Nanoparticle-Based Immunotherapy for Cancer

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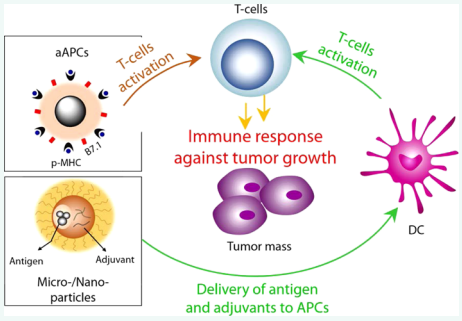
ABSTRACT The design of nanovaccines capable of triggering effective antitumor immunity requires an understanding of how the immune system senses and responds to threats, including pathogens and tumors. Equally important is an understanding of the mechanisms employed by tumor cells to evade immunity and an appreciation of the deleterious effects that antitumor immune responses can have on tumor growth, such as by skewing tumor cell composition toward immunologically silent tumor cell variants. The immune system and tumors engage in a tug-of-war driven by competition where promoting antitumor immunity or tumor cell death alone may be therapeutically insufficient. Nanotechnology affords a unique opportunity to develop therapeutic compounds than can simultaneously tackle both aspects, favoring tumor eradication. Here, we review the current status of nanoparticle-based immunotherapeutic strategies for the treatment of cancer, ranging from antigen/adjuvant delivery vehicles (to professional antigen-presenting cell types of the immune system) to direct tumor antigen-specific T-lymphocyte-targeting compounds and their combinations thereof.



KEYWORDS: cancer immunotherapy · tumor immune surveillance · tumor immune evasion · tumor immunoediting · tumor-associated antigens · danger-associated molecular patterns · T-lymphocytes · lymphocyte co-stimulators · vaccines · nanoparticles · targeted delivery · environment-responsive nanoparticles

The immune system embodies what can be referred to as the "police force" of the organism, primarily responsible for detecting and destroying foreign invaders (*i.e.*, pathogens). This function is executed by the coordinated action of two distinct cellular compartments, referred to as the "innate" and "adaptive" arms of the immune system. The innate immunity compartment is largely composed of phagocytes (macrophages and dendritic cells) and granulocytes (neutrophils, eosinophils, basophils, and mast cells) and contributes the first line of defense. These cell types recognize general molecular patterns on pathogens or "danger" signals from within and are quickly activated and recruited to sites of infection, inflammation, or tissue damage. The lectin and alternate pathways of the complement system are additional components of the innate immune compartment. The lectin pathway, for example, is triggered by recognition of microbial molecules (*e.g.*, mannose residues) by soluble lectins (*e.g.*, mannose-binding lectin). The adaptive immune compartment, ruled by T- and B-lymphocytes, affords both specificity (*i.e.*, against specific antigens rather than molecular patterns) and "memory" to the immune response (*i.e.*, the capacity to mount very rapid and highly specific immune responses to specific molecules—antigens—expressed by pathogens seen in the past). These two attributes of the adaptive immune response are enabled by random rearrangement of extensive arrays of gene cassettes in loci encoding the antigen receptor molecular complexes expressed on the surface of lymphocytes. This random rearrangement process can generate a huge repertoire of T- and B-cell receptors for antigen (TCR and BCR, respectively) and essentially limitless repertoires of T- and B-cell specificities (one antigen-specific receptor for each T- or B-cell) in a single individual. An initial encounter with a pathogen triggers the rapid recruitment of

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non-antigen-specific innate immune cells as well as a more elaborate and time-consuming activation, expansion, and recruitment of antigen-specific T- and B-cells. The sequential engagement of innate and adaptive immunity will collectively promote not only clearance of the pathogen but also generation of pools of long-lived, pathogen-specific memory lymphocytes that can quickly respond in a very specific way to repeated encounters with pathogens seen before.

However, the immune system does a lot more than just protect us against infections. In 1909, Paul Ehrlich proposed that the immune system not only patrolled the "self/non-self" frontier but also had policing duties preventing the propagation of potentially lethal rebellions of "self" in the form of tumors. Cancer can be considered a genetic disease that arises when genes responsible for regulating the proliferation and terminal differentiation of normal tissue cells mutate, affording individual cells the ability to proliferate out of control and migrate (metastasize) to distal anatomic sites, compromising organ integrity and function. Ehrlich's hypothesis is supported by a number of observations. For example, genetic T-cell deficiencies or loss-of-function mutations in genes encoding molecules responsible for lymphocyte effector function (*i.e.*, killing of tumor cells) result in increased susceptibility to spontaneous and induced forms of cancer in mice.[1] Likewise, acquired immunodeficiency syndrome (AIDS) and transplant immunosuppressive therapy in humans are associated with increased risk to both viral- and non-viral-induced types of cancer.[2,3]

Tumor Immune Surveillance. The immune system must be able to differentiate between cancer and normal cells to effectively fight cancer. Lymphocyte development in the thymus (for T-lymphocytes) and bone marrow (for B-lymphocytes) is associated with a strict process of clonal selection whereby lymphocyte clones expressing antigen receptors targeting self-antigens with high affinity are eliminated from the mature, peripheral repertoire (a process known as "negative selection"). Although negative selection protects the organism from harmful autoreactivity, it also depletes the peripheral repertoire of lymphocyte specificities that could potentially suppress tumor growth. Fortunately, cancer cells are not identical to normal cells and may express various types of tumor-associated antigens (TAAs).[4] The most specific TAAs are those arising *de novo* exclusively in cancer cells (also known as "neoantigens") (Figure 1, left). Mutated gene products, proteins encoded by chromosomal aberrations (*i.e.*, fusions encoded by chromosomal translocations), or proteins displaying altered post-translational modifications are some examples. In other instances, normal self-proteins that are not expressed in adult, differentiated cell types or that are only expressed at very low levels (*i.e.*, embryonic or cell-differentiation antigens) become immunogenic TAAs if they are expressed or overexpressed by cancer cells. Viral antigens expressed by viral-induced forms of cancer can also be considered neoantigenic TAAs.

VOCABULARY: **Antigens** - molecules that can be recognized by lymphocytes with a high degree of specificity; **Tumor immunotherapy** - approaches capable of triggering lymphocyte responses against tumor-associated antigens; **Tumor immune surveillance** - the immune system capacity to detect and eliminate cancer cells; **Tumor immune evasion** - responses of the tumor that render it resistant or invisible to immune surveillance; **Tumor immunoediting** - the immune response against a tumor promotes the selection and survival of tumor cells that are resistant or invisible to the antitumor immune response; **Nanoparticles as vehicles for drug delivery** - nanoparticle-based compounds used as carriers to deliver chemotherapeutic agents or immune stimulating molecules to specific cell types, excluding an active role for the nanoparticle structure in biological activity; **Direct T-lymphocyte targeting/activating nanoparticle compounds** - nanoparticle-based compounds in which the nanoparticle component plays an active role in biological activity by assembling lymphocyte-activating molecules in the appropriate configuration; **Targeted delivery** - nanoparticle-mediated delivery of therapeutic compounds to specific anatomic locations, tissues, or cell types; **Nanovaccines** - nanoparticle-based compounds designed to trigger antigen-specific immune responses.

