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REVIEW ARTICLE

Nanovaccines for remodeling the suppressive tumor microenvironment: New horizons in cancer immunotherapy

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Abstract Despite limited successes in clinical development, therapeutic cancer vaccines have experienced resurgence in recent years due to an enhanced emphasis upon co-mitigating factors underlying immune response. Specifically, reversing the immune-suppressive effects of the tumor microenvironment, mediated by a variety of cellular and molecular signaling mechanisms, has become fundamental toward enhancing therapeutic efficacy. Therein, our lab has implemented various nano-vaccines based on the lipid-coated calcium phosphate platform for combined immunotherapy, in which antigenic, epitope-associated peptides as well as immune-suppression inhibitors can be co-delivered, often functioning through the same formulation. In probing the mechanism of action of such systems *in vitro* and *in vivo*, an improved effect synergy can be elucidated, inspiring future preclinical efforts and hope for clinical success.

Keywords vaccine, nanoparticle, tumor, immunotherapy, microenvironment

1 Introduction

Because of the biological characteristics of metastasis, resistance, and recurrence for malignant tumors, the effects of traditional surgery, radiotherapy and chemotherapy for tumor treatment remain limited. In recent years, due to rapid development in biotechnology and research of the molecular mechanism underlying the occurrence and development of cancers, the active immunotherapy of malignant tumor based on adoptive transfer of dendritic cell (DC) vaccines has attracted increased attention [1,2]. In this treatment model, tumor-associated antigens are loaded into DCs by various methods and presented to $CD8^+$ T-cells *in situ*, which are induced toward proliferation and differentiation into specific cytotoxic T-cells, so as to play an active role in tumor cell killing. Compared with traditional methods, immune therapy through DC-based tumor vaccines presents the advantages of longer effect duration, stronger specificity, and fewer adverse reactions, particularly for metastatic recurrence caused by residual lesions [3,4].

Despite considerable exploration of cancer vaccines at various stages of clinical development for treatment of melanoma, lung cancer, breast cancer, prostate cancer and other malignancies, results have fallen far below expectations, with the clinical effectiveness rate below 30% [5,6]. For example, the average survival time of patients after vaccination by Provenge, the only therapeutic vaccine for prostate cancer that is currently approved by the FDA, can only be extended for 4 months, underscoring a limited treatment effect which limits potential survival benefits [7].

Traditional efforts toward enhancing cancer vaccine efficacy focused predominantly on direct effects, most notably through the antigen presentation process. Isolated DCs loaded with tumor antigen *ex vivo* and administered as a cellular vaccine have been found to induce protective and therapeutic anti-tumor immunity [8]. Another alternative is to encapsulate antigens into certain vehicles, such as nanoparticles [9], micelles [10] and liposomes [11], with the subsequent presentation *in vivo* performed by targeting to DCs either local to the injection site or through the lymphatic drainage of such formulations and accumulation at locally accessible nodes [12]. Although these methods can overcome the problems of low efficiency of tumor antigen presentation and insufficient populations of effector lymphocytes, the anti-tumor effect *in vivo* has not been notably improved.

A number of studies have shown the immune inhibitory effect of the tumour microenvironment (TME) to be one of

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the key factors in limiting the realization of a potent T-cell response [13,14]. As the obvious site of treatment effect execution, the TME can negatively regulate immune-effector cells through direct tumor and stromal cell signaling as well as through their secreted cytokines [15,16]. These immunosuppressive cytokines, such as IL-6, IL-10, TGF-β, VEGF, etc., lead to a direct inhibitory effect on the activation of cytotoxic T lymphocytes (CTLs) and the differentiation and maturation of DCs [17]. On the other hand, they promote the recruitment and activation of immunosuppressive cells in the TME, such as myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs) and regulatory T-cells (Tregs) [18]. In turn, these activated negative regulators are induced to release more immunosuppressive factors, thus forming a vicious cycle of immune suppression in the TME [19,20].

Therefore, reversing the immune suppression within the TME represents a key issue toward establishing a meaningful clinical efficacy for tumor vaccines. In the following sections, we will outline major factors which contribute to such immune suppression, and discuss the benefits of carrier-based strategies, particularly through the LCP formulation, in providing a capable combination approach to immune cell activation and microenvironment suppression.

