Nanoparticulate immunotherapy for cancer

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ABSTRACT

Although surgery, radiation therapy, and chemotherapy have significantly improved as treatments for cancer, they can rarely control metastatic disease and cures remain scarce. Promising recent developments suggest that cancer immunotherapy may become a powerful new therapy that clinicians can offer cancer patients. The opportunity to orchestrate the body’s own immune system to target, fight, and eradicate cancer cells without destroying healthy cells makes this an extremely attractive treatment modality. Our increased knowledge in anti-tumor immunity and the immunosuppressive tumor microenvironment (TME) has provided many therapeutic strategies to battle cancer. That combined with advancements in the field of particulate delivery systems provide a mechanism to deliver these immunotherapeutics to their specific targeted cells and the TME. In this review we will focus on the current status of immunotherapy and the potential advantages of utilizing nanocarriers within the field.

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1. Introduction to tumor immunobiology

1.1. Innate/adaptive immunity

The immune system fights against pathogenic infections via innate and adaptive mechanisms for immediate defense and long-lasting protection. Innate immune cells, such as macrophages, dendritic cells (DCs), natural killer (NK) cells, etc., provide the initial, “first line” of protection by recognizing conserved pathogen-associated molecular patterns (PAMPs) via pattern-recognition receptors (PRRs) [1], including C-type lectin receptors (CLRs), Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and cytosolic DNA sensors [2,3]. Adaptive immunity usually proceeds the innate immune response and requires activation of T and B lymphocytes. This activation requires recognition of specific antigens by T and B cell receptors and, subsequently results into the generation of antigen-specific effector T cells and/or antibody secreting plasma cells. Importantly, adaptive immunity also features production of ‘memory’ T and B cells that exist in a state of readiness to mount a more rapid attack upon the second encounter of a pathogen. Effective activation of adaptive immunity depends on the sensing of microbes by PRRs expressed on antigen-presenting cells (APCs) in particular DCs [4].

1.2. Cross talk between tumor cells and immune system

Growing evidence has shown that the immune system interacts with tumors throughout tumor development, including initiation, progression, invasion, and metastasis. It is also becoming clear that the complex cross talk between the immune system and cancer cells can both inhibit and enhance tumor growth, which has become a hallmark of cancer [5]. A cancer immunoediting model [6,7] was developed to understand the apparently paradoxical functions of host immunity on cancer, based on the temporal occurrence during tumor progression: an early elimination phase (elimination of tumor cells by a competent immune system), an equilibrium phase (a balance phase when tumor progression is still controlled by the immune system but sporadic tumor cells that manage to survive immune destruction; immune editing occurs) and an escape phase (when the tumor evades immune surveillance and an immunosuppressive tumor microenvironment is established). Immune editing is believed one of the key aspects why tumors evade surveillance and lie dormant in patients for years through “equilibrium” and “senescence” before re-emerging [8].

2. Immune cells and mediators in tumors

Elimination of cancer cells via the immune system is mainly mediated by immune effector cells, such as CD8+ cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and natural killer T (NKT) cells. These
cells have been found within various types of tumors and studies involving cancer patients revealed that the presence of CD3+ or CD8+ tumor-infiltrating lymphocytes (TILs) was associated with increased overall survival [9]. CD8+ CTL is the major anti-tumor player of adaptive immunity. Recognition and elimination of cancer cells by CD8+ T cells require two signals: 1) a signal provided by the engagement of tumor antigenic peptide/class I MHC complex on antigen presenting cells, in particular DCs, with antigen-specific T cell receptor (TCR), and 2) stimulatory signals mediated by interactions between accessory molecules (e.g., CD80, CD86, LFA3) on APCs and their cognate receptors on CTLs (e.g., CD2, CD28, LFA1) [10]. Activated CD8+ CTLs kill tumor cells by releasing cytotoxic proteins (perforin, granzymes, and granulysin) or engagement of Fas ligand (FasL) on T cells and Fas on target cells, and subsequent recruitment of the death-induced signaling complex (DISC). NK cells are innate immune effector cells that recognize non-antigen-specific surface receptors [11] and trigger target cell death through release of cytotoxic granules and secretion of cytokines and chemokines to promote subsequent adaptive immune responses [12]. NKT cells (also invariant NKT or iNKT cell), another member of innate immune system, express a semi-invariant TCR that recognizes lipid antigens (e.g., α-GalCer) presented by CD1d (antigen presenting molecules) [13]. Upon activation, NKT cells rapidly produce large amounts of IFN-γ, which can profoundly modulate innate and adaptive arms of the immune system for tumor rejection. NKT cells may also directly mediate tumor lysis via Fas–FasL engagement or release of perforin [14].

In contrast to immune effector cells, CD25+ regulatory T cells (Tregs) and myeloid derived suppressor cells (CD14+ HLA-DR+ MDSCs) limit inflammation and immune activation [15] and help to maintain self-tolerance [16] (Fig. 1). Tregs have been found at high frequencies in various neoplastic malignancies such as breast, lung, liver, GI tumors, and melanoma contributing to an immune-suppressive TME [17,18]. Increased recruitment of Tregs is correlated with reduced survival and increased progression in pancreatic and ovarian cancers [19].

Fig. 1. Immunosuppressive regulators in tumor microenvironment. Tumors escape immune surveillance by various mechanisms that operate in parallel with anti-tumor immunity. Anti-tumor immunity can be suppressed by various cell types including tumor cells, stromal cells and immune cells such as MDSCs, Tregs and TAMs. These immunosuppressive cells secrete numerous soluble mediators such as arginase, prostaglandin E2, TGF-β, IDO, adenosine and NOS2. Arginase and IDO limit T-cell functions by depleting arginine and consuming tryptophan. TGF-β, IDO and IL-10 suppress the activity of T cells and natural killer cells as well as cause the expansion of Tregs. TGF-β can also suppress or alter activation, maturation and differentiation of DCs, CD4+ and CD8+ T cells. Moreover, due to changes in epigenetic machinery of tumor cells, expression of MHC-I/II molecules, proteins associated with APM and costimulatory molecules (CD80/CD86) is down-regulated which prevents successful antigen presentation and tumor detection. Moreover, tumor cells also express surface molecules such as PD-L1/PD-L2 that engage PD-1 receptor on the surface of activated T cells which cause the anergy and exhaustion of T cells. CTLA-4 receptor on tumor binds to co-stimulatory molecules on APCs and prevents antigen presentation. Collectively, tumors escape immune surveillance via inhibitory mechanisms utilized by all of these cell types.

carnomas [19,20]. MDSCs also down-regulate both innate and adaptive arms of the immune system via a variety of mechanisms, including release of IL-10, activation of Tregs, and sequestration of cysteine needed for T cell protein synthesis and suppress CD8+ T cell function [21]. Analogous to associations between Tregs and outcome, elevated circulating MDSCs correlate with poor prognosis in pancreatic, esophageal, and gastric cancers [22].

Macrophages are divided into two categories based on their functions: classical M1 and alternative M2 macrophages. The M1 macrophage is involved in the inflammatory response, pathogen clearance, and antitumor immunity while M2 macrophages influence an anti-inflammatory response, wound healing, and pro-tumorigenic properties. During tumor progression, large numbers of monocytes are recruited to the tumor site which then differentiate into tumor-associated macrophages (TAMs) [23]. In response to various signals generated from tumor and stromal cells, TAMs are predominantly polarized toward a M2-like phenotype and subsequently promote tumor growth, angiogenesis, invasion, and metastasis. Clinical studies have suggested that TAM accumulation in tumors correlates with a poor clinical outcome [24].

In addition to immune cells, immune mediator molecules are also an important part of immunoeediting process (Fig. 1). Cytokines such as interferons (IFN), specifically IFN-γ and IFN-α, as well as interleukins (IL) (IL-2, IL-12), and granulocyte macrophage colony-stimulating factor (GM-CSF) etc., have shown anti-tumor capability and act directly on tumors or enhance functions of effector cells. For example, IFN-γ is one of the major anti-cancer immune mediators and is produced by activated Th1 cells (a subset of CD4 + T-helper cells), CD8 + T cells, NK cells and NKT cells. IFN-γ is a potent activator of macrophages, NK cells, neutrophil phagocytic activity, and promotes synthesis of Class I and II MHC molecules that enhance antigen presentation [25]. Genetic deficiencies in IFN-γ (or of its receptor) result in spontaneous tumor development implying that IFN-γ plays a crucial role in immunosurveillance and elimination of neoplastic cells [26]. On the other hand, a range of inflammation mediators [cytokines, chemokines, free radicals, prosta-
glandins, transcription factors, microRNAs, and enzymes such as, arginase (ARG1), indoleamine 2,3-dioxygenase (IDO), cyclooxygenase and matrix metalloproteinase (MMP), nitric oxide synthase (NOS2)] are released by or reside in cancer cells and immune cells, collectively act to create a favorable microenvironment for the development of tumors. Transforming growth factor β (TGF-β) and IL-10 are the major immune suppressive cytokines secreted by tumor cells, MDSC, and TAMs while several proinflammatory cytokines (IL-1, IL-6, TNF-α) that mediate chronic inflammation in the tumor, significantly contribute to tumorogenesis and progression [27].