The rapid growth of tumors is often associated with high rates of tumor cell death (*i.e.*, due to hypoxia or to accumulation of mutations that compromise cell viability). Tumor necrosis results in the release of both TAAs and "danger" signals (also referred to as danger-associated molecular patterns or DAMPs). DAMPs function as stimuli for the activation and recruitment of phagocytes and professional antigen-presenting cells (APCs), which can process TAA-derived peptides for presentation to tumor-specific lymphocytes in the context of membrane-bound major histocompatibility complex (MHC) class I or class II molecules (the APC "hands" that present TAA-derived peptides to antigen receptors on CD8+ and CD4+ T-lymphocytes, respectively) (Figure 1, left). Several DAMPs have been suggested to play a role in the activation of APCs in cancer, including filaments of F-actin,[5] DNA[6,7] and adenosine triphosphate or high-mobility group B1 protein[8] among others. Activated TAA-specific CD4+ T-cells produce cytokines such as interferon-gamma and tumor necrosis factor alpha that can both suppress tumor survival and upregulate the expression of MHC class I molecules by the tumor cells, facilitating their targeted recognition by TAA-specific cytotoxic CD8+ T-lymphocytes (CTLs) (Figure 1, left).

Tumor Immune Evasion. The activation of the immune system as an attempt to blunt tumor growth leads to an "arms race" wherein immunity suppresses and fast

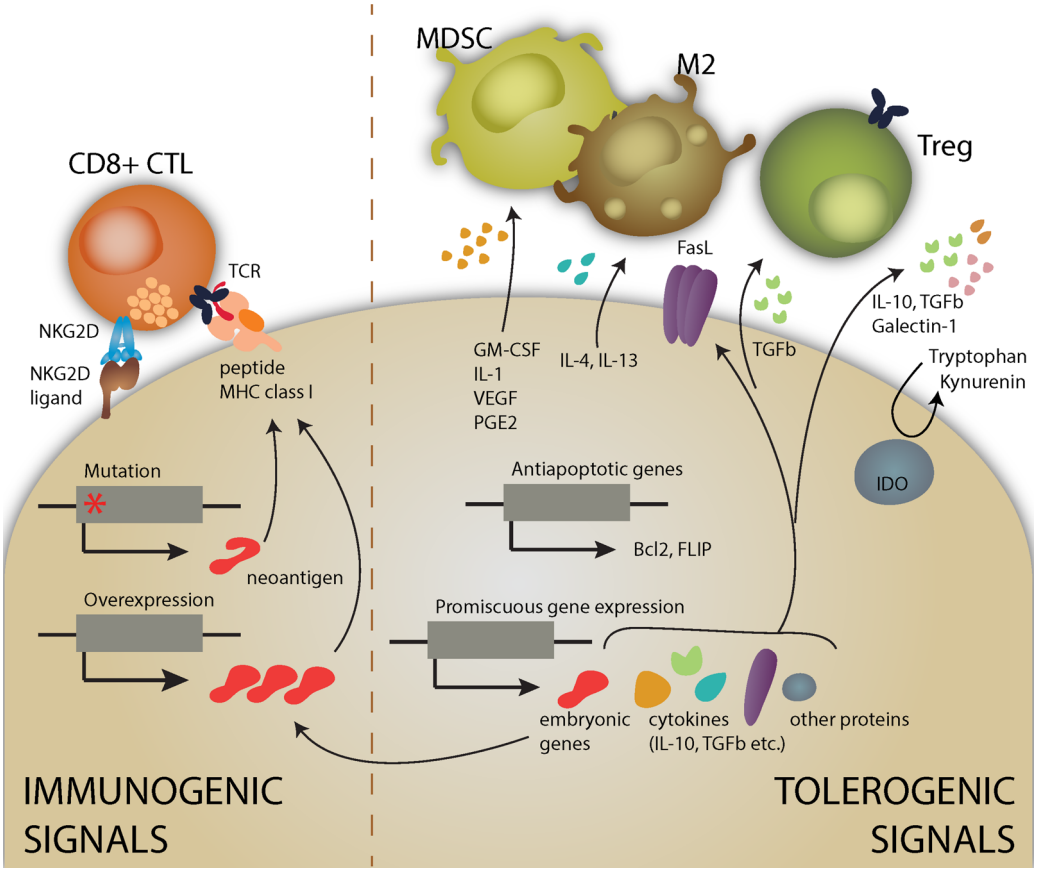

Figure 1. Tumor-derived signals that promote (immunogenic) or suppress (tolerogenic) antitumor immune responses. Expression of various types of tumor-associated antigens, including mutant gene products and overexpressed differentiation proteins, render tumor cells recognizable by TAA-reactive T-lymphocytes. TAA-activated CD8+ cytotoxic T-lymphocytes can target and kill tumor cells *via* TCR-mediated recognition of TAA-derived epitopes displayed by tumor MHC class I molecules or *via* the engagement of NKG2D receptor expressed by activated CD8+ T-cells and NKG2D ligands expressed by tumor cells. However, tumor cells can also suppress antitumor immune responses by expressing the immunosuppressive enzyme IDO and various cytokines that promote the recruitment of immunosuppressive cells, including MDSC, M2 macrophages, and Treg cells, or by upregulating antiapoptotic pathways, such as Bcl2 and FLIP.

tumor cell replication and mutation rates favor tumor growth. Importantly, the immune system exerts a selective force that can alter the cell composition of the tumor, wherein immunity ultimately promotes the survival of the fittest and least immunogenic tumor cells, a process known as "tumor immunoediting".[9] In some cases, the immune response will eradicate the tumor; in other cases, it will just contain tumor growth, leading to a delicate stalemate that can be disrupted by temporal states of immunosuppression promoting tumor recurrence. In the worst-case scenario, mutations in tumor cells render them insensitive to the immune system—the outgrowth of these "immunologically silent" cells will invariably result in the most aggressive and lethal tumors.

Tumors can evade recognition by the immune system *via* several mechanisms (Figure 1, right). At one level, tumor cells may gain a survival advantage by downregulating MHC class I expression. Although this effectively compromises tumor cell killing by TAA-specific CTLs (which recognize peptides presented by MHC class I molecules), it also facilitates the recognition and killing by natural killer (NK) cells, a subset of lymphocytes that become activated when the total levels of MHC class I (expressed by all nucleated cell types of the body) fall below a certain threshold. Changes in MHC class I expression on tumor cells can be caused by direct mutations in the MHC genes themselves or by mutations in molecules associated with TAA antigen processing and presentation, such as proteasome subunits or endoplasmic reticulum peptide transporters. Additionally, mutations can also alter the expression levels and molecular identity of TAAs, resulting in antigenic shifts and drifts.

At another level, tumors can evade immune surveillance by developing resistance to CTL-mediated killing mechanisms (Figure 1, right). Tumor cells can inhibit the perforin/granzyme pathway of tumor cell killing by expressing granzyme-specific serine proteases (serpins).[10,11] Tumors may also express decoy receptors for death receptors like Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), such as soluble Fas, decoy receptor 3 (DcR3), DcR4, and osteoprotegerin.[12,13] In addition, tumors may express increased levels of

antiapoptotic and prosurvival/oncogenic molecules, such as B-cell lymphoma 2 (BcL-2), signal transducer and activator of transcription 3 (STAT3), cellular FLICE—Fas-associated protein with death domain-like interleukin-1 β-converting enzyme—inhibitory protein (c-FLIP) (which inhibits the Fas and TRAIL pathways) or B-cell lymphoma extra-large (Bcl-$_{XL}$).

