptor I inhibitor—SB505124, a hydrophobic small molecule drug, leading to inhibition of TGF-$\beta$ receptor I and subsequent expansion of T cells and NK cells by blocking key immunosuppressive pathways.[70] Similarly, by delivering PD-L1 siRNA using polyethylenimine (PEI) liposomes, PD-L1 levels have been knocked down leading to immunosuppressive to immunostimulatory phenotype changes in human and mouse ovarian cancer-associated DCs with subsequent increase in tumor-reactive $CD8^+$ T cell numbers and improved mice survival.[71] Liposomal delivery of IL-2 has also showed enhanced therapeutic effects with reduced toxicities in a variety of other tumors including liver and lung cancers leading to significant reduction in tumor growth.[72,73]

In addition to their use for systemic delivery and homing to tumors, nanoparticle formulations show benefits for intratumoral delivery of immunomodulators: based on their size, the nanoparticle-based formulation of immunotherapies limits their escape into systemic circulation, thus minimizing off-target effects and maximizing local immunostimulation. For example, immunostimulatory liposomes conjugated with IL-2 and anti-CD137 antibodies targeting activated T cells led to increased IL-2 dosing within the tumor when delivered directly via intratumoral vs. systemic injections. The intratumoral treatment resulted in a higher ratio of tumor-infiltrating $CD8^+$ T cells over regulatory T cells in established melanomas.[46] Likewise, PEGylated liposome formulation have been used to deliver agonistic anti-CD40 antibodies and TLR agonist CpG molecules using intratumoral administration resulting in significant tumor inhibition while sequestering the immunostimulatory payload in targeted tissues and reducing its systemic leakage, thus minimizing off-target inflammatory effects.[45] Similarly, intratumoral administration of CpG payloads on gold nanoparticles has shown to induce significant macrophage and DC infiltration in tumors and significantly affected tumor growth by concentrating the CpG oligonucleotides in the tumor tissue and lowering the high dose requirements of systemic administrations.[74]

Passive tumor homing and intratumoral administration of nanoparticles are accompanied by their natural tropism towards phagocytic cells of the innate immune system *in vivo* including monocytes, neutrophils, macrophages and dendritic cells. Such affinity towards immune cells can also be used to deliver immunomodulating payloads to tumor-infiltrating immune cell populations and thereby reprogramming the tumor microenvironment. For example, nanocomplexes encapsulating CpG oligonucleotide and anti-IL-10 and anti-IL-10 receptor antisense oligonucleotides were efficiently captured by tumor-associated macrophages (TAMs) and resulted in altered macrophage phenotypes, leading to a significant anti-tumor effect in a hepatoma murine model.[75] To improve the partition in TAMs over macrophages associated with the mononuclear phagocyte system (MPS), mannose-modified polymeric micelles containing acid-sensitive PEG modifications were developed. The PEG shielding reduced uptake by the MPS

in circulation at neutral pH and improved tumor accumulation through persistent circulation. The acidic pH in the tumor resulted in PEG shedding and uptake by TAMs and their subsequent reprogramming.[76]

However, nanoparticles have also been targeted to immune cells in circulation to deliver payloads to the tumor tissue. RGD-targeted single walled carbon nanotubes (SWCNTs) have showed enhanced tumor accumulation via hitchhiking Ly6C$^{hi}$ monocytes in the circulation that are recruited to the site of the tumor in response to inflammation.[77] Also, in a recent study, gold nanoparticles combining mouse vascular endothelial growth factor (VEGF)-siRNA with TAM-targeting M2 peptide have been used to inhibit both TAMs and cancer cells by targeting the VEGF pathway in both cell populations. Such synergistic inhibitory effects on both cancer cells and the immunosuppressive TAM population resulted in significant tumor regression and disease control for extended periods in orthotopic lung cancer in mice.[78] These examples highlight the utility of nanoparticles to target phagocytic cells for immune activation; tissue- and cell-specificity can be further tailored by altering nanoparticle shape, size, charge, hydrophobicity and surface chemistry.[33,79,80]