Tumors exploit several immunological processes to escape immune surveillance, such as increasing Treg cell functionality, down-regulating expression of tumor-associated antigens, antigen processing machinery (APM) and accessory/co-stimulatory molecules through epigenetic reprogramming and modifying production of immune suppressive mediators. Our growing understanding of the complex interplay between tumor cells and the immune system has provided pharmacological targeting opportunities for cancer immunotherapy (Fig. 2). Depending on the approach, immunotherapy should strike more specifically against the tumor, thus lowering the damage to healthy tissue and preventing debilitating side effects that are nearly unavoidable with radiation, chemotherapy, and surgery.

Activated and tumor-specific immune cells can reach areas that a surgeon cannot, and the immune system may, when appropriately stimulated, target even microscopic disease and disseminated metastases. Further, immunotherapy should not result in temporary benefits or have to deal with multidrug resistance as do chemotherapy and radiation therapy. Also, immunotherapy could more efficiently target cancer cells that are slowly dividing or quiescent-characteristics associated with cancer stem cells. Finally, memory cells elicited by immunotherapy may suppress the re-emergence of cancer. This potential of long-term control or even complete eradication of the cancer is possibly the most promising aspect of immunotherapy since induced anti-tumor

![Image](https://example.com/fig2.png)

**Fig. 2.** Immunotherapeutic strategies for cancer. A growing understanding of the complex interplay between tumor cells and immune system has provided pharmacological targeting opportunities for cancer immunotherapy. Antigen expression and presentation of tumor cells can be increased by delivering drugs that manipulate epigenetic machinery (5-Aza, SAHA, etc.). Tumor antigen specific effector cells can be generated via cell based or subunit vaccines targeting DCs and the delivery of cytokines and growth factors. Blocking of CTLA-4 or PD-1 pathways via antibodies (Anti CTLA-4 or Anti PD-1) can restore T cell exhaustion in TME. Immunosuppression can be reversed by many mechanisms — such as depleting MDSCs (via All-trans-retinoic acid (ATRA), PDE-5 inhibitors and nitroaspirin), depleting Tregs, and TAMs (via bisphosphonates and legumain vaccine) and inhibiting regulatory mediators (via siRNA, antibodies or small molecules inhibitors against TGF-β, IDO, PDE-5 and COX-2).
responses have sometimes proven durable over many years (at least in a subset of patients).

3. Current immunotherapies

Cancer immunotherapy is a rapidly moving field and represents a novel approach to cancer treatment. Table 1 provides examples of immunotherapies currently in clinical trials that are investigating the potential of providing lifesaving treatments to more patients with more types of cancer. This section will also provide additional detail to the most promising immune-based therapies.

3.1. Targeting tumor cells via antibody therapy

Over the past 15 years, monoclonal antibodies (mAbs) have emerged as a promising strategy to fight cancer [45,46]. Cancer targeting mAbs can be designed to bind molecules on the surface of cancer cells and act as a marker for the body's immune system to destroy them. An example is alemtuzumab (Campath®), which is used to treat chronic lymphocytic leukemia (CLL). Alemtuzumab binds to the CD52 antigen that is overexpressed on leukemia cells. Once attached, the antibody “flags” the leukemia cells for destruction by immune cells [47]. Other cancer targeting mAbs can attach to and block antigens that are important signals for the growth and metastasis of cancer cells. For example, trastuzumab (Herceptin®) is an antibody against the HER2 protein, which is overexpressed on some types of cancer, and when activated allows for cancer cells to proliferate. Trastuzumab binds to these proteins, blocks down-stream HER2 signaling and subsequently inhibits proliferation of these cells [47]. Antibody therapies have been reviewed extensively [46], and therefore will not be a focus of this review.

3.2. Improvement of antigen expression and presentation pathways

3.2.1. Epigenetic modulators

One major mechanism of tumor immune evasion is to alter tumor antigen expression and presentation to immune effectors. It has been determined that tumors can down regulate expression of cancer testis (CT) antigens, MHC class I/II, co-stimulatory molecules (CD80, CD86 and CD40) and genes involved with antigen processing machine (APM) [48]. Emerging evidence suggests that epigenetic changes defined as mechanisms that change gene expression by ‘marking’ DNA or its associated proteins without changing the DNA sequence) are responsible for the down regulation of these genes [49,50]. For example, methylation of the DNA encoding MHC class I/II heavy chain gene and histone acetylation of APM genes have been shown to be responsible for their inhibition [48]. DNA methyl transferases inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi) can reverse immune resistance by restoring the defective functions of cancer cells. The anti-tumor effect of this type of drugs, 5-azacytidine (5-AC) and 5-aza-2’-deoxcytidine (decitabine) is being evaluated in clinical trials in combination with IL-2 therapy [51–53]. The effect of IL-2 producing cellular vaccine or CpG ODN was augmented when MHC-I deficient HPV-16 associated tumor was treated with 5-AC, which increased the expression of MHC-I and co-stimulatory molecules such as CD80 and B7-H1, and synergistically regressed tumor growth [54]. Data from this study suggests that epigenetic therapy is a promising approach when used in combination with cancer vaccines, for treating solid tumors that are deficient of MHC-I and APM.

3.2.2. Dendritic cell therapy

Dendritic cells are professional antigen-presenting cells that play an essential role in generating robust antigen-specific T cell immune responses against cancer. Immunotherapeutic strategies have attempted to utilize the ability of dendritic cells to deliver antigens as a means of therapeutic vaccination in individuals with advanced malignancies [55–57]. In 1995, the first clinical trial was carried out to investigate therapeutic dendritic cell cancer vaccines for the treatment of melanoma [55]. For these studies, DCs were generated ex vivo by culturing patient’s own hematopoietic progenitor cells with cytokine combinations, pulsed with tumor antigens, followed by ex vivo maturation, and then administered back into the patient to elicit an immune response against the cancer cells carrying the antigens. Sipuleucel-T (Provenge®) was the first DC-based therapeutic cancer vaccine approved in 2010 by the FDA, for the treatment of prostate cancer [58]. As a personalized therapy, DC vaccines are highly labor intensive, requiring skilled technicians to isolate and expand cells from each patient, a process that can take between 4–16 weeks, which might not be feasible for patients with highly progressive diseases. Furthermore, storage, transportation, and reconstitution of these cellular vaccines are problematic. These issues combined make these cellular vaccines very expensive [59]. In addition, even though the vaccination resulted in antigen-specific CTL responses at the immunization and metastatic disease sites, no major therapeutic response was demonstrated in many

Table 1
Summary of current clinical trials for cancer immunotherapy.