Tumors can also suppress immunity actively by turning off activated T-cells *via* PD-L1, which ligates the negative co-stimulator PD1 on the lymphocyte's membrane,[11,14,15] or by secreting immunosuppressive molecules, such as transforming growth factor-beta (TGFβ), a cytokine that inhibits the activation and differentiation of T-cells and APCs (Figure 1, right). Some tumors express inducible enzymes with immunoregulatory roles, such as cyclooxigenase-2, which promotes the conversion of arachidonic acid to prostaglandin H_2 (PGH_2 a precursor of PGE_2), or indoleamine 2,3-dioxygenase (IDO), which catabolizes the essential amino acid tryptophan and generates kynurenines.[16,17] Both PGE_2 and kynurenines can suppress T-cell activation. The production of these and other molecules can also promote the generation of professional regulatory (formerly known as "suppressor") cells with immunosuppressive roles, such as Foxp3+ Tregs (*via* TGFβ and PGE_2), myeloid-derived suppressor cells (MDSCs; *via* GM-CSF and PGE_2),[18] or M2-type macrophages (*via* interleukin (IL)-4 and IL-13).[19]

Tumor Immunotherapy: Promises, Disappointments, and Lessons. Over 100 years ago, Coley used bacteria (Coley's toxins) as an attempt to trigger bystander antitumor inflammatory responses. This approach only had sporadic and difficult to reproduce success. Identification of TAAs in the 1990s enabled the development of tumor-specific therapies, such as peptide-, protein-, or cell-based vaccination approaches, potentially capable of stimulating pre-existing antitumor immunity or of inducing *de novo* antigenic responses. However, after decades of intensive pursuit, this remains a challenging goal. Classical vaccination approaches have been extensively tested and found to be largely inefficient.[20,21] Peptide-based tumor vaccination, for example, is compounded by inefficient uptake, processing, and presentation of the delivered epitopes by activated professional APCs at the site of immunization. In addition, properly processed and presented peptides may not be sufficiently immunogenic. Furthermore, even when CD4+ and CD8+ T-cell populations elicited by peptide vaccination were detectable in patients, the clinical outcome was often not correlated with the magnitude of the responses. Animal studies have suggested that peptide vaccination may preferentially elicit tumor-specific T-cell responses of low avidity and low cytotoxic activity.[22]

Recent years have witnessed the testing of modified vaccination approaches using long peptides or whole proteins given along with powerful adjuvants (agents capable of stimulating innate immune cells, including professional APCs, or of accelerating or magnifying the immunogen-induced adaptive T-cell response), such as cytokines like IL-2 or GM-CSF, Toll-like receptor (TLR) agonists (recognized by molecular pattern recognition receptors), or recombinant lymphocyte co-stimulatory molecules such as CD80 (B7.1).[23,24] These approaches also failed to induce clinically significant antitumor immune responses[21,25] for two main reasons. First, many of the TAAs that were used in these trials were normal differentiation antigens.[26,27] Expression of these TAAs by undifferentiated cell types during development triggers the deletion (negative selection) of lymphocytes expressing high-affinity antigen receptors, depleting the immune system of these specificities. Second, the resulting low-avidity immune responses against these differentiation TAAs cannot overcome tumor immunoediting responses.[9,22]

Notwithstanding the negative results of these trials, there is substantial evidence in both patients and animal models that spontaneous high-avidity TAA-specific T-cell responses can control tumor growth.[28−31] In addition, tumor-infiltrating lymphocytes expanded *ex vivo* can significantly reduce tumor burden in patients and animal models upon adoptive transfer.[32,33] Collectively, these observations suggest that induction and expansion of fully differentiated high-avidity tumor-specific CTLs may be a *sine qua non* requirement for effective tumor immunotherapy.[34] Likewise, generation of memory T-cell responses against multiple TAAs and epitopes may be required for long-lasting protection against tumor recurrence and metastases.[35]

De novo induction and expansion of high-avidity tumor-specific CTLs is a challenging undertaking that requires efficient antigen capture, processing, and presentation by professional APCs, activation/maturation of these APCs into T-cell-activating units, and the productive activation, differentiation, and expansion of MHC class I- and class II-restricted tumor-specific T-cell pools (Figure 2). The recent clinical success of approaches that block negative regulators of T-cell activation, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4), demonstrate the importance of sustained T-cell activation and expansion for effective tumor immunotherapy. However, animal experimentation has also demonstrated that approaches aimed at suppressing molecular and cellular regulators of T-cell activation are only effective if delivered in the presence of effector-memory T-cell populations arising in response to repeated TAA-induced stimulation.[35−38] Thus, whereas current vaccine design paradigms can effectively generate prophylactic and therapeutic immunities against foreign pathogens, they may be ill-suited as platforms with which to build cancer-fighting vaccines. Accordingly, new therapeutic platforms capable of inducing, expanding, and sustaining

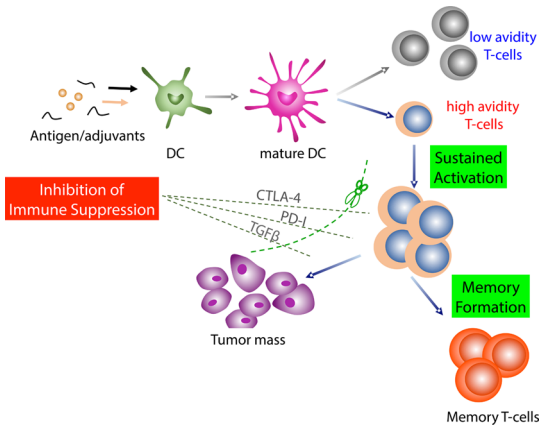

Figure 2. Requirements for successful antitumor immune responses. Successful antitumor immune therapy requires efficient delivery of both tumor-associated antigens and adjuvants to professional APCs, such as dendritic cells (DCs), and efficient presentation of TAA-derived epitopes by tumor-associated MHC class I molecules to cognate T-cells. Sustained activation and expansion of high-avidity tumor-specific T-cells, efficient memory T-cell formation, inhibition of T-cell-intrinsic and tumor-derived negative regulators of T-cell activation are key factors for therapeutic efficacy.

high-avidity effector and memory T-cell responses to TAAs will need to be developed. These new approaches will need to overcome tumor immune editing responses and obviate the severe adverse effects caused by systemic inflammation that are seen in patients treated with general immune activators, such as anti-CTLA4 mAb.