2 Factors inducing immune suppression in TME

2.1 Cell-mediated immune suppression

A variety of cells can inhibit the immune response in the TME, including Tregs and MDSCs. Tregs ($CD4^+CD25^+$ $Foxp3^+$) are a class of IL-10 expressing immune regulatory cells derived from the thymus. Studies have indicated that Tregs are widely distributed in various tumor tissues (such as lung cancer, breast cancer, gastric cancer, and colorectal cancer) and play an important role in immune suppression [21]. Several mechanisms are involved in their negative regulation, including secretion of cytokines to inhibit the function of effector cells, secretion of granzyme and perforin to kill effector cells and interfering with the metabolic function of effector cells [22,23].

In addition to Tregs, MDSCs also play a role of immune suppression in the TME. MDSCs are a group of myeloid derived heterogeneous cells, with $Gr\text{-}1^+CD11b^+$ as the main surface marker. Studies show their central role in immune suppression in both tumor-bearing mouse models as well as cancer patients [24]. These cells can inhibit the function of T-cells by up-regulating the expression of arginine 1 and nitric oxide synthase [25]. MDSCs can further be differentiated into tumor associated macrophages (TAMs), secrete Th-2 type cytokines, promote tumor angiogenesis and tumor metastasis, and promote tumor cells to escape immune surveillance. In addition, such Th-2 type cytokine secretion is known to facilitate angiogenesis and tumor metastasis [26].

2.2 Cytokine-mediated immune suppression

In the TME, both tumor cells and stromal cells secrete a variety of cytokines (including IL-6, IL-10, TGF-β and VEGF), among which IL-10 and TGF-β are the most significant [27]. The expression of IL-10 is significantly increased in almost all malignant tumors, and its immunosuppressive function is recognized through prevention of DC-mediated differentiation of $CD8^+$ T-cells into CTLs, as well as in inhibiting the function of antigen presenting cells (APC) directly [28].

Further, TGF-β plays a dual role in promoting and inhibiting cell proliferation. In the systemic immune system, it can induce immune tolerance by inhibiting the expression of MHC antigen and interfering with the differentiation and maturation of DCs [29]. In the microenvironment of tumor tissue, TGF-β exerts non-specific effector inhibition across T-cells, macrophages, natural killer cells, and the like. In addition, TGF-β can induce the amplification of Tregs, and the amplification of Tregs can further secrete TGF-β, thus forming a vicious cycle of immune suppression in the TME [30].

2.3 Enzyme-mediated immune suppression

Indoleamine-2,3-dioxygenase (IDO) is a metabolic enzyme, rate-limiting in tryptophan metabolism, which is directly related to tumor immune suppression [31]. IDO is mainly secreted by stromal cells in the TME, such as endothelial cells, macrophages, and dendritic cells. Studies show that IDO can induce immune tolerance in TME through several mechanisms. Overexpression of IDO results in depletion of tryptophan, which limits the capacity for T-cell clonal expansion. Secondly, the toxic metabolites of tryptophan, L-kynurenine and picolinic acid, can directly inhibit T-cell activation, and even induce the apoptosis of T-cells. In addition, IDO can inhibit the immune function of activated T-cells by inducing the proliferation of Tregs [32]. Recently, IDO has become one of the most important regulated target for antitumor immunotherapy. There are 3 IDO inhibitors into clinical research, including NewLink Genetics's indoximod and NLG919, Incyte's INCB024360 [33] .

2.4 Ligand-mediated immune suppression

Limitations in T-cell activation at the cellular level also can function to limit overall vaccine response. The activation of T-cells requires two signals from APCs functioning in concert to elicit the desired response. The first comes from the combination of the antigen peptide-MHC complex and the T-cell surface receptor, and the second comes from the binding of the co-stimulatory molecules of the APCs with

their corresponding receptors on the T-cell surface [34]. A lack of co-stimulant signaling can prevent or elicit incomplete activation, and induce a state of incompetence or apoptosis, thereby limiting the potential to facilitate a CTL response. The binding between B7 family molecules on the APC and CD28 family molecules of T-cells provides both positive and negative co-stimulatory signals, which will balance the immune response to a foreign antigen and immune tolerance to self-antigen. T-cells can express a class of inhibitory receptors, such as CTLA-4, which can negatively regulate the function of T-cells, and inhibit the function of T-cells after binding with B7 [35].