<table>
<thead>
<tr>
<th>Clinical trial</th>
<th>Immunotherapy</th>
<th>Type of cancer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase II</td>
<td>Herceptin and NeuVax™ vaccine (immunodominant nonapeptide derived from the extracellular domain of the HER2 protein with GM-CSF)</td>
<td>HER2-expressing breast cancer</td>
<td>[28]</td>
</tr>
<tr>
<td>Phase I</td>
<td>CEA(6D)/carinoembryogenic antigen / VRP (virus-like replicon particle) vaccine (AVX701)</td>
<td>Stage III colon cancer</td>
<td>[29]</td>
</tr>
<tr>
<td>Phase II/III</td>
<td>Tergenpumatucel-L (consists of 3 non-small cell lung cancer (NSCLC) cell lines that have been genetically modified to express alpha-gal carbohydrates on cell surface molecules)</td>
<td>Progressive or relapsed NSCLC</td>
<td>[30]</td>
</tr>
<tr>
<td>Phase II</td>
<td>CAR T cell receptor immunotherapy targeting VEGFR2</td>
<td>Metastatic cancer</td>
<td>[31]</td>
</tr>
<tr>
<td>Phase II</td>
<td>Azacitidine (DNMTi) and Entinostat (HDACi)</td>
<td>Advanced NSCLC</td>
<td>[32]</td>
</tr>
<tr>
<td>Phase II</td>
<td>FOLFIRINOX (leucovorin + 5-fluorouracil + irinotecan + oxaliplatnin) + GVAX (allogeneic GM-CSF-transduced pancreatic tumor cell vaccine) + Ipilimumab</td>
<td>Pancreatic cancer</td>
<td>[33]</td>
</tr>
<tr>
<td>Phase II</td>
<td>AZD9150, a STAT3 antisense oligonucleotide</td>
<td>Malignant ascites</td>
<td>[34]</td>
</tr>
<tr>
<td>Phase II</td>
<td>Tafadafil</td>
<td>Head and neck squamous cell carcinoma</td>
<td>[35]</td>
</tr>
<tr>
<td>Phase II</td>
<td>Iplimumab and all-trans retinoic acid</td>
<td>Stage IV melanoma</td>
<td>[36]</td>
</tr>
<tr>
<td>Phase II</td>
<td>Zoledronate</td>
<td>In triple negative breast cancer</td>
<td>[37]</td>
</tr>
<tr>
<td>Phase II</td>
<td>Romidepsin (HDACi) and Lenalidomide (immunomodulatory)</td>
<td>Peripheral T-cell lymphoma</td>
<td>[38]</td>
</tr>
<tr>
<td>Phase II</td>
<td>Lenalidomide (Revlimpl)</td>
<td>Chronic lymphocytic leukemia (CLL)</td>
<td>[39]</td>
</tr>
<tr>
<td>Phase I/I a</td>
<td>PLX3397 (a CSF1R inhibitor) with Pembrozulam (an anti-PD-1 Ab)-double-immune suppression blockade</td>
<td>Advanced melanoma and other solid tumors</td>
<td>[40]</td>
</tr>
<tr>
<td>Phase II</td>
<td>TG01 (a collection of 7 mutated RAS peptides) and GM-CSF</td>
<td>Resected pancreatic cancer</td>
<td>[41]</td>
</tr>
<tr>
<td>Phase I/I</td>
<td>Anti-OX40 Ab, Cyclophoshamide (CTX) and Radiation</td>
<td>Progressive metastatic prostate cancer</td>
<td>[42]</td>
</tr>
<tr>
<td>Phase II/III</td>
<td>Celecoxib</td>
<td>Bladder cancer</td>
<td>[43]</td>
</tr>
<tr>
<td>Phase II</td>
<td>LY2157299 (TGF-β receptor inhibitor) and Enzalutamide (anti-androgen)</td>
<td>Metastatic castration-resistant prostate cancer</td>
<td>[44]</td>
</tr>
</tbody>
</table>
advanced tumors [55]. One hypothesis regarding the lack of response was that immune inhibitory pathways elicited by the tumor/tumor micro-environment prevented CTLs from exerting their functions. Therefore, as discussed in more detail in Sections 3.3 and 3.4, inhibiting immune checkpoint receptors CTLA-4 and PD-1 (involved in the negative regulation of CTL function) and modulating immunosuppressive environment may be good companion therapies for DC vaccines.

3.2.3. Cancer vaccines

In contrast to cellular vaccines, specific components of subunit vaccines (viral or non-viral-based recombinant antigen proteins, antigenic peptides, formulated with or without adjuvants such as TLR agonists) can be directly administered to the patient, to induce high numbers of antigen-specific effector and memory T cells. These vaccines rely on the patients' endogenous DCs for their uptake and antigen presentation. Components of subunit vaccines can be easily designed based on recombinant technology and epitope focusing and these vaccines can be easily stored and transported. Peptide based vaccines for cancer have been in clinical trials since 1995 [60,61]. There have been durable clinical responses in some patients that receive melanoma vaccines, however overall clinical response rates were low. In an effort to improve subunit vaccines, researchers have moved away from using short peptides (which have little or no tertiary structure and thus undergo rapid degradation in tissue and serum) to longer peptides [60,61]. These longer peptides prevent degradation by exopeptidase and provide extra “handles” to pro tease and APM to present with MHC-I. Additionally the longer peptides have the potential to induce memory CD8+ T cells. Currently there are several Phase III clinical trials of subunit cancer vaccines with multiple tumor types, such as peptide-based Gp100, IMA901, NeuVax, etc., viral-based TC-2010, ProstAtak™, Prostvac®-V/F-TRICOM™, etc., and antigen-expressing whole cell vaccines Algenpantucel-L and Tergenpantucel-L, etc., which have been reviewed in detail elsewhere [62].

As for DC vaccines, elicitation of high numbers of antigen specific T cells by subunit vaccines does not necessarily ensure an effective vaccine. Therefore, it is anticipated that therapeutic vaccines would be more effective in combination with other therapies that enhance tumor infiltration, and reverse suppressive TME.

3.2.4 T cell therapy

The isolation, stimulation, and reinfusion of patients' T lymphocytes for the treatment of disease, termed adoptive cell transfer (ACT), were initially reported in the 1980s. ACT has been utilized for the stimulation and expansion of potent antigen-specific T cells that can kill cancer cells. The primary challenges in this field are identifying tumor-specific targets and avoiding off-target toxicities. Using this approach a personalized treatment can be achieved based on growing tumor-infiltrating lymphocytes (TIL) ex vivo from surgically excised tumor specimens of patients and then adoptively transferring them back into the patient [63]. This treatment is often coupled with IL-2 therapy and has been used on a number of different cancers — renal cell carcinoma, breast cancer, oral squamous cell carcinoma, and non-small cell lung cancer [63,64]. A similar therapy utilizes genetically modified T cells, which target cancer through a chimeric antigen receptor (CAR), T cells are isolated, modified with T cell signalizing domains that are fused with antibody derived targeting domains, and then infused back into patients. This therapy redirects the effector function of T cells toward specific TAA's, without the requirement of antigen processing or presentation. CAR T cell therapy has been successful in treating patients with hematologic malignancies, however it has been less effective in treating solid tumors [65]. Although these therapies have great potential to be efficacious — it is extremely difficult to offer as a widely available therapy since the cell culture process requires extensive manipulation by highly skilled scientists/technicians.

3.2.5. Cytokine therapy

Immune modifying agents such as cytokines can be used to enhance the body's immune response against cancer [45,66]. Cytokines are a group of signaling proteins that help regulate immune system responses. There are two types of cytokines that are currently used to treat patients with cancer — interferon (IFN) and interleukins (IL). In 1995, IFN-α was the first cytokine to be approved to treat patients with leukemia and advanced melanoma, and remains the first choice treatment for patients with metastatic melanoma [66]. IFN-γ is a pleiotropic cytokine with immunoregulatory, antiproliferative, differentiation-inducing, apoptotic, and anti-angiogenic properties [66]. Although it is involved in the upregulation of MHC-I molecules to aid in the priming and presentation of antigens in APCs, it has also demonstrated to be a potent enhancer of tumor colonization [66]. Depending on the context, IFN-γ can have cytotoxic or cytokinoprotective effects [67]. In 1998, interleukin-2 (IL-2) was approved as a cancer treatment, and was shown to be effective in treatment of metastatic melanoma and renal cell carcinoma [66]. IL-2 is a lymphocyte growth factor and is essential in activation of T cells and triggering innate immunity by stimulating functions of NK cells and macrophages. A few other lymphokines (IL-7, IL-12, IL-15, IL-21) are also currently being evaluated for their anticancer properties [45,66]. Another cytokine under investigation is GM-CSF (granulocyte-macrophage colony stimulating factor), which promotes growth and differentiation of granulocytes/macrophages and DCs, potentially contributing to improved antigen presentation [66]. In clinical trials, GM-CSF was potentially effective against acute myeloid leukemia when combined with primed lymphocytes [68]. Tumor necrosis factor (TNF) is a multifunctional cytokine that plays divergent roles in cell survival, proliferation, differentiation, and death. Though TNF-α has shown antitumor activity, its use in cancer therapy is controversial [69]. Furthermore, systemic administration of TNF-α induced serious adverse side effects (systemic shock and wide spread inflammatory responses) and therefore its use is restricted [69].