Applications of Nanotechnology to Cancer Therapy: Nanoparticles as Vehicles for Drug Delivery. A variety of nanoparticle structures have been used as vehicles to deliver imaging tags or cytostatic/cytotoxic drugs to sites of tumor growth. These nanodevices can carry a wide spectrum of molecular cargos, stabilizing their biological activity and increasing their solubility in biological fluids.[39−41] During the last two decades, several nanoparticle-based compounds delivering encapsulated or conjugated cytotoxic drugs have reached the clinical trial stage.[40,42,43] Nanoparticles prolong the circulation time of the cargo by protecting it from degradation and promote its preferential accumulation (hence local concentration) in tumors, due to their abnormal vascular architecture and enhanced permeability and retention (EPR) effects.[44] Upon extravasation into tumors, they have a tendency to accumulate *in situ* due to impaired lymphatic drainage (Figure 3).[45] Passive tumor-targeted delivery enhances the therapeutic index of the delivered chemotherapeutic agents, minimizing their off-target systemic toxicity as compared to their non-nanoparticle-delivered counterparts.[46−51] The efficacy of these first-generation antitumor nanomedicines essentially relies on EPR effects; however, EPR effects vary across tumor types, owing to differences in vascular anatomy and permeability.[43,52] Therefore, EPR-dependent passive targeting is inefficient and often results in unpredictable clinical outcomes, particularly in the context of metastatic cancer, where tumor cells grow in different vascular beds.[44,47,53] Nanoparticle size and surface properties were identified as playing critical roles in the half-life and biodistribution of these compounds. Nanoparticles larger than 7 nm in hydrodynamic diameter evade renal filtration and urinary excretion.[54] On the other hand, particles larger than 100 nm are rapidly eliminated from the circulation by phagocytes of the reticulo-endothelial system.[55,56] At tumor sites, only the former manage to rapidly penetrate deep into the tumor matrix.[57,58]

Addition of polyethylene glycol (PEGylation) or other polymers to the nanoparticle surface lengthens the circulation time of these compounds by inhibiting phagocyte uptake and promotes their recruitment to and accumulation into tumors.[46,57,59] Unfortunately, these modifications also inhibit the uptake of these compounds by the tumor cells themselves. To overcome this limitation, "active" tumor-targeting approaches involving the conjugation of tumor-specific ligands to the nanoparticle surface have been developed.[60−65] Coating ligands for tumor receptors, such as herceptin, folate, or transferrin, on the nanoparticle surface improves the delivery and uptake of nanoparticles by tumor cells. Recent studies in animal models using peptides as brain-tumor-targeting ligands have provided encouraging results, but the clinical significance of these approaches remains unclear.[66,67] In general, however, only less than 5% of the total administered dose accumulates in tumors using current delivery approaches.[47,68]

Another critical consideration is the structural stability of nanoparticles in serum. Most organic nanoparticles are eliminated in the circulation shortly after i.v. administration. For instance, polymeric micelles may not be able to maintain their structural integrity due to rapid dilution and structural dissociation after i.v. injection, resulting in the loss of cargo.[69] Environment or stimuli-sensitive (pH, redox, temperature, and UV light) drug-releasing strategies have been incorporated into the nanoparticle design to overcome this problem, with promising antitumor effects.[70,71]

Collectively, these studies have enhanced our understanding of the physical/chemical properties impacting on biodistribution, pharmacokinetics, and toxicology of tumor-targeting nanomedicines and have paved the way for developing next-generation compounds with superior therapeutic activity (Figure 3). Nanoparticles of reduced size (<100 nm), adequately sheltered from phagocyte uptake, with high structural integrity in the circulation and long circulation time, capable of accumulating at sites of tumor growth, able to penetrate deep into the tumor mass, and capable of selectively targeting, delivering, and releasing cytotoxic payloads within tumor cells are some of the desired properties. Importantly, the knowledge generated by

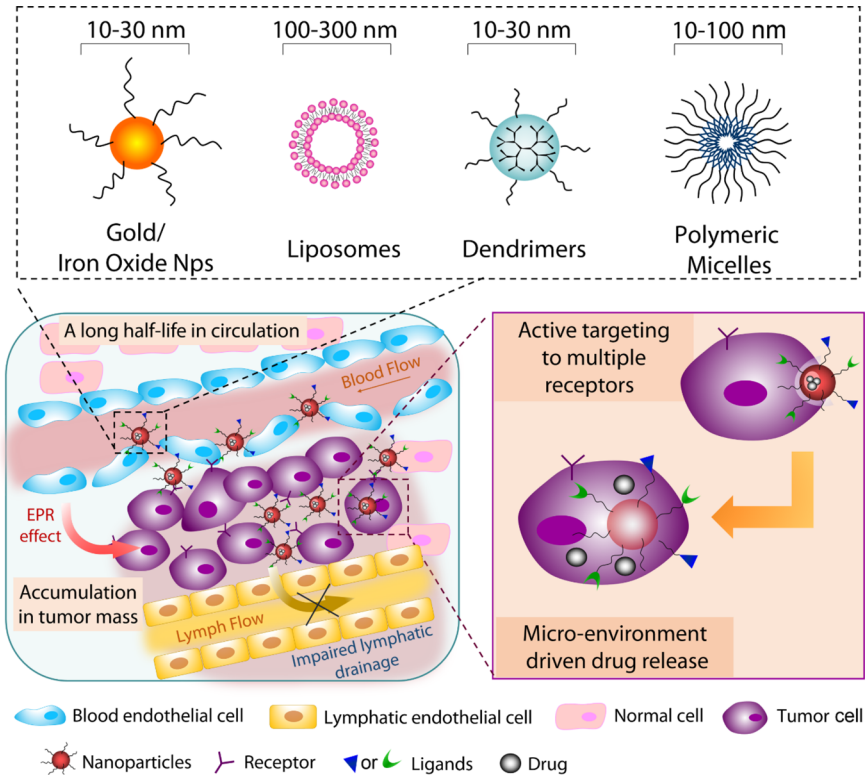

Figure 3. Optimal design principles for nanoparticle-based, tumor-cytotoxic vehicles. Different types of nanoparticles can serve as vehicles for targeted delivery of tumor-cytotoxic drugs. Size, surface chemistry, structural stability, circulating half-life, vascular permeability, extravasation, and retention into the tumor due to impaired lymphatic drainage, tumor cell binding, and internalization *via* receptor- or transporter-mediated interactions, and intracellular delivery of payload in response to intracellular stimuli are some of the key parameters.

these studies will facilitate not only the development of next-generation nanocarriers for drug delivery but also the development of nanoparticle-based therapeutic approaches to elicit antitumor immunity.

Nanoparticle-Based Approaches To Elicit Antitumor Immunity. Efficient and targeted delivery of immunomodulatory and immunostimulatory molecules to appropriate cells is key to successful development of nanovaccine formulations.[72] Compared to conventional approaches, nanoparticles can protect the payload (antigen/adjuvant) from the surrounding biological milieu, increase its half-life, minimize its systemic toxicity, promote its delivery to APCs, or even directly trigger the activation of TAA-specific T-cells.

Nanoparticles have also been used as vehicles to deliver antigens to professional APCs *in vivo*, to elicit adaptive immune responses (Figure 4 and Table 1). Antigens and adjuvants bound to and/or encapsulated within nanoparticles have been shown to trigger T- and B-cell responses of increased magnitude as compared to soluble antigens given with various adjuvant types, suggesting that these approaches might also have therapeutic significance in cancer.[73,74] Clearly, however, the engineering principles governing the therapeutic efficacy of tumor-specific nanovaccines will undoubtedly differ from those guiding the design of nanoparticles used as vehicles for drug delivery; whereas the former seek professional APCs, the latter aim to unload their payload into tumor cells, bypassing APCs and other phagocytes.