In addition to their natural interactions with mononuclear phagocyte cells, nanoparticles carrying immunostimulatory payloads have also been utilized to modulate the functioning of T and B cells for therapeutic interventions. Adaptive T cell immunotherapy plays a central role in cancer therapy; cancer antigen-specific T cells can be expanded using vaccines (*in vivo*) or through ACT, the latter requires the *ex vivo* expansion of T cells followed by infusion into patient. While effective, ACT has many drawbacks because the methods are expensive, cumbersome and personalized.[22] To enhance the technology, in one study, dextran-coated iron oxide particles with surface-coupled MHC-Ig dimers and anti-CD28 antibodies were designed to allow magnetic field-based aggregation of particles bound to T cell receptors (TCRs). *Ex vivo* stimulation of T cells with these particles in the presence of a magnetic field-enhanced TCR clustering reduced the threshold of activation of T cells and improved the efficacy of adaptive T cell therapy.[81] Moreover, nanotechnology opens the door for the *in vivo* targeting, priming and expansion of T cells. For example, *in vivo* loading of T cells with lipid nanoparticle ''backpacks'' carrying stimulatory cytokines was demonstrated. The nanoparticle-mediated *in vivo* priming resulted in 80-fold increased T cell expansion and significant enhancements in the efficacy of ACT without systemic toxicity.[82] Similarly, circulating adaptive T cells were targeted *in vivo* by IL-2 loaded liposomes via anti-Thy1 antibodies, resulting in enhanced T cell proliferation more effectively compared to administration of soluble cytokines.[83] These approaches overcome a decline in function of transplanted T cells following infusion, particularly in the setting of solid cancers with a highly immunosuppressive microenvironment.

Finally, we recently demonstrated the use of virus-based nanoparticles as an immunotherapeutic, where the properties of the nanoparticle itself unlocked a potent anti-tumor immune response. We have demonstrated that plant-derived virus-like particles stimulate a potent immune-mediated anti-tumor response when introduced into the tumor microenvironment after tumors are established: VLPs from cowpea mosaic virus (CPMV), without nucleic acids, LPS, or any other recognized immune adjuvants, generated an effective anti-tumor immune response in mouse models of multiple tumor types, including triple negative breast cancer, disseminated ovarian cancer and primary and metastatic melanoma. The particles are not cytolytic to tumor cells and the effects are immune mediated. Most importantly, preliminary data indicate that the effect is systemic and durable, resulting in immune memory protecting mice from re-challenge.[84] The immunotherapy follows an *in situ* vaccination approach in which immune-stimulatory reagents (here CPMV) are applied directly into the suspected metastatic site or into an identified primary tumor. This approach modulates the local microenvironment to relieve immunosuppression and potentiate anti-tumor immunity against antigens expressed by the tumor. This approach not only offers the potential for new therapeutics but also may lead to new levels of understanding how the immune system defines ''danger signals''.

## Improving cancer vaccines – mediators of adaptive immune response

A wide range of cancer vaccines has been evaluated for a variety of human malignancies.[85,86] The overarching goal is to deliver tumor-associated antigens to professional antigen presenting cells (APCs) to elicit adaptive immune responses mediated by tumor-specific cytotoxic T cells and antibodies. As an active immunotherapeutic approach aimed at stimulating endogenous anti-tumor immune response, cancer vaccines offer effective long-term protection against recurring and residual tumors. However, development of an effective therapeutic vaccine against established disease is challenging, and despite decades of pursuit, establishment of successful vaccination strategies based on proteins, peptides, autologous dendritic cells or tumor cells have largely been unsuccessful.[18,87] While vaccines based on autologous cells are costly and technically challenging, peptide-based cancer vaccination suffers from inefficient uptake, processing and presentation of the delivered epitopes by activated professional APCs.[23,51,87–90] Moreover, such vaccination strategies have led to the generation of low avidity tumor-specific T cell responses. Whole protein vaccination with powerful and often poorly tolerated adjuvants, immunostimulatory cytokines such as IL-2 or GM-CSF and/or TLR agonists have failed to induce clinically significant anti-tumor responses.[91–93] However, spontaneous tumor antigen-specific and high avidity T cell response has been shown to control tumor progression. Together with the demonstration of significantly reduced tumor burden in both patients and animal models following ACT, this suggests that high avidity tumor-specific CTL response could lead to long-lasting immunoprotection against relapsing or residual cancer.[85,86,94]