3.3. Immune checkpoint blockade agents

Immune checkpoints refer to a multitude of inhibitory pathways crucial for the immune system to maintain self-tolerance and modulate duration and amplitude of an immune response. Tumors are known to co-opt certain immune-checkpoint pathways, particularly against T cells that are specific for tumor antigens, as a mechanism of immune resistance. Since many of the immune checkpoints are initiated by ligand-receptor interactions, they can be readily blocked by antibodies or modulated by recombinant forms of ligands or receptors [46,70]. In 2011 the FDA approved the first immune checkpoint antibody, Ipilimumab (Yervoy, Bristol-Myers Squibb) for the treatment of unresectable or metastatic melanoma. Ipilimumab is a fully human antibody which binds and blocks the 'checkpoint' protein CTLA-4 (cytotoxic T lymphocyte antigen 4) which is expressed on activated T cells, where it serves as an inhibitor of T cell activation [70]. In some cases Ipilimumab resulted in long-lasting responses in patients with melanoma. However, in other instances, it caused T cells to attack healthy tissue in the form of autoimmune reactions (serious adverse side effect) [70,71]. Pembrolizumab (Keytruda, Merck) and Nivolumab (Opdivo, Bristol-Myers Squibb) are other examples of checkpoint blockade antibodies that were recently approved in 2014 by the FDA to treat advanced melanoma [70]. The mode of action for both involves binding and blocking PD-1 (programmed death-1). PD-1 is a surface protein on T cells in inflamed tissues and tumors and when bound to PD-L1 (a protein that is overexpressed on some cancers) can inhibit T cell proliferation, cytokine production, as well as cause exhaustion of T cells [70]. Therefore, drugs that target either PD-1 or PD-L1 can boost the immune response against cancer.

3.4. Therapies to target tumor microenvironment (TME)

One mechanism by which tumors evade an immune response is the lack of recognition of the tumor as a 'non-self-antigen'. This occurs because tumor cells release soluble (cytokine) mediators such as
adeno sine, prostaglandin-E2 (PGE-2), TGF-β, IDO and vascular endo-
theial growth factor-A (VEGF-A), which exert multiple direct and indi-
rect immunosuppressive activities. As described in Section 1, antitumor immu nity in TME can also be suppressed by various cellular mediators such as MDSC, Tregs and alternatively activated type-2 (M2) macro-
phages. Collectively, because of inhibitory mechanism of soluble and cellular mediators, tumors can manipulate the immune system and es-
cape immune surveillance. By regaining control of the TME, tumor in-
duced immunosuppression could be eliminated, thereby increasing the efficacy of tumor immunotherapy.

3.4.1. Manipulation of soluble mediators in TME

Immunosuppression caused by TGF-β signaling is well studied in tis-
tue culture and animal tumor models. TGF-β is over expressed in mela-
noma, breast, colon, esophagus, stomach, liver, lung, pancreas, and prostate cancers, as well as in hematologic malignancies [72]. TGF-β can suppress or alter the activation, maturation and differentiation of NK cells, DCs, macrophages, neutrophils, and CD4+ and CD8+ T cells. Furthermore, it can induce Tregs which can lead to immune tolerance [73]. Inhibition of the TGF-β signaling pathway may promote the na-
tural immune response against tumors. Potent TGF-β inhibitors have been developed by GSK, Biogen Idec, Scios Inc., Genzyme, Eli Lilly & Co., and NC/NIH; however, many investigated TGF-β inhibitors in pre-clinical studies and clinical trials have revealed poor pharmacokinetics, low tumor penetration, and high toxicity [74]. Bhola et al. have shown that inhibition of TGF-β by small molecular inhibitor LY-2157299 enhances efficacy of paclitaxel in triple-negative breast cancer [75]. However, they did not explore reversion of immunosuppression behind TGF-β signaling pathways. Minimal effort has been focused for design-
ing formulations to deliver small molecular inhibitors or siRNA therapeutics targeting TGF-β and receptors, “leaving the door wide open” for the de-
velopment of efficient drug delivery strategies to overcome these chal-
 lenges and effectively abolish the immunosuppression in the tumor microenvironment.

3.4.2. Manipulation of cellular mediators in TME

3.4.2.1. Blocking differentiation and recruitment of MDSCs. MDSCs are het-
erogeneous cell populations that increase as a result of cancer, inflam-
 nation and infection. MDSCs are generated in bone marrow in re-
 sponse to cancer-derived growth factors such as, granulocyte colony 
stimulating factor (G-CSF), IL-6, GM-CSF, TNF-α and VEGF; and are rec-
tained in tumor sites by different chemokines such as CCL2, CXCL5 and CXCL12 [76]. Once activated, they release ARG1, NOS2, IDO, and im-
 munosuppressive cytokines that inhibit the functions of CTLs, DCs, and NK cells as well as give rise to Treg cells. MDSCs are potential targets 
for cancer immunotherapy and by inhibiting or depleting MDSCs, the ef-
 cacy of other cancer therapies may be enhanced.

Phosphodiesterase-5 (PDE-5) inhibitors such as sildenafil and 
tadalafil inhibit degradation of cyclic guanosine monophosphate 
(cGMP) leading to reduction in ARG1 and NOS2 expression [76]. Intrap-
eritoneal (ip.) injections of PDE-5 inhibitors in mice reduced the ability 
of MDSCs to inhibit CD8+ T cells, resulting in enhanced intratumoral T cell infiltration and activation, reduced tumor growth, and improved the antitumor efficacy of adoptive T cell therapy. Moreover, N-hy-
droxy-ι-Arginine (NOHA) and N (G)-Nitro-ι-Arginine Methyle Ester (L-NAME) inhibit ARG1 activity. When L-NAME was administered in-
traperitoneal in C26GM colon carcinoma bearing mice, immunosup-
pressive activity of MDSCs was suppressed resulting in slower tumor growth and improved tumor specific immune response [77]. Although, PDE-5 inhibitors reduce tumor burden by deactivating MDSCs, the same class of compounds is clinically approved to block the effect of PDE-5 in smooth muscle cells for treatment of erectile dysfunction. Therefore, de-
 livery vehicle must be used to direct their use in TME to inhibit MDSCs, to increase their degradation half-life and to avoid off-target interac-
tions [78]. Another mechanism by which MDSCs suppress T cell function

is through NO (nitric oxide), which increases nitration of T cell recep-
tors, CCL2 and STAT 1 [76]. Nitro-aspirin is a classic aspirin molecule co-
valently linked to a NO donor group, which restores T cell function by inhibiting NOS enzyme produce by MDSCs [79]. C26 colon carcinoma 
bearing mice treated with nitro-aspirin resulted in reduced MDSC at tumor sites, improved T cell function and inhibition of tumor growth as compared to control mice [80]. In many cancers, expression of COX-
2 and production of PGE-2 are associated with tumorigenesis and tumor progression [81]. MDSCs also have high expression of PGE-2 re-
 ceptors. COX-2 inhibitors reduce MDSCs suppressive function by reduc-
ing production of arginase. COX-2 inhibitors, celecoxib have synergistically increased the efficacy of DC based immunotherapies by preventing expansion of MDSCs and reversing T cell tolerance, resulting in improved survival of mice with mesothelioma [82].

3.4.2.2. Targeting regulatory T cells.OX40 is a cell surface receptor whose expression is induced on activated CD4+ and CD8+ T cells by stimula-
tion through CD28 signaling. Upon binding to OX40L on the surface of B cells, DCs and macrophages, OX40–OX40L signaling enhances prolifera-
tion of T cells, leading to larger expansion and larger pool of effector and memory (CD4+ and CD8+ ) T cells. It also inhibits generation and ac-
tivity of inducible Treg cells in the TME [83]. Administration of OX40 ag-
onist may be very effective at increasing the pool of effector and memory T cells and indirectly inhibiting Tregs. Combined treatment of agonistic Ab with IL-2, GM-CSF, DC vaccine or adoptive T cell transfer therapy resulted in increased numbers and function of CD8+ T cells and memory cells, and decreased the numbers of FoxP3+ Tregs in various pre-clinical tumor models such as sarcoma, melanoma, lung carci-
noma, thymoma, breast and prostate cancers [84]. Recently, small-
molecule based modulators of the OX40 pathway have been designed and exhibit similar efficacy in cellular assays [85].

Functions of regulatory T cells can also be suppressed by targeting TLR receptors. As discussed previously, PGE-2 can promote induction of Tregs at the tumor site. Targeting PGE-2 via COX-2 blocker agents re-
sulted in reduction of infiltrating Tregs at the tumor site [86]. New class of immunomodulatory drugs (IMIDs) such as thalidomide, lenalidomide, and pomalidomide, can increase the expansion of DC stim-
ulated NK cells and inhibit IL-2 mediated generation of FoxP3+ CD25+ 
Tregs in multiple myeloma. Co-administration of IMIDs with cancer vac-
cine may be a great approach to modulate anti-tumor immunity [87].