Nanoparticle-Based Delivery TAAs to Professional APCs. It has been shown that certain nanoparticle designs possess immunostimulatory properties, and that antigens delivered by these nanoparticle types can induce T- and B-cell responses in the absence of exogenously added adjuvants.[75,76] In an attempt to generate therapeutic antitumor immunity, model tumor antigens were conjugated to various types of particles and injected into OVA-expressing melanoma, thymoma, or lymphoma-bearing mice.[77,78] Delivery of model antigens bound to naked nanoparticles induced potent T-cell and antibody responses against OVA-expressing lymphoma or colon adenocarcinoma, delaying tumor growth and lengthening the survival of animals.[78−80] Particle size appears to be an important variable affecting the biological activity of these compounds. Small virus-sized particles (≤40 nm) readily reach lymph nodes draining the site of injection, facilitating their uptake by DCs, the presentation of peptides arising from the coated antigens by the DCs' MHC class I molecules, and the activation of TAA-specific CD8+ T-cells carrying antigen receptors for these peptide−MHC (pMHC) class I complexes.[81,82] Endocytosis of such virus-like particles by DCs likely triggers the activation of danger-sensing pathways in

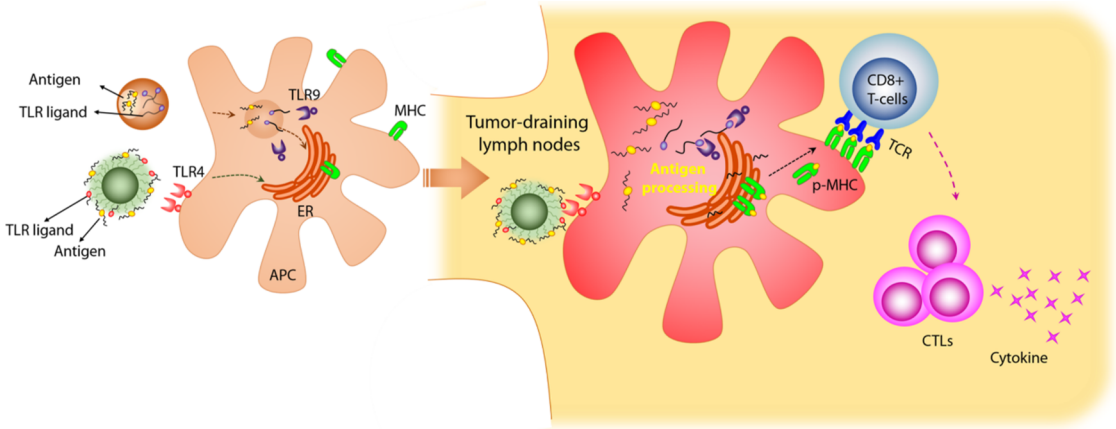

Figure 4. Nanoparticle-based delivery of TAAs and/or adjuvants to tumor-draining lymph nodes. Nanoparticles have also been used as vehicles for delivery of TAAs and/or adjuvants (*i.e.*, TLR ligands). Capture of these compounds by professional APCs promotes peptide−MHC class I formation and APC maturation. Recognition of the nanoparticle-delivered/TAA-derived epitopes displayed on the APC membrane in the context of the individual's MHC class I molecules by cognate CD8+ T-cells results in the generation of tumor-cytotoxic CTLs.

DCs, promoting DC activation and maturation into immunogenic APCs.[83] Conjugation of the antigens to nanoparticle structures not only contributes to their structural integrity but also to slowing down the rate of antigen release within DCs, enhancing their immunogenicity.

Delivery of Nonspecific DC Stimuli (Adjuvants) to Tumor-Draining Lymph Nodes (TDLNs). Tumor antigens drain into TDLN, where they are uptaken by professional APC types, including DCs, and then presented to T-cells. High numbers of tumor-specific T-cells have been found in TDLNs using tumor-specific peptide−human leukocyte antigen tetramers (four identical biotinylated pMHC complexes tetramerized by a fluorochrome-conjugated streptavidin molecule) as detection reagents.[103] However, DCs residing in TDLNs generally display an immature/nonactivated phenotype, which compromises their ability to productively activate antitumor T-cell responses.[14,104−110]

Recent studies have explored the ability of nanocarriers of DC stimulatory molecules to promote the activation and maturation of TDLN DCs. Subcutaneous injection of nanoparticle-bound cytosine-phosphate-guanine (CpG) oligonucleotides (an adjuvant) resulted in the preferential accumulation of these molecules in the draining lymph nodes in a melanoma model.[84] CpG−nanoparticle complexes were quickly uptaken by APCs and triggered the release of the cytokines IL-12 and IL-6,[85] which contribute to the activation of effector CD4+ T-helper-type 1 cells.[111] Injection of CpG-coated nanoparticles into a tumor proximal site (to target TDLNs) resulted in strong antitumor immune responses, slowing the growth of a subcutaneous tumor. TDLN-targeted, CpG-coupled nanoparticles increased the number of DCs producing IL-12 and expressing the T-cell-activating molecule CD40 (which binds to its receptor CD154 on CD4+ T-helper cells) as well as the number of effector CD4+ T-cells in the TDLNs, leading to an increase in the number of effector T-cells within the melanoma itself and to tumor regression.[86,87] Similar results were obtained with different types of nanoparticles and nanoparticle sizes, including gelatin-, liposome-, and pyridyl disulfide-based compounds ranging from 25 to 270 nm in diameter, with a drop in TLDN targeting efficiency for nanoparticles larger than 100 nm in diameter.[81,84−88] Additionally, simultaneous delivery of CpG ODN and IL10 siRNA using PLGA microparticles afforded protection in a prophylactic murine model of B-cell lymphoma.[89] Likewise, anti-CD40/CpG-carrying liposomes triggered robust antimelanoma responses with minimal systemic side effects.[90]

Co-delivery of Antigen and Adjuvant. Another approach that has been tested involves administering particles delivering both antigens and adjuvants together. Liposome-based particles delivering a model tumor antigen (OVA or OVA-derived peptides) in the context of CpG or other TLR agonists, including lipopolysaccharide, Zymosan, or R848, had superior immunogenic activity against melanoma than conventional vaccination approaches.[91] Such antigen/TLR agonist-loaded liposomes migrated to the lymph nodes draining the site of injection, induced the activation of antigen-specific CD4+ and CD8+ T-cells, and slowed the growth of a subcutaneous melanoma. Studies using liposomes of varying sizes (60−120, 200, and 500 nm) indicated that the immunogenic properties of these compounds increased with particle size, suggesting the contribution of phagocytes other than DCs.[92,93] In a recent study, delivery of OVA and CpG *via* 30 nm diameter micellar nanoparticles triggered immune responses that were several fold greater than those induced by nanoparticles carrying only OVA, indicating that the role of nanoparticle size varies as a function of nanoparticle type.[94] Although these micellar nanoparticles delivering both OVA and CpG were pH-sensitive and likely released their payload in endosomes, it is not clear to what extent this contributed to immunogenicity.