Nanoparticle-based vaccine approaches offer multiple advantages that could fulfill the stringent requirements for the generation of such high avidity tumor-reactive

T cells.[95,96] Presenting antigens/epitopes on nanoparticulate carriers not only provides the requisite stability and longevity, it also facilitates efficient interactions with key immune cell populations.[88,97] Clearly, nanoparticle engineering principles for vaccine platforms are different from those targeted for delivery of immunomodulators. While the former seeks interaction with APCs and other phagocytes, the latter aims to generally avoid phagocytic clearance thus prolonging circulation and improved tumor penetration. Nanoparticle-based vaccine formulations can improve the resulting immunostimulation by promoting multivalent receptor cross-linking, by altering intracellular processing and presentation or by colocalizing synergistic cues from the antigen, adjuvants and costimulatory molecules within the same cellular populations. The particulate nature of nanoparticles mimic pathogen-associated molecular patterns (PAMPs) that are perceived as danger signals and drive protective immunity.[98] Such patterns are recognized by the pattern recognizing receptors (PRRs) such as TLRs on immune cells, specifically APCs, and facilitate enhanced uptake of nanoparticle-based vaccines by these cells.[99,100] Activation of PPRs provides immunogenic cues to the immune system instructing it to launch specific response to the antigens carried by the nanoformulation.

B cells have evolved to recognize multivalent display of antigens on microbial surfaces and play a major role in vaccine-mediated antibody responses, thus enhancement of their engagement is crucial for immunotherapies based on cancer vaccines. Nanoparticle-based antigen display that mimic pathogenic structural features are highly efficient in engaging B cell receptors (BCRs) to promote greater signaling, antigen internalization and processing of antigens for presentation to $CD4^+$ T cells.[101] Multivalent display of antigens on nanoparticles also leads to TLR stimulation promoting strong humoral responses with long-lived high avidity antibody responses mediated by secretion of co-stimulatory cytokines such as IFN-$\alpha$ and IL-12.[102] For example, we have demonstrated that potato virus X (PVX) displaying HER2-derived B-cell epitopes effectively generates HER2-specific antibodies.[103] Similarly, plant viral nanoparticles coupled to a weak idiotypic (Id) tumor antigen have been used as a conjugate vaccine to induce antibody formation against a murine B-cell malignancy.[104] Other virus-like particles have similarly been evaluated as efficient vaccine carriers.[105–108] For a detailed review, we would like to refer the reader to our recent article (Lee, 2016).[147].

Professional APCs, particularly dendritic cells, are critical initiators of adaptive immune response, comprising both humoral and cellular responses, and are therefore an important target for anti-cancer nanomedicine. Nanoparticle-based vaccines are readily taken up by DCs and are associated with enhanced anti-tumor response as compared to soluble antigens.[109] Following processing in endolysosomal compartments, soluble exogenous antigens are exclusively presented on MHC-II molecules to activate $CD4^+$ helper T cells that in turn stimulate B cell-mediated antibody response. However, via a process called cross-presentation, nanoparticle-mediated delivery of antigenic peptides also results in cytosolic release of antigens where MHC-I loading can occur, resulting in $CD8^+$ T cell priming and ensuing cytotoxic T cell response.[110] Such cross-presentation of cancer antigens have been demonstrated for a wide range of distinct nanoparticles and new strategies are being developed to improve the efficiency of such cross-presentation.[111–113] These include strategies employed for cytosolic drug delivery of membrane-impermeable molecules such as via endolysosomal disruption through the proton sponge effect using biodegradable nanogels,[114] endolysosomolytic and pH-responsive micelles,[115] as well as endoplasmic reticulum (ER) targeting approaches where nanoparticles shuttle to cytosol following endosome-ER fusion.[116]