3.4.2.3. Targeting tumor associated macrophages (TAMs). TAMs play a critical role in helping the tumor escape immune surveillance. During tumor immune evasion phase, TAMs increase in numbers and suppress T cells by releasing immunosuppressive factors such as IL-10 and TGF-β. TAMs also express PD-L1 and produce chemokine CCL22, which further inhibits T cell proliferation and promotes Treg trafficking to the tumor. Evidence suggests that the presence of TAMs is associated with poor tumor progression and survival [88].

DNA vaccines against legumain (a member of the asparaginyl endo-
peptidase family functioning as a stress protein, overexpressed by T
M cells, DCs and macrophages, tumors) are a promising strategy to induce CD8+ T cells against TAMs, which can reduce the numbers of TAMs and decrease the production of TGF-β, TNF-α, MMP-9, and VEGF in the TME. In murine models of metastatic breast, colon, and non-small cell lung cancers, 75% of mice survived tumor challenges and 62% of mice was completely free of me-
tastasis when treated with legumain DNA vaccine [89]. Macrophage 
oligomer-stimulating factor 1 is a major survival factor for TAMs. Inhibition of CSF-1 receptor (CSF-1R) via humanized mAb (RG7155) has reduced the macrophage population in phase-I clinical trial of diffuse-type giant tumor [90]. Additionally, polarizing M2 phenotype into M1 phenotype via TLR agonist is another strategy to improve efficacy in anti-tumor immu
notherapy. For example, utilizing the CpG (TLR-9 agonist) in con-
junction with anti-CD40 Abs has been shown to rapidly induce produc-
tion of pro-inflammatory cytokines leading to polarization of

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M2 to M1 macrophages and subsequently, accelerated suppression of tumor growth [91].

3.4.3. Targeting tumor vasculature

Rapid growth of tumor makes it important to develop new blood vessels to enable blood supply of oxygen and nutrients and elimination of waste. Proangiogenic factors, mainly vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), are widely overexpressed in tumors and stimulate vasculogenesis and angiogenesis, making them the central target for anti-angiogenesis cancer therapy. VEGF-A produced by tumor cells not only plays a key role in tumor angiogenesis, but also modulates the immune environment in tumor, such as reduces lymphocyte infiltration, prevents DC maturation, induces MDSC, increases tumor accumulation of regulatory T cells and recruits monocytes/macrophages to tumor, all of which are known to contribute to tumor growth and survival [92]. At present, antiangiogenesis therapy is essentially anti-VEGF/VEGFR therapy. Multiple anti-VEGF drugs have been approved by FDA for treatment of different types of tumors which either target receptor signal pathways of proangiogenic molecules (tyrosine kinase inhibitors, for example - sunitinib, sorafenib, pazopanib, lapatinib), or neutralizing circulating proangiogenic factors or their receptors (monoclonal antibodies and derivatives, bevacizumab, ranibizumab) [93]. Bevacizumab, the first VEGF inhibitor approved by the FDA for cancer treatment, is a humanized monoclonal antibody against VEGF-A, and is currently used in patients with metastatic CRC, non-small cell lung cancer and metastatic breast cancer in combination with chemotherapies [93,94]. The mechanisms of action for anti-VEGF therapy include inhibition of new blood vessel growth, apoptosis of endothelial cells, modulation of immune environment, etc. [95,96]. Although highly effective in treating many mouse cancers, anti-VEGF drugs have not been as promising in the clinic. The benefits are at best transitory and are followed by a restoration of tumor growth and progression [97]. Heterogeneity of the tumor vasculature as well as multiple other mechanisms has been proposed to explain the ineffectiveness in patients [95,98,99]. Later studies into the consequences of VEGF inhibitor use have shown that, although they can reduce the growth of primary tumors, VEGF inhibitors can concomitantly promote invasiveness and metastasis of tumors [100,101].

4. Nanoparticulate carriers for delivery of tumor immunotherapies

The key advantages of using nanoparticulate carriers are improved solubility and bioavailability of the cargo. They can be loaded with a variety of cargos such as siRNA, peptides, proteins, and small molecule therapeutics. Importantly, by associating the cargo with a nanoparticulate carrier, the cargo can be protected from degradation, which can increase its half-life, enhancing potential efficacy. Furthermore, these systems can be modified for targeted site-specific delivery, mitigating systemic toxicity issues. To date, there are 45 nanoparticulate formulations approved for clinical use including liposomes for cancer therapeutics and diagnostic agents, polymer-protein conjugates of IFN-α, GM-CSF and anti-TNF-α Ab for tumor therapy, and virosomes for flu vaccines [102].

There are a multitude of methods for fabricating nanoparticles – varying from formation of micelles [103], liposomes [104], emulsions [105], or through a template/particle molding techniques [106,107]. Using these techniques, nanoparticles can be composed of an assortment of different materials, with varying sizes, shapes, and chemical and surface properties. Possessing the manufacturing platforms to control and design nanoparticles possessing specific parameters allows investigators the unique ability to match optimized nanoparticles to the specific delivery requirements. For example, the design parameters for the delivery of immunotherapy agents that require systemic (intravenous) delivery and accumulation and release at the tumor site, will differ from those that require local delivery to tissue resident APC’s or to draining lymph nodes. These design parameters are outlined below.

4.1. Particle design parameters for systemic delivery to tumor

In the last 20 years much effort has been put forth for the development of nanoparticles for the systemic delivery of cancer therapeutics. This first generation of nanotherapeutics relied heavily on passive accumulation on nanoparticles in tumors due to the enhanced permeability and retention (EPR) effect. The EPR effect is mainly due to permeable tumor vasculature, which allows macromolecules and nanoparticles to enter the tumor, while the lack of effective lymphatic drainage and elevated interstitial fluid pressure prevents them from being removed [108]. These studies have not only improved our understanding of the physical/chemical properties that affect nanoparticle biodistribution, and pharmacokinetics – but they have also laid the foundation for the development of next-generation nanocarriers for tumor-targeted immunotherapies.

In general, when nanoparticles are administered systemically, they are coated with plasma proteins upon injection (opsonization), which mark them for clearance by the mononuclear phagocyte system, (MPS) [109–111]. Since extended circulation half-life is pertinent for utilizing the EPR effect, much research has been devoted to the development of long-circulating nanoparticles that avoid the MPS [112,113]. Particles should be either neutral or negatively charged, within a size range of 8 to 200 nm to avoid kidney, liver and splenic filtrations [109]. Longer circulation half-life can be achieved through surface coating with a stealthing agent, such as polyethylene glycol (PEG) [110]. Once long circulation is achieved the next barrier is extravasation of the particles from the tumor blood vessels and into the tumor. For this to occur, margination (the ability of a particle to flow toward the blood vessel walls) is important. Particle shape can play a major role in margination and it is generally accepted that non-spherical particles with higher aspect ratios can marginate more readily than spherical particles [114,115]. Once the particles are at the blood vessel wall they have the opportunity to extravasate through the leaky tumor vasculature and penetrate into the tumor mass. This can be achieved by decreasing nanoparticle size to sub 100 nm, and a recent work has shown that nonspherical particles are capable of penetrating tumors more rapidly than spherical particles [116–119]. After extravasation into the tumor bed, particles then need to release their cargo. Maintaining controlled release of the drug from the nanocarrier is crucial for improved efficacy and reduced toxicity. Ideally, the carrier should allow minimal premature cargo release before it reaches the intended site, but sufficient release after it reaches the target. Cargo release from the particle carrier can be dictated by particle erosion and diffusion (as with biodegradable particle matrices) or by external (including light, ultrasound, and magnetic fields) or physiological (pH gradients and redox states) stimuli [120–123]. Depending on the cargo and the particle matrix, this may occur via extracellular or intracellular means (or a combination of both). If intracellular delivery is necessary, particle internalization can be enhanced either by modifying particle shape (we have shown that cellular internalization can be enhanced by increasing particle aspect ratio) or by incorporating active targeting ligands to encourage receptor-mediated endocytosis [124–126].