TABLE 1. Nanoparticle-Based Approaches to Elicit Anti-Tumor Immunity

Strategy	Ref#	Micro/Nano-particles	Size	Surface chemical composition	Payload		Tumor model	Effect
					Coated	Encapsulated		
	77	Iron oxide beads	0.5-1.5 mm (?)	Amino group	OVA	---	OVA-expressing melanoma (*s.c.*) or thymoma (*i.v.*)	Protected animals from a tumor challenge.
Delivery of TAAs to APCs	78	Polystyrene microspheres	20 nm-2 μm	Carboxyl group	OVA	---	OVA-expressing T cell lymphoma EG7 (*s.c.*)	A single dose of Ag-conjugated beads protected mice from tumors and resulted in rapid clearance of established tumors.
	79	Poly(γ-glutamic acid) (γ-PGA)	---	Carboxyl group	OVA	listerolysin (LLO)	---	Efficiently taken up by DCs and induced strong cellular and humoral immunity.
	80	iron oxide–zinc oxide	15 nm	Zinc oxide	Carcinoembryonic antigen (CEA)	---	Colon adenocarcinoma (MC38)	Enhanced tumor antigen-specific T-cell responses, delayed tumor growth and improved survival versus controls.
Delivery of adjuvants to TDLNs	84	Cationized gelatin	272 nm	Quaternary amino group	CpG	---	Melanoma (*s.c.*)	Significantly decreased tumor size and prolonged survival time (~40 days) compared to control mice (25-30 days).
	85	Cationized gelatin	134 nm /~250 nm	Quaternary amino group	CpG	---	---	Enhanced the uptake and immunostimulatory activity of CpG ODN both *in vitro* and *in vivo*.
	86	Pluronic-stabilized poly(propylene sulfide) (PPS)	30 nm	Naked	CpG	---	Melanoma (*s.c.*)	Slowed down tumor growth vs. control mice in association with increase in CD8+/CD4+ T-cell ratios and activated (CD25+) CD8+ T-cell frequencies in the TDLN.
	87	Pluronic-stabilized PPS	30 nm	Naked	TRP-2 or CpG	---	Melanoma (*i.d.*)	Strong anti-tumor response in terms of tumor growth, survival, and effector CD8+ T-cell responses.
	88	Pluronic-stabilized PPS	25 nm	Polyhydroxylated	OVA	---	---	Generated humoral and cellular immunity in mice in a size- and complement-dependent manner.
	89	PLGA	1.18 μm	Branched PEI	CpG/IL10 siRNA	---	A20 B-cell lymphoma (*i.p.*)	PMPs can be used to modulate TLR ligand-mediated immune-stimulation in DCs, through co-delivery of cytokine-silencing siRNAs and Boost antitumor immunity.
	90	Liposome	150 nm	Maleimide group	CpG/anti-CD40	---	Melanoma (*i.m.*)	Induced robust anti-tumor responses with minimal side effects
Co-delivery of antigen and adjuvant	91	Cationic liposomes	---	Naked	---	TLR agonists (LPS, zymosan, R848, CpG) and OVA	Melanoma (*s.c.*)	Elicited CD4+ and CD8+ T cell responses and controlled the growth of established B16 melanoma tumors.
	92	Cationic liposomes	500 nm -1.5 μm	Naked	---	OVA, pIC and CpG	---	Induced potent CD8+ T cell responses without the need of additional stimuli.
	93	Cationic liposomes	130-260 nm	Naked	---	OVA and PAM_3CSK_4 (PAM) or CpG	---	Resulted in IFN-γ production by re-stimulated splenocytes from immunized mice.
	94	pH-responsive polymeric micelles	23 nm	PEGylated	OVA	CpG	---	Enhanced CD8+ T cell responses and increased CD4+IFN-γ+ (Th1) responses, eliciting a balanced IgG1/IgG2c antibody response.
	95	PLGA	248 nm	Naked	anti-DEC205 Ab	α-GalCer and OVA	OVA-expressing melanoma (*i.v.*) or EG7 (*s.c.*)	Co-delivery of α-GalCer and protein Ag to CD8α+ DCs triggered Ag-specific Ab and cytotoxic CD8+ T cell responses, leading to a potent antitumor response.
	96	Cancer cell membrane coated PLGA	110 nm	Cancer cell membrane	Tumor-associated or homotypic binding antigen	---	Melanoma	Promoted a tumor-specific immune response.
	97	Lipid-calcium-phosphate (LCP)	40-45 nm	Mannose modified	---	Trp2 peptide and CpG	Melanoma (*s.c.* or *i.v.*)	Exhibited superior inhibition of tumor growth in both subcutaneous melanoma and lung metastasis models.
	98	Liposome-protamine-hyaluronic acid (LPH)	50 nm	PEGylated	Anisamide	siRNA and HA	Melanoma (*s.c.*)	TGF-β down-regulation boosted the vaccine efficacy and inhibited tumor growth, owing to increased numbers of tumor infiltrating CD8+ T cells and decreased numbers of regulatory T cells.
Direct activation of TAA-specific T-cells	99	Microbeads	---	Naked	HLA-Ig and anti CD28	---	---	Robust generation of antigen-specific CTLs that recognized endogenous antigen−MHC complexes presented on melanoma cells.
	100	Polystyrene latex beads	5 μm	Sulfated	anti-His_6 mAb, anti-CD28 and anti CD137 (4-1BB) mAb	---	Melanoma (*s.c.*)	Efficiently inhibited subcutaneous tumor growth and markedly delayed tumor progression in tumor-bearing mice.
	101	Magnetic beads	---	Naked	---	^{pep}MHC-Ig dimers and B7.1-Ig	Melanoma (*s.c.* or *i.v.*)	Induced significant tumor reduction in a mouse telomerase antigen system, and led to complete tumor eradication in a mouse TRP-2 antigen system.
	102	iron-dextran particles/ quantum dot nanocrystals	50-100 nm/ 30 nm	Dextran/ Avidin	MHC-Ig dimer and anti CD28	---	Melanoma (*s.c.*)	Induced antigen-specific T cell proliferation from mouse splenocytes and human peripheral blood T-cells and enhanced tumor rejection.

CD8+ DCs are a specialized subset of DCs with enhanced capacity for "antigen cross-presentation" (presentation of peptides derived from captured antigens by the DCs' MHC class I molecules to CD8+ T-cells). The endocytic C-type lectin receptor DEC205[112] has been used to specifically deliver antigen-coated particles to this DC subset. One study coated an anti-CD205 antibody onto PLGA nanoparticles that were loaded with the model antigen OVA and α-GalCer, an antigenic ligand for invariant natural killer T-cells (*i*NKT), which are a subset of T-lymphocytes different than conventional CD4+ and CD8+ T-cells.[95] It has been shown that simultaneous presentation of α-GalCer and tumor antigen by DCs enhances cytotoxic T-cell responses against the tumor.[113] Indeed, anti-CD205 mAb-coated nanoparticles were preferentially uptaken by CD8+ DCs, and this led to robust OVA-specific CD8+ T-cell responses and reduced growth of OVA-expressing melanoma and EG7.[95]

In an effort to deliver a full spectrum of tumor antigens to DCs for induction of tumor-specific T-cell responses, a study investigated the therapeutic activity of PLGA nanoparticles delivering tumor cell membranes in the context of the Toll-like receptor 4 (TLR4) agonist monophosphoryl lipid A (MPLA).[96]

These membrane-loaded PLGA nanoparticles (100 nm in diameter) were readily uptaken by DCs, which were activated by MPLA, triggering the activation of CD8+ T-cells specific for a known tumor epitope in a melanoma model. However, whether these tumor membrane-loaded nanoparticles can simultaneously induce immune responses against different TAAs was not evaluated.