A significant advantage of nanoformulations is the control over their transport kinetics that facilitates tissue-specific delivery of antigens. Tumor antigens drain into tumor draining lymph nodes (TDLNs), where they are taken up by professional APCs, including DCs leading to subsequent presentation and priming of T cells. TDLNs are rich in phenotypically and functionally immature APCs and are therefore key targets for priming APCs using cancer vaccines and the subsequent adaptive immune response. The size-dependent lymphatic drainage of nanoformulations has been established and is an important design consideration for developing cancer vaccines: Nanoparticles between 20 and 45 nm optimally drain to lymph nodes and are retained.[117] While smaller particles are likely to be flushed out of lymph nodes, those larger than 100 nm are less efficiently transported from the peripheral injection sites, generally via cell-mediated transport.[118] Upon subcutaneous injection, only a small fraction of the vaccine is delivered to DCs whereas the majority is cleared by the body or engulfed by other immune cells.

By targeting DCs, vaccine efficiencies can be further improved by overcoming non-specific uptake of nanoparticle-based vaccines. For example, cancer vaccines based on biodegradable poly(lactic-co-glycolic acid) (PLGA) nanoparticles when coated with an agonistic αCD40-mAb (NP-CD40), showed highly efficient and selective delivery to DCs *in vivo* and improved priming of $CD8^+$ T cells against two independent tumor-associated antigens.[119] Additionally, targeting a specific subset of DCs could also define the type of stimulated immune response.[120] For example, TLR7 and TLR9 agonists can convert the tolerogenic plasmacytoid DCs to innate immunostimulatory types, whereas targeting various C-type lectins can modulate variable adaptive response. For instance, DC-SIGN, DEC-205, DNGR-1 and Langerin favors $CD8^+$ T cell cellular (Th1) responses while $CD4^+$ and B cell humoral (Th2) responses are achieved by targeting of DCIR2.[121] The targeting of multiple DCs subtypes, in particular, holds great potential to enhance vaccine efficiencies.

### Sustained immunostimulation through *in situ* depots and artificial APCs

Nanoparticles-based depots have recently gained attraction as a source of sustainable immune stimulus. For example, cytokine depots composed of liposomes or polymeric

particles carrying pro-inflammatory cytokines have been developed for anticancer vaccines and for intratumoral administration for therapy. These cytokine-loaded particles can enhance tumor-specific immune response in conjugation with tumor antigen from irradiated tumor cells or co-encapsulated with the cytokines.[55] Using these strategies, GM-CSF encapsulated in polymer particles or IFN- $\gamma$/IL-2 in liposomes caused increased leukocyte infiltration/increased humoral response and enhanced cytolytic capacity of $CD8^+$ T cells resulting in higher fraction of mice surviving melanoma challenge.[122] Similar studies extended in human trials involved incorporation of tumor-specific idiotype isolated form follicular lymphoma patient into IL-2 containing liposomes and monthly vaccinations which resulted in sustained tumor-specific $CD4^+$ and $CD8^+$ T cell response and continuous remission.[123]

On the other hand, cytokine depots have been also employed to treat primary tumors with peri- or intratumoral injections. Here, primary tumors serve as the source of antigen while cytokine depot activate leukocytes in tumor microenvironment and promote immunotherapy against primary tumor and metastasized tumor cells. Local injection of polymeric microparticles loaded with IL-2 for the treatment of brain or liver tumors has showed that this approach was more effective at treating tumors and protecting against rechallenge than tumor cells engineered to express IL-2.[124] Compared to treatment with soluble IL-2, liposomal IL-2 treatment of B16 melanoma bearing mice resulted in higher survival rates, slower tumor growth rates prior to resection and increased recruitment and protected mice against rechallenge with melanoma.[124]

Further studies using this strategy have involved particles with combinations of various cytokines including IL-12, TNF-$\alpha$, GM-CSF or IL-18. Treatment of established tumors with IL-12 loaded particles prior to surgical resections promoted systemic anti-tumor immunity that prevented recurrence and metastasis.[125] Furthermore, particles with combination of IL-12 and GM-CSF showed eradication of primary tumors through $CD4^+$ and $CD8^+$ cells, while the effect on metastasis was through NK/NKT cells.[126]