4.2. Particle design parameters for local delivery to immune cells

The development of nanoparticles to target immune cells is a newer field, however much work has gone into designing particles to deliver cargo to tissue resident APC’s or drain and trigger activation of immune cells residing in lymph node, as required for vaccine administrations. For these applications particles are administered subcutaneously, intradermally, intramuscularly, intraperitoneally, etc. Since the adaptive
immune response is mainly initiated in secondary lymphoid organs, transport of nanoparticulate vaccine to the draining lymph node (dLN) is an important factor in designing these nanocarriers. The size of nanoparticulate carrier plays an important role in shaping an immune response; it not only influences cellular uptake and intracellular trafficking, but also affects lymphatic trafficking. NPs from 5 nm to 100 nm in size, transport via convective force and diffuse deeper into the extracellular matrix and are able to travel to dLNs by afferent lymphatic vesicles. Particles greater than 500 nm remain trapped in extracellular matrices [127]. These larger particles can potentially be taken up by resident APCs and then trafficked to the dLNs [128].

Once they reach the dLNs, retention of NPs is also dependent on their size. Larger particles will be taken up by subcapsular macrophages whereas smaller particles can directly access T cell region and can be taken up by immature DCs residing within LNs [127]. Moreover, in the dLNs, smaller nanoparticles can target larger numbers of immature DCs, B cells and T cells [129]. Reddy et al. have shown higher lymphatic drainage and lymph node retention of 20 nm and 45 nm polypropylene disulfide (PPS) NPs as compared to 100 nm NPs after intradermal injections. Retention of smaller sized particles was seen up to 5 days [130]. Mueller et al. have also shown the importance of particle size for targeting LNs and generating a better humoral response. In comparing non-draining 1 × 1 μm cylindrical particles, to rapidly draining 80 × 180 nm rod-shaped particles, smaller particles were able to sustain prolonged antigen presentation to APCs and elicited a stronger humoral response than the non-draining 1 × 1 μm NPs [131]. Furthermore, Fisis et al. also demonstrated the significance of size in generating anti-tumor immune response utilizing 40 nm and 100 nm ovalbumin coated particles. The 40 nm particles were able to drain to LNs and localized with residing DCs to a higher extent than 100 nm particles, and were able to induce prophylactic as well as therapeutic immunization responses against the tumor. These studies indicate that targeting immature DCs residing LNs is a successful strategy to elicit antigen specific immune response [132]. This concept is further supported by positive results from human clinical trials utilizing 40 nm ISOMATRIX particle which induced potent CD8+ T cell [133].

Particle surface charge can also affect lymphatic drainage and lymphatic retention. The extracellular matrix (ECM) is made up of collagen, whereas smaller particles can directly access T cell region and can be taken up by immature DCs residing within LNs [127]. Moreover, in the dLNs, smaller nanoparticles can target larger numbers of immature DCs, B cells and T cells [129]. Reddy et al. have shown higher lymphatic drainage and lymph node retention of 20 nm and 45 nm polypropylene disulfide (PPS) NPs as compared to 100 nm NPs after intradermal injections. Retention of smaller sized particles was seen up to 5 days [130].

Particle surface charge can also affect lymphatic drainage and lymphatic retention. The extracellular matrix (ECM) is made up of collagen fibers and negatively charged proteins (glycosaminoglycans), therefore positively charged (cationic) particles remain trapped at the injection site and possibly phagocytosed by APCs and then trafficked to the LNs [134]. In contrast, particles that are negatively charged and neutral/surface pacified with PEG have limited interaction with the ECM, which facilitates their traffic to dLN either through enhanced trafficking in lymphatic vesicles or through internalization by migratory DCs [135]. Furthermore, surface lipid coatings are highly important in determining the outcome of particles. APCs and B cells are able to recognize pathogens by their surfaces, which are densely covered with proteins, lipids and polysaccharides. By mimicking these highly repetitive patterns (HRP) of biomolecules on the surface of particulate carriers, we can enhance multivalent antigen presentation and induce more robust and potent immune response. In multivalent ligand vaccines, increasing the valency of ligands on NPs increases avidity and apparent binding, which influences cell surface receptor clustering for signal transduction [134]. More specifically, 15-20 hapten molecules that are spaced 5–10 nm apart (similar to the average spacing of viral coat proteins) are an ideal special arrangement to efficiently activate B cell receptors [136]. Highly repetitive patterns of antigen/adjuvants on nanoparticulate carriers allow efficient binding of natural IgM antibodies through high-avidity interactions, leading to recruitment and activation of complement component 1q (C1q) and the classical pathway of the complement cascade [136]. Furthermore, hydrophilic nanoparticle surfaces such as polyhydroxyalkyl (–OH) pluronic-coated NPs, can activate alternative pathways of complement activation [137]. Therefore changing chemical groups on particle surface (by changing chemistry of polymer or linkers) may allow us to manipulate their capacity to trigger the complement activation cascade and opsonization profile [138].

5. Nanoparticle assisted immunotherapy

5.1. Improvement of antigen expression and presentation pathways

5.1.1. Particulate delivery of epigenetic modulators

As discussed earlier, epigenetic changes in tumor cells are responsible for lower antigen expression and lower expression of MHC-I/II, costimulatory molecules, as well as proteins involved in antigen processing pathways. Targeting tumor cells via inhibitors of epigenetic machinery could synergistically increase anti-tumor immunity when combined with cancer vaccines by facilitating more efficient antigen presentation and processing. Epigenetic inhibitors are efficacious in preclinical studies and in clinical trials against various solid tumors like melanoma, pancreatic, prostate, breast, and colon cancers [51–53,139]. However their lower solubility, shorter half-life, peripheral degradation, low permeability, and non-specific toxicity limit their use as immune modulators, and necessitate a need to design efficient delivery systems. Recently, much effort has been devoted to designing delivery carriers for vorinostat (HDACi), for example using copolymer micelles of poly (ethylene oxide) (PEO)–polyactic acid (PLA). These carriers have improved vorinostat solubility up to 40-fold, improved the pharmacokinetics, extended circulation half-life, and decreased clearance [140,141]. Furthermore, the therapeutic index of vorinostat was improved by developing acid sensitive delivery system out of a pro-drug of vorinostat [142]. Moreover, it was discovered that epigenetic drugs can sensitize tumors to chemotherapy to enhance their efficacy. For example, NF-κB decatibiting (DNMTi) was able to increase the sensitivity of breast cancer cells to doxorubicin (DOX) [143].

5.1.2. Nanoparticulate delivery of subunit vaccine

Subunit vaccines offer a safer and more specific approach to generate immunity, by administering specific components of pathogenic organisms (e.g. bacterial coat proteins, peptides, carbohydrates or lipids) to stimulate the immune system. In the soluble form these biomolecules suffer from poor immunogenicity as well as short in vivo half-lives that limit their ability to reach target cells. This necessitates design of nanoparticle carriers to deliver subunit vaccines that target APCs and specific cellular compartments. Specifically, cytosolic delivery of exogenous antigen into MHC class I presentation pathway of APCs is required to induce potent CTLs response. Lipid–calcium–phosphate (LCP) nanoparticles represent a new class of intracellular delivery systems for sending cell membrane impermeable antigens to cytosol to induce CTLs response in cancer immunotherapy. Xu et al. have co-encapsulated Trp-2 peptide and Cpg ODN in mannose decorated LCP nanoparticles which resulted in higher cargo deposition to LNs and superior inhibition of tumor growth in both B16F10 melanoma subcutaneous and lung metastasis models [144]. Interestingly, Vasieievich et al. reported that a cancer vaccine with Trp-2 (tyrosinase related protein-2, melanoma associated antigen) peptide and a cationic lipid (R-DOTAP (1, 2-dioleoyl-3-trimethylammonium-propane) formulated into nanocomplexes was able to elicit high population of functionally active tumor-infiltrating lymphocytes and break the T cell tolerance in tumor in a murine melanoma model, achieving significantly delayed tumor growth [145].

Since cancerous cells can escape immune surveillance via multiple mechanisms, utilizing a single epitope peptide restricted to MHC-I might not be sufficient to generate efficient anti-tumor immune response. Nanoparticulate delivery systems offer the opportunity to deliver multiple epitope peptide vaccines to a single cell. Tan et al., have shown that combinational delivery of multiple NPs carrying different tumor-associated antigen (TAA) peptides generated a better anti-tumor response than nanoparticles loaded with single peptide epitope. Specifically, delivery of PLGA emulsion carrying TAA peptides Trp-2, gp100, and immunosuppressive retroviral protein epitope p15E had
significantly better tumor regression and survival in B16F10 murine melanoma model as compared to PLGA emulsion loaded with only a single TAA peptide [146].