Heo *et al.* evaluated the ability of PLGA nanoparticles carrying both a TLR agonist and a STAT3-specific siRNA or the antigen OVA and a suppressor of cytokine signaling 1-specific siRNA to promote DC maturation *ex vivo*.[114,115] The relatively large size (~150 nm in diameter) and structural instability of the loaded compounds (~15 h after production) make it difficult to test the immunological properties of these structures *in vivo*. Small nanoparticles are usually stable but have a reduced payload capacity. Simultaneous administration of each of these molecules on separate small nanoparticles may be a potential solution. This approach has been tested recently using LCP (lipid-calcium-phosphate, 30 nm) nanoparticles loaded with the tumor peptide Trp2 and CpG[98] and LPH (liposome-protamine-hyaluronic acid, 40 nm) nanoparticles loaded with anti-CD47-specific siRNA and coated with anisamide, a ligand to target sigma receptor-overexpressing melanoma cells.[116] Although injection of LCP nanoparticles elicited antigen-specific CTL responses that effectively eliminated Trp2 peptide-loaded splenocytes in tumor-bearing mice, this approach had negligible effects on the growth of advanced tumors. Administration of LPH nanoparticles reduced TGFβ expression by tumor cells, as expected, augmenting the anti-melanoma effects of LCP vaccination.[98] Thus, combination of different nanoparticle types having complementary biological activities is an approach that deserves consideration.

Direct Activation of TAA-Specific T-Cells by Micro- and Nanoparticle-Based Approaches. The nanoparticle compounds described above were designed with the classical vaccination paradigm in mind: as vehicles to simultaneously deliver antigens and "danger" signals to DCs to promote the activation and recruitment of effector TAA-reactive CD4+ and CD8+ T-cells to the tumor site. Experimental data to date do indeed suggest that these nanoparticle-based structures are more immunogenic than the non-nanoparticle-based peptide vaccination approaches tested previously, which have been largely unsuccessful in the clinic.[20,117] Nevertheless, these approaches suffer from a number of limitations that cannot be readily overcome. One of these relates to the specificity and efficiency of antigen delivery to immunogenic (rather than tolerogenic) APC types (*i.e.*, certain DC subtypes as opposed to all DCs, regardless of phenotype, or other phagocyte types), which cannot be adequately controlled by these approaches. Furthermore, whereas activated DCs express immunogenic cytokines and T-cell co-stimulatory molecules capable of triggering T-cell activation, they can also express ligands for co-inhibitory receptors expressed on activated T-cells, as a mechanism to tune down the magnitude of, and eventually terminate, antigen-induced T-cell responses (in the course of normal immune responses). Furthermore, activated T-cells upregulate the expression of co-inhibitory receptors (*e.g.*, CTLA-4) that are triggered by the ligation of *co-stimulatory* molecules expressed by immunogenic DCs (*e.g.*, CD80 and CD86, which are normally ligated by the co-stimulatory receptor CD28 on naive T-cells). Since CTLA-4 binds to these otherwise *co-stimulatory* ligands with higher affinity than CD28, future approaches relying on antigen delivery and activation of DCs will have to overcome these negative feedback regulatory loops that prevent the development of exaggerated immune responses (precisely the opposite of what is needed to control tumor growth). The significance of these considerations is highlighted by the ability of blocking anti-CTLA-4 mAb therapy to both promote the rejection of several types of established transplantable tumors in mice, including colon carcinoma, fibrosarcoma, prostatic carcinoma, lymphoma, and renal carcinoma,[118,119] and elicit significant clinical responses in human clinical trials, albeit at the expense of causing systemic inflammation.[36,120] Nanoparticle-based approaches that can directly trigger the activation of antigen-specific T-cells without the need of cellular intermediaries may represent a potential solution to this challenge (Figure 5).

One such approach involves the delivery of microspheres displaying TAA-relevant pMHC complexes and anti-CD28 mAb (which ligates and triggers the T-cell co-stimulator CD28, a receptor for the ligands CD80 and CD86 on professional APCs) (also referred to as "artificial APCs"). Cell-size (several micrometers in diameter) polystyrene latex- or iron-oxide-based microparticles delivering both pMHC and co-stimulator could trigger the activation of cognate T-cells both *in vitro* and *in vivo*.[99,121] When such artificial APCs were given to tumor-bearing mice that were adoptively transfused with large numbers of T-cells specific for the corresponding TAA (from mice expressing a transgenic TCR for the corresponding peptide−MHC class I complex), enhanced antitumor responses were observed.[100,101,122] However, evidence supporting the ability of these compounds to trigger the activation, expansion, and recruitment of TAA-specific tumor-cytotoxic T-cells from the endogenous T-cell repertoire in non-T-cell-transfused mice (circulating at extremely low—virtually undetectable—peripheral frequencies) is lacking. Furthermore, these cell-sized structures have the potential to aggregate in small blood capillaries, particularly after repetitive administration.[123]

Several prototypes of artificial APCs have been tested *in vitro*. In one design, avidin-coupled PLGA-based

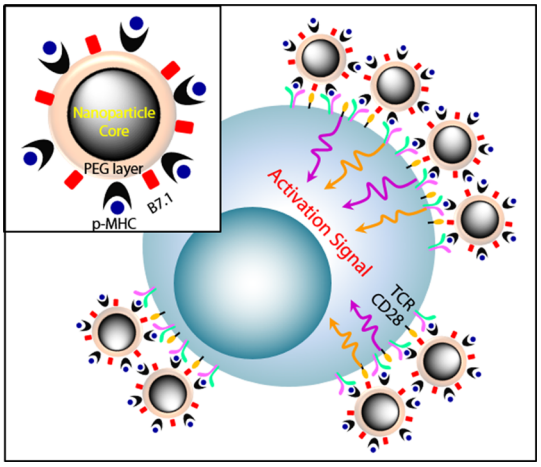

Figure 5. Direct T-cell-triggering micro/nanoparticle structures. Particles displaying TAA-relevant pMHC complexes and T-cell co-stimulatory molecules such as anti-CD28 mAb or B7.1 have the capacity to trigger T-cell activation. It remains to be determined whether these compounds can trigger the activation of tumor-specific T-cells contained within the peripheral T-cell repertoire of unmanipulated (non-T-cell transfused) tumor-bearing hosts.

micro- (8 μm diameter) or nanoparticles (~130 nm diameter) were conjugated with a biotinylated anti-CD28 mAb and a biotinylated pMHC complex (an OVA-derived peptide presented by the murine MHC class I molecule K^b fused to the constant portion of immunoglobulin).[124] When cognate CD8+ T-cells (ovalbumin-specific MHC class I-restricted CD8+ T-cells—OT-I—, expressing the corresponding T-cell receptor specificity) were cultured with these compounds, they underwent activation and produced the cytokine IL-2 (T-cell growth factor). Addition of IL-2 to these particles' payload enhanced their T-cell stimulatory potency.[125] The use of linker molecules that afforded spatial flexibility to the pMHC complexes coated onto the surface of these particles also enhanced their immunological activity, indicating that the *in vitro* biological activity of these compounds is affected by the coat design.[121] These observations provided proof-of-principle that this type of compound can directly trigger cognate T-cells, bypassing the need for a processing intermediary (an APC, for example). Although PLGA particles are biocompatible and easy to produce, it is unclear whether they are sufficiently stable and can be loaded with the sufficient number of pMHC, anti-CD28 mAb, and IL-2 molecules (the payload) to induce clinically relevant T-cell activation *in vivo*, particularly in hosts carrying an unmanipulated lymphocyte repertoire.