The concept of immunotherapeutic depot has also been similarly used in therapeutic vaccine DepoVax$^{TM}$ (DPX-0907), which employs a liposome-based platform harboring custom formulated mixtures of $CD8^+$ T-cell peptide epitopes, a tetanus toxoid derived Th epitope, and an adjuvant of choice (such as a toll-like receptor agonist) to provide signals for improved antigen presentation. The liposomes carry incorporated hydrophilic antigens and adjuvant directly into an oil medium such as Montanide ISA51 VG, entrapping all vaccine ingredients in a form suitable for efficient uptake, processing and presentation by antigen-presenting cells (APCs). Such DPX formulated vaccines have been shown to induce effective immune responses after a single-dose administration.[127]

Another development in this area is the design and engineering of artificial APCs (aAPCs) that are synthetic mimics of natural antigen presenting cells, to promote T cell activation and subsequent expansion, both *ex vivo* and *in vivo*. Essentially, aAPCs are particles to which proteins required for T cell activation, such as MHC-epitope complexes, agonist anti-CD3 and agonist anti-CD28, have been conjugated.[128] Both spatial and temporal organization of these signals during aAPC/T cell contact is important for efficient T cell activation. The first generation aAPCs were composed of solid, micron-sized polystyrene beads or with iron oxide cores and were used for *ex vivo* expansion of T cells. Their large size provided a large area of contact between aAPCs and T cells. However, the second generation of aAPCs engineered for *in vivo* applications were smaller particles at the nanometer size scale (<100 nm) that showed favorable distribution to T-cell-rich regions such as spleens and lymph nodes upon systemic administration.[129] Shape is again a key design parameter here. $CD8^+$ T cells migrated preferentially to the long axis of ellipsoidal aAPCs and the extended length of contact resulted in enhanced proliferation and anti-tumor response.[130]

## Challenges and opportunities for nanotechnology-based immunotherapies

Despite almost a decade-long research, preclinical and clinical, into the immunostimulatory potential of nanotechnology-based platforms, only a handful of approaches thus far have received clinical approval. A success story in this regard is T-VEC. An attenuated version of herpes simplex virus, T-VEC is a genetically engineered oncolytic virus that specifically replicates in cancer cells and leads to anti-cancer response by secreting cytokine GM-CSF. With successful phase III trials, T-VEC has been recently approved for treatment of melanoma patients as an injectable formulation.[131]

However, the translational gap highlights not only the complex relationship of cancer and immunology, but also underlines poor understanding of the *in vivo* behavior of nanoparticles and its safety concerns. Undesirable immunotoxicity of nanoparticles, adverse interactions with biomolecules, long-term accumulation at off target sites, remain challenging. Generation of pro-inflammatory cytokines (such as IL-6 and TNF-$\alpha$) and inflammasome response contribute to immunotoxicity. For example, mesoporous hollow silica nanoparticles and carbon nanotubes have been associated with the induction of pro-inflammatory cytokines, liver damage and activation of Kupffer cells.[132,133] Similarly, nanosize $TiO_2$ particles lead to oxidative stress, neutrophil activation and inflammation in lungs,[134] while carbon nanotubes have been implicated for inflammatory fibrogenic pulmonary response.[135] On the other hand, MPS clearance resulting from adsorption of serum proteins, complements and immunoglobulins results in off-target accumulations.[136]

As highlighted in the above discussions, the combination of physical and chemical properties of nanomaterials influences their biological interactions and hence their applicability for specific immunotherapeutic interventions. Therefore, depending on the immunostimulatory pathways and mechanisms to be perturbed, the nanoparticle platform and route of administration must be selected based on overall biodistribution, pharmacokinetics, range of cellular interactions and toxicological risk assessments. Extensive

preclinical evaluation of various nanoparticle platforms, both *in vitro* and *in vivo*, is therefore critical.