Moreover, particulate carriers can provide sustained release of antigens to induce more potent cellular responses and immune memory. PLGAs is a commonly used biodegradable polymer material in nanoparticle synthesis, with physicochemical properties readily tunable to achieve various degradation profiles and cargo release kinetics. Liposomes are another commonly used particulate carrier, in which cargo release is achieved after disruption of lipid bi-layer. In comparing release kinetics, liposomes typically release their cargo at a faster rate than the PLGA nanoparticles. In comparing these release kinetics, it was found that a prolonged and sustained release from PLGA NPs resulted in persistent antibody titers, stronger cellular responses and higher frequency of effector T cells as compared to liposomal formulation [147].

5.1.3. Targeting DCs via nanoparticulate vaccine

Dendritic cells are the major class of APCs. Targeting DCs via antibodies against their cell surface receptor such as DC205, CD40 or CD1c, for delivery of subunit vaccine components can increase the efficiency of cross-presentation and induce more potent CD8+ T cells. Cruz et al. have evaluated the efficiency of these different targeting strategies to activate DCs and elicit a potent CD8+ T cell response. Model antigen protein ovalubumin (OVA), TL3 ligand Poly I:C and TL7/8 ligand R848 were encapsulated in PLGA NPs decorated with different antibodies against surface receptors, DC205, CD1c, and CD40. All targeted NPs were stimulated in vitro DCs for expression of co-stimulatory molecules and production of IL-12, and induced proliferation of antigen specific IFN-γ producing CD8 + T cells. Subcutaneous vaccination of CD40, DEC205 and CD1c targeted NPs in C57BL/6 mice induced significantly higher frequency of CTLs as compared to non-targeted NPs [148]. In another study, Rosalia et al. have improved the delivery of OVA protein to DCs and induced potent anti-tumor immune response in B16-OVA melanoma model in mice via co-delivery of OVA, Pan3CSK4 (TL3 agonist) and poly I:C (TL3 receptor agonist) in anti-CD40 Ab decorated PLGA NPs [149].

DCs can also up-regulate co-inhibitory molecules including PD-L1 and PD-L2. The balance in expression level and activation of co-stimulatory molecules and inhibitory molecules determine the activation state of T cells. Hobo et al. have achieved efficient knockdown of PD-L expression on human monocyte derived DC and superior induction of ex vivo antigen-specific T cells via intracellular delivery of PD-L1 and PD-L2 siRNA using cationic lipid nanoparticles (LNP) and delivery of antigen peptide mRNA via electroporation [150]. Moreover, similar groups have also developed DOPE based NPs to delivery PD-L1 and PD-L2 siRNA which increased transfection efficiency and suppression of PD-L1 and PD-L2 [151]. In another study, Cubillos-Ruiz et al. have shown increased uptake of NPs to tumor associated regulatory DCs via delivery of siRNA–PEG complexes. Delivery of siRNA via PEI complexes converted regulatory DCs to antigen presenting DCs and also enhanced tumoricidal activity of DCs via TLRS stimulation. Moreover, siRNA delivery via PEI complexes significantly reduced tumor growth and improved survival of ovarian carcinoma bearing mice [152]. Similarly, Teo et al., have increased siRNA uptake and reduced toxicity of NPs, when PD-L1 siRNA was delivered to PD-L1 overexpressed epithelial ovarian cells via PEI–PEG–siRNA complexes. Delivery of PD-L1 siRNA via PEI nanocomplexes resulted into 40% to 50% of PD-L1 knockdown with two fold increased sensitivity of SKOV-3 to T cell killing as compared to scrambled siRNA sequence [153].

5.1.4. Nanoparticulate delivery of cytokines

Cytokines are potent modulator of immune system in state of cancer. Because of their highly immunostimulatory nature, TNF-α, IFN-γ and IL-2 have been approved by the FDA for the treatment of melanoma, renal cell carcinoma and leukemia. Systemic delivery of cytokines resulted into sub-therapeutic effect because of their rapid excretion and enzymatic degradation. High concentrations of cytokines are required due to their rapid clearance and paracrine effect, which results in toxic side effects. To overcome these problems, lipid and polymer-based particulate delivery systems have been developed for delivery of IFN-γ, TNF-α, IL-2, as well as other potential therapeutic cytokines such as IL-12, GM-CSF, IL-4 and IL-6 [154]. Utilizing particulate carrier systems to deliver cytokines systemically prevents their degradation and in vivo neutralization, increases their plasma residence time and allows for site specific delivery [155–158]. Intraperitoneal delivery of liposomal IL-2 and microsphere IL-12 stimulated CTLs against tumor cells and decreased hepatic and subcutaneous tumor metastasis, respectively [159, 160]. Filder et al. have taken advantage of the ability of mononuclear phagocyte systemic (MPS) to clear NPs and targeted alveolar macrophages via liposomal delivery of lymphokines, muramyl dipeptide (MDP), TNF-α and IL-1x to clear lung metastasis of melanoma [161–164]. Subsequently, significant effort has been put forward to design PEG coated liposomes to decrease their opsonization and increase circulation half-life, to target them to the tumor site. By utilizing stealth properties of liposomes, systemic delivery of nanoparticle IL-2 has significantly reduced tumor growth compared to soluble IL-2 [165]. Moreover, systemic delivery of pegylated liposomal TNF-α resulted in synergistic antitumor activity and reduced toxicity in combination with liposomal doxorubicin (Doxil) in soft tissue sarcoma-bearing rats [166]. In combination with cancer vaccines, cytokine therapy can increase the number and functionalities of effector T cells, as well as increase the memory T cell population. Increased infiltration of CD8+ and CD4+ T cells and regression of tumor growth was achieved when liposomal IL-2 and tumor specific liposomal antigen was injected in a patients with follicular lymphoma in phase-4 clinical trial [167,168].

5.2. Nanoparticulate immunotherapy to target TME

MDSCs, Tregs and TAMS are major players that act against the anti-tumor immunity. Together with tumor cells, they secrete soluble mediators and through direct interaction inversely affect the function and survival of DCs, T cells and NK cells. As shown in Fig. 2, developing inhibitors of soluble cytokines and enzymes or inhibiting pro-tumorogenic functions of these soluble and cellular immunosuppressive mediators would improve therapeutic efficacy of cancer vaccines and other immunotherapies.

5.2.1. Soluble mediators

Particulate delivery of a TGF-β receptor inhibitor to tumors was shown to increase the number of tumor infiltrating CD8 + T cells, NK cells, and synergistically regressed tumor growth [169]. In a recently published preclinical study Park J. et al. developed biodegradable nanolipogels (nLGs) of cyclodextrin for simultaneous and sustained delivery of hydrophilic IL-2 and a hydrophobic small molecule inhibitor of TGF-β (SB505124) to the TME [169]. Testing this system in a B16F10 murine melanoma model demonstrated increased number of tumor infiltrating CD8 + T cells, NK cells, and decreased tumor growth with improved survival [169]. Moreover, Xu et al. found that LCP nanoparticle vaccine carrying Trp-2 peptide and CpG ODN was less efficacious against the late stage of B16F10 melanoma [170]. For the treatment of advanced melanoma, a liposome–protamine–hyaluronic acid (LPH) acid NP carrying TGF-β siRNA was co-delivered with a cancer vaccine which was able to knock down TGF-β by about 50%. This combined immunotherapeutic approach increased the level of tumor infiltrating CD8 + T cells, and decreased the level of Tregs as well as suppressed tumor growth by 52%, as compared with vaccine treatment alone [170].

5.2.2. Suppression of cellular mediators via nanoparticle-based immunotherapies

5.2.2.1. Targeting MDSCs. Although multiple small molecule NO donors have been designed to target MDSC in solid tumors, their poor
permeability and retention, and byproduct toxicity of in vivo metabo-
lites limits their clinical use. Drug delivery carriers may help overcome
these limitations and salvage NO-based therapies to control MDSCs in
TME. Ellen V. S. et al. reported that silica nanoparticles encapsulated
with NO-releasing derivative of pyrrolidone could inhibit the growth
of ovarian cancer cells in vitro [171]. Elevated expression of COX-2 re-
ceptor on MDSCs is associated with their immunosuppressive functions.
Ursolic acid (UA), a potent Cox-2 inhibitor, has been reported for its
anti-inflammatory, antitumor and hepatoprotective action. UA inhibits
growth of colon cancer, endometrial cancer, and melanoma via effec-
tively inducing apoptosis and inhibiting angiogenesis by inhibition of
COX-2 expression [172]. However, UA has limited water solubility
which leads to lower bioavailability and poor pharmacokinetics which
restricts its clinical use as a Cox-2 inhibitor. To improve its solubility
and cell uptake, UA loaded mPEG-PCL (methyl-poly (ethylene) glycol-
poly-caprolactone block copolymers) nanoparticles were prepared via
nanoprecipitation. Significant difference in apoptosis of gastric cancer
cells-SGC7901 was found when treated with polymeric NPs as com-
pared to free UA, which was correlated with enhanced inhibition of
COX-2 activity [173]. Biodegradable PLGA NPs incorporated with anoth-
er Cox-2 inhibitor celecoxib have also shown improved antitumor activ-
ity against glioma tumor cell line U87MG [174].