These types of T-cell-targeting particle structures have been recently tested *in vivo*. Commercially available avidin-coated iron oxide nanoparticles (50−100 nm in diameter) or quantum dots (30 nm in diameter) and microbeads (4.5 μm in diameter) were coated with biotinylated peptide−MHC−Ig along with biotinylated CD80−Ig or anti-CD28 mAb. *In vitro*, these different structures had similar immunological activity. *In vivo*, however, whereas the nanoparticle-based structures penetrated efficiently into tissues, enabling rapid access to peripheral lymphoid organs, the microparticle-based compounds experienced significant degrees of retention at the site of injection. To test the therapeutic potential of these compounds, C57BL/6 mice that had been previously transfused with melanoma antigen-specific (TCR-transgenic) CD8+ T-cells (hence carrying a contrived repertoire artificially enriched for melanoma-specific T-cells) were given a subcutaneous injection of B16 melanoma cells. One dose of quantum dots co-delivering both cognate peptide−MHC−Ig and anti-CD28 mAb, given along with non-nanoparticle-bound IL-2, was clearly able to slow melanoma growth.[102] Similar effects were seen in mice treated with two doses of iron oxide nanoparticles co-delivering the same T-cell stimulatory molecules given 7 days apart and *via* different routes (i.v. and s.c.), in the absence of soluble IL-2.[102] Although the therapeutic activity of these compounds in wild-type mice remains untested (or unreported), the results of these studies are intriguing enough to warrant further exploration.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Particle-based tumor immunotherapy remains at an infant stage of development but is clearly an approach that holds tremendous potential. Some studies have harnessed both micro- and nanoparticles as vehicles for coordinated delivery of tumor antigens and immune stimulatory molecules to DCs and other professional APC types. In general, these approaches have yielded improved outcomes as compared to conventional, non-particle-based tumor antigen vaccination approaches, resulting in augmented CD4+ and CD8+ T-cell responses against the tumor. Likewise, nanoparticle-based approaches aimed at overcoming tumor-driven immunosuppressive signals and milieus have also provided preliminary evidence of efficacy. Collectively, these studies have identified several parameters that will need to be taken into account in future studies. In addition to design principles affecting compound stability, biodistribution, pharmacokinetics, and toxicity, nanoparticle size considerations are a common thread in many of these studies. Ideally, small nanoparticles, ranging in diameter from 10 to 40 nm, displaying optimized surface chemistries, can travel longer distances both within and between immune organs, therefore increasing the likelihood that they will be internalized by the desired APC type. This can be accomplished by encapsulating antigens and/or adjuvants within nanoparticle cargo compartments and/or by conjugating these molecules to the nanoparticle surface. Although small nanoparticles cannot deliver the same payloads as their larger counterparts,

they are more readily internalized by DCs as opposed to macrophages, which have a preference for larger particulate material. Although nanoparticles designed to overcome T-cell inhibitory signals derived from the tumor itself (*i.e.*, TGFβ) or expressed by chronically activated T-cells as a negative feedback regulatory loop (*i.e.*, CTLA-4) may result in various forms of systemic toxicity and may end-up being "wasted" by APC capture (not the desired cellular destination), they may be able to boost the magnitude of immune responses induced by nanoparticle-based immunization approaches.

Direct T-cell targeting is emerging as an attractive alternative approach in nanoparticle-based tumor immunotherapy. Development and clinical translation of this approach will benefit from optimization of nanoparticle engineering and the design of nanoparticle coats capable of packing pMHC and co-stimulatory ligands at high densities. Only a few (mostly commercially available) nanoparticle designs that are either not biocompatible or have inadequate ligand-binding/packing capacity have been evaluated. Although the immunological activity and therapeutic efficacy of these compounds remain limited, they will undoubtedly improve with the advancement of next-generation designs. These compounds have the theoretical capacity of being able to trigger the sustained activation/expansion of high-avidity TAA-specific T-cells without inducing bystander immune responses. These compounds can display different combinations of pMHC and co-stimulatory molecules to activate specialized subsets of effector and/or memory TAA-specific T-cells. They can deliver one or more pMHC types along with different combinations of co-stimulatory molecules, cytokines, and blockers of suppressors of TCR signaling at different stages of tumor progression. We can foresee compounds aimed at promoting the activation of naive tumor-specific T-cells, compounds aimed at promoting the differentiation of these T-cells into long-living memory T-cells, compounds designed to sustain the survival and systemic expansion of these memory T-cells, compounds capable of priming T-helper CD4+ and tumor-cytotoxic CD8+ T-cell responses simultaneously to promote the formation of "helped" effector memory CD8+ T-cells, and even compounds capable of expanding other types of tumor-fighting immune cell types, including NK cells and iNK T-cells, among others. We can envision combinations of nanoparticles delivering different tumor-specific pMHC complexes to contain the tumor-editing effects of therapy with individual pMHC specificities. The combinatorial possibilities are endless.

Clinical translation of the most effective approaches/combinations will undoubtedly require the optimization of the corresponding nanostructures. Most of the studies reported to date used commercially available nanoparticles. Although positive results were reported, it will be crucial to define the optimal chemical and physical properties of these compounds, including nanoparticle size, surface chemistry and density, and payload quality and quantity, not only in terms of therapeutic efficacy but also in terms of toxicity, pharmacokinetics, and biodistribution. We have recently shown that repeated administration of nanoparticles displaying autoimmune disease-relevant pMHC complexes expands pools of cognate (antigen-specific) memory-like regulatory T-cells that can broadly suppress polyclonal autoimmune inflammation without compromising systemic immunity[126,127] (and our unpublished data). More recently, we have developed a novel PEGylated iron oxide nanoparticle design that has high pMHC-binding/packing capacity (our unpublished observations). These compounds are functionalized to enable the directional ligation of multiple copies of pMHCs and other ligands and are small (~40 nm in hydrodynamic diameter), highly stable after synthesis, fully biocompatible, nontoxic, nonimmunogenic, and useful for repeated administration. These properties make these nanoparticles an excellent candidate as a platform with which to build next-generation T-cell-targeting/stimulating nanomedicines to both suppress (in autoimmunity) and potentiate (in cancer) immunity.

Conflict of Interest: The authors declare the following competing financial interest(s): P.S. is Scientific Founder and Officer of Parvus Therapeutics Inc.

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