Some key aspects of the physiological behavior of nanoparticles that have been driving forces behind their applications in nanomedicine are still fairly ineffective.[137] Delivering therapeutic payload to tumors via systemic administration remains challenging. The EPR effect is critical for nanoparticle homing into solid tumors for drug delivery or immunomodulation of the tumor microenvironment.[67] However, adequacy of the EPR effect remains a controversial subject amongst the scientific community and varies greatly between various cancer types and from primary tumors to metastatic sites. EPR is also a function of nanocarrier morphology and results in only a small fraction homing into tumors; a large fraction is still non-specifically delivered to off-target sites and could lead to severe side effects.[138] Even after reaching tumor sites, penetration of nanoparticle therapeutics in tumor mass may be impaired because of barriers created by abnormal tumor physiology including abnormal tumor structures, such as physically compromised vasculature, abnormal ECM, and high interstitial fluid pressure.

Targeting tumor vasculatures or cancer cell receptors could increase uptake and retention of nanoparticles in endothelial and cancer cells. However, from an immunotherapeutic point of view, targeting immunomodulators to immune cell populations within the tumor stroma could be a more effective strategy as even if a small population of these cells is activated, they would proliferate through a cascade of specific mechanisms leading to effective coordinated adaptive anti-tumor response. Also, keeping in mind the broad spectrum of immune cells that could potentially be activated by therapeutic immunomodulators, systemic toxicity remains a significant concern. Concentrating the immunotherapeutic drugs into nanoparticles followed by tissue- and cell-targeted delivery has been shown to overcome dose-limiting toxicities by minimizing off-target accumulation. Similarly, improving nanoparticle stability in