All-trans retinoic acid (ATRA) is an active metabolite of vitamin A
and exerts potent effect on cell growth, differentiation and apoptosis
via agonistic binding to nuclear receptors retinoic acid α and β (RAR
α/β)). ATRA eliminates MDSCs via up regulation of (cSS) glutathione
synthase in MDSCs. This results in accumulation of GSH in cells which
neutralizes reactive oxidative species produced by MDSCs [175]. Be-
cause of its tumor protective role, ATRA is being included in therapeutic
schemes for Kaposi’s sarcoma, head and neck squamous cell carcinoma,
ovarian carcinoma, bladder cancer, and neuroblastoma [176]. In vivo
administration of ATRA dramatically reduced the presence of immature
myeloid cells (mMcs) by promoting their differentiation into mature
dendritic cells, macrophages, and granulocytes. Decreased presence of
Mmcs in tumor-bearing mice noticeably improved CD4+ and CD8+
mediated tumor-specific immune response. These data suggest that
elimination of mMc with ATRA may open an opportunity to improve
the efficacy of cancer vaccine [177]. ATRA can also act as a powerful can-
cer stem-cell (CSC) differentiating agent. Sun et al., have shown that si-
multaneous delivery of ATRA and doxorubicin (DOX) through
nanoemulsion, induced breast CSC differentiation, decreased number of
breast CSCs in tumor and suppressed tumor growth by attenuating
their tumor initiating ability [178]. Wang et al., have investigated the
role of pH sensitive nanoparticles in delivery of ATRA. Proliferation of
hepatocellular carcinoma cell line (HepG2) was inhibited and differenti-
tation was enhanced when ATRA was delivered to intracellular com-
partment via pH sensitive dendrimers of poly (amidoamine) (PAMAM) [179].

5.2.2.2. Targeting Tregs. Manipulating functions of Tregs in TME is a desir-
able immunotherapy approach. However, no delivery system is avail-
able to selectively target Tregs in the TME. To target intratumoral
Tregs, Sacchetti et al., conjugated glucocorticoid-induced TNFR-related
receptor (GITR) ligands to pegylated single-walled carbon nanotubes
(PEG-SWCNTs). In vivo investigations of PEG-SWCNTs armed with
GITR ligands in B16F10 melanoma model found higher accumulation of
SWCNTs in intratumoral Tregs, as compared to accumulation within
intratumoral non-Tregs or splenic Tregs [180]. This kind of innovative
strategies to target cellular mediators in TME could pave the way for
novel anti-tumor immunotherapy.

5.2.2.3. Targeting TAMs. Bisphosphonate (BP) molecules such as clodronate,
zolindronic acid (ZOL) and pamidronate are prescribed in
osteoporosis to prevent bone loss. Recently, BPs have been found to in-
duce apoptosis in cancer cells and TAMs [181]. However, due to short in
vivo half-life, coupled with rapid uptake and accumulation in bone, their
use as antitumor agent for extra-skeletal malignancies is limited. Deliv-
ery of BPs via nanoparticulate delivery vehicles is a promising approach
to improve their half-life and to target them to the TME. Clodronate was
encapsulated in lipid vesicles of phosphatidycholine and cholesterol. It
was found that melanoma tumors mice treated with clodronate lipo-
somes, were depleted in TAM at TME, and tumor growth was inhibited
[182]. Moreover, liposomal clodronate also efficiently depleted phagoc-
cytic cells and inhibited tumor growth in the murine tetratocarcinoma
(F9) and human rhabdomyosarcoma (A673) xenografts in mice. When clodronate was combined with VEGF-neutralizing Ab, stronger
anti-tumor response was observed, suggesting that cancer therapy
aimed at hematopoietic precursor cells can be a novel therapeutic ap-
proach for tumor regression [183]. ZOL is another BP drug that has short half-life and high bone accumulation. PEGylated self-assembled
NPs of ZOL were developed to overcome its pharmacokinetics limita-
tions. ZOL-NPs induced reduction in TAMs and complete remission of
prostate cancer (PC3) tumors which resulted improved overall survival
[184,185]. Furthermore, Sabatino et al., confirmed the ability of ZOL to
deplete the macrophage in vivo via administering ZOL in a red blood
cell (RBC) encapsulated formulation. A single administration of ZOL
loaded RBCs caused approximately 30% and 70% depletion of hepatic
macrophages and spleen macrophages respectively [186]. Since macro-
phages have high expression of mannose receptors, macrophages can
also be targeted via mannose decorated NPs. Zhu et al., developed a
sheddable PEG, mannose-modified nanoparticle platform to improve
TAM targeted delivery. Sheddable PEG was conjugated to mannose dec-
orated PLGA NPs via an acid sensitive linker. In a murine melanoma
model (B16F10) they found improved accumulation of NPs in TAMs as
compared to NPs conjugated to non-sheddable PEG. This is mainly due
to the PEG preventing opsonization at physiological pH, however, at
lower intratumoral pH of 6.8, the acid sensitive linker was cleaved, re-
leasing PEG allowing the mannose labeled NPs to be taken up by
TAMs [187].

6. Conclusion and future directions

Advances in our understanding of the dynamic and complex interac-
tions of immune regulation and the interplay of tumor cells and the im-
une system have provided a scientifically sound foundation for
advancing cancer immunotherapy leading to innovative therapeutic
strategies. The clinical success of genome-targeted agents has laid the
foundation and provided impetus for other cancer therapies, including
the prerequisite to identify predictive biomarkers for selection of pa-
ients for treatment. The ability to block key pathways by which tumor
cells seek to evade or suppress the immune response is critical to
realizing the potential of cancer immunotherapy. Fortunately, im-
mune checkpoint therapies and combination strategies with immu-
notherapy have provided cancer patients with novel treatments that
have the potential to elicit durable control of disease, improvement in
quality of life and even possible cures in some cancer types. However,
many challenges remain if durable tumor eradication with minimal tox-
icity is to be achieved in a broader population of cancer patients.

The manipulation of the immune system through administration of
immuno-modulating therapies such as monoclonal antibodies, cyto-
kines, growth factors, TLR agonist, and/or a vaccine to direct an effective
and long-lasting immune response against cancer cells are attractive
strategies. Armies of cancer

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subcutaneously), dLNs, or to modulate cellular and soluble mediators in the TME. The continued advancements in the field of particle-based drug delivery, such as optimizing size, shape, surface properties and/or physicochemical properties will allow to develop and design improved formulations of immunotherapeutics. Safer and more specific antibodies and tumor-associated antigens have been designed due to our growing understanding in the field of epitope focusing, DNA recombinant engineering and genome based screening. Because of advancement in the field of medicinal chemistry and high-throughput screening, more potent and specific small molecular inhibitors against soluble and cellular mediators are also being designed. It is therefore possible to design particle delivery systems which can target IDO in the TME, suppress depletion of tryptophan, or inhibit OX-40 on surface of Tregs to re-install anti-tumor immunity.

Optimizing the effectiveness of immunotherapy will require targeting the anti-tumor immune response at multiple levels, and this may be achieved through synergistic combinations. Examples include combining immune checkpoint inhibitors or epigenetic immune modulators with cancer vaccines, or by combining chemotherapy and cancer vaccines with targeted drug delivery vehicles carrying inhibitors/modulators of immunosuppressive TME. The ability of an activated immune response to generate: 1) a diverse T cell repertoire that adapts to heterogeneous and genetically unstable tumors, 2) persistent memory T cells with specificity for tumor antigens, which provide efficient recall responses against recurrent disease, and 3) re-establish the “normal cellular environment” makes it absolutely essential to expand our efforts to identify and develop rational combinations to unleash antitumor immune responses for the benefit of cancer patients. The high versatility of nanoparticle delivery systems will not only help enable individual nanoparticle interventions, but also the combination therapies to greatly improve the therapeutic benefits. Given the advancements in the field of nanoparticle delivery systems and parallel innovations in related fields, it seems likely that more effective treatments and/or cures for many types of cancer will become reality.

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