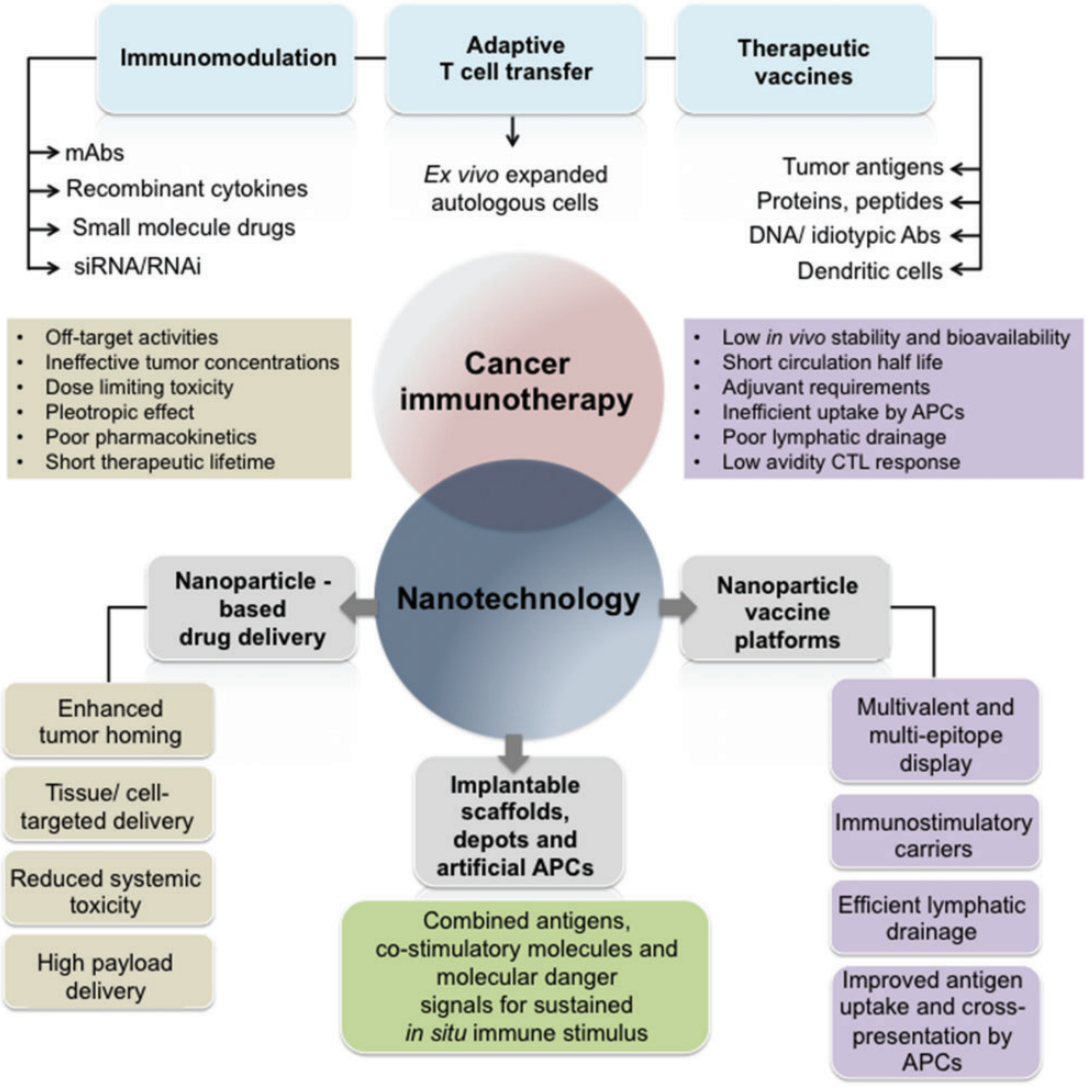


**Figure 1** Cancer immunotherapeutic strategies and nanotechnology-based intervention to overcome challenges of current immunotherapy approaches. (A color version of this figure is available in the online journal.)

circulation, upon serum protein conjugations and under varying pH and redox potentials should also be key to rule out premature release of such broad-spectrum cargo.[139] At the same time, emphasizing the development of safer biodegradable nanomaterials will facilitate earlier translation of such platform technologies.

As briefly mentioned earlier, design principles for nanoparticle-based vaccines targeting cancer antigen-specific adaptive immune responses are essentially contrasting to those for delivery of therapeutic immunomodulators. The former requires effective uptake and processing by professional antigen presenting cells in secondary lymphoid tissues, while the latter specifically requires avoidance of phagocytosis by APCs. Thus, selection of platform technology becomes critical. Also, given the consistently evolving nature of the tumor, multi-epitope vaccines are likely to be more effective because combinations of antigens may reduce the window for the tumor to down-regulate the antigens and escape immune system detection.[140] Therefore, nanoparticles that facilitate presentation of high payloads of multiple epitopes are preferable. Cancer vaccines are also likely to be more efficient under conditions of minimal residual disease or in combination with immunomodulators reducing the immune suppression of established tumors.

Nanomanufacturing and quality control and assurance must be considered along the translational pathway. Improved scaled-up production of nanoparticles of interest with excellent reproducibility and consistency of composition, physical properties and chemical addressability is a critical bottleneck for a large range of contemporary nanoparticle platform technologies. Advances such as PRINT™ technology[141] and biology-inspired materials such as plant virus-based technologies that can be manufactured in plants[142] are some of the examples where such criteria have been met. Protein-based platform technologies based on plant viruses and bacteriophages have added benefits of genetic programmability, whereby genes for proteins, targeting peptides and antigenic motifs of interests can be inserted into the viral genome and expressed as a fusion product to the coat proteins.[143,144] Similarly, specific chemical functionalities in the form of amino acid side chains can be coded into the viral genome with precise spatial control to expand the contemporary virus-based and virus-like nanoparticle libraries with altered reactivities.[143] Once genetically engineered in such manner, large-scale production of identical clones through propagation in their natural plant and bacterial hosts provides excellent control over the nanomanufacturing process, leading to excellent structural and functional monodispersity. Moreover, engineering interventions during the self-assembly process of such viral-based materials also provides another level of control over their structural and functional properties by altering their aspect ratios/size[145] or by loading non-natural cargos within the viral capsids.[146] Such control over the structure-function relationship is currently unattainable with most synthetic nanomaterials and is immensely desirable towards development of improved platform technologies.

In summary, cancer immunotherapy holds great promise in cancer therapy. Collaboration between cancer immunologists and engineers will further the understanding of the complex and underlying immunology, therefore driving technological development. With emerging nanotechnological interventions, efficacy of many such immunotherapies could be drastically improved and a merger of these two rapidly growing fields of science could facilitate clinical translation of many cancer immunotherapies.

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#### DECLARATION OF CONFLICTING INTERESTS

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