Recently, it was discovered that members of the CD28-B7 family, programmed death-1 (PD-1) and its ligands PD-L1 and PD-L2 play an important role in negative immune regulation [36]. PD-1 is an immune inhibitory receptor by means of interaction with its ligand, thereby playing a negative role in the immune response. The combination of PD-1 and PD-L2/PD-L1 can selectively stimulate the IL-10-secreting T-cell subsets, which leads to down-regulation of cellular immune function and facilitates T-cell apoptosis [37]. Further, PD-L1 can up-regulate the expression of the anti-CD3 monoclonal antibody-activated Fas and FasL on the T-cells, promoting the secretion of IL-10 and ultimately leading to the apoptosis of specific CTL populations [38]. Moreover, PD-L1 can affect the differentiation, development and maturation of dendritic cells and their effects on specific CTL induction, while up-regulation of PD-L1 expression on myeloid DCs in TME can inhibit the immune response of T-cells as well [39]. Therefore, PD1/PD-L1 pathway inhibitor (known as "checkpoint blocker") can restore the activity of T-cells and enhanced immune response through inhibiting the combination of PD1 and PD-L1 and blocking the negative regulatory signals [40].

3 Nanovaccine based combined immunotherapy

Given the fundamental importance of the TME in mediating immune-suppressive effects, one would envision an ideal therapeutic strategy to be two-fold: to design a potent cancer vaccine which can stimulate effective CTL activation against a target tumor antigen, while concomitantly delivering therapeutic agents which can modulate the microenvironment to support effector cell function. Over the past half-decade, our lab has explored these concepts through our lipid-coated calcium phosphate (LCP) nanoparticle platform, which is capable of stably encapsulating a variety of therapeutic materials, delivering them to cells of interest in a targeted fashion, and releasing their contents into the cytoplasm in a pH-dependent and cationic lipid-mediated mechanism. In the next sections, we will outline both the synthesis of such nanoparticles and the therapies employed through which to enhance CTL function in cancer vaccine therapy through such a combination approach.

3.1 Construction of nanovaccine

After internalization into cells by endocytosis, the nanoparticles will be included in the early endosomes, which will eventually progress toward late lysosomes [41,42]. The lysosomal environment is acidic and contains a large number of enzymes, which can lead to drug degradation or degeneration, and reduce drug efficacy. Proton pumps on endosomes membrane serve to maintain stable pH in endosomes [43]. Proton absorption and buffering materials possess the function of increasing thechanging osmotic pressure of endosomes due to counterion influx, which will swell and destroy the endosomes to release drug into the cytoplasm, thus improving therapeutic bioavailability for a wide array of intracellular functions. Therefore there are clinical situations in which novel formulations with capability of effective delivering vaccines and insignificant potential toxicological issues are needed.

Among the possible strategies that have been reported (as seen in Table 1), calcium phosphate nanoparticles (LCP NPs) serves to tackle this specific consideration both *in vitro* and *in vivo* [67,68]. Calcium phosphate is a natural biological material which possesses good biological safety and biodegradability. In addition, CaP is an acid sensitive material, which can itself contribute to osmolarity increases in order to rupture endosomes. CaP is one of the earliest DNA transfection materials [69], employed due to favorable interactions between divalent calcium and the anionic phosphate backbone of DNA in the process of precipitation. When internalized into cells, CaP nanoparticles will be degraded in the endosome and release the loaded cargoes into the cytoplasm with sufficient osmolarity-driven membrane rupture.

Size-controllable CaP nanoparticles were prepared by reverse microemulsion method, obtained by dispersing aqueous solution into an oil phase containing Igepal CO-520 and cyclohexane [68]. Separate solutions of calcium chloride plus therapeutic, and sodium phosphate, were established within respective emulsions. After mixing the two phases, the exchange reaction between the microemulsions produce CaP precipitates on the nanoscale, a process in which such nanoparticles are surface-stabilized with an amphiphilic phospholipid dioleoyl phosphatydic acid (DOPA) (see Fig. 1). It is expected that the CaP core should be coated with DOPA because (1) excess calcium should be available on the core surface, given the high calcium:phosphate conditions present in the combined microemulsion, (2) precipitation with the divalent phosphate head-group of DOPA is competitive with the core precipitate, and (3) the amphiphilic DOPA is expected to locate preferentially at the microemulsion interface, creating structural uniformity in precipitation and encap-

Table 1 Various delivery systems for therapeutic tumor vaccines

Vehicles	Payload	Tumor model	Ref.
Liposome			
DOTAP/DOPE	HER-2/CpG	TUBO	[44]
DOPC/DOPE/DOPG	TERT peptide	Murine lymphoma RMA-HHD	[45]
DPPC/Man3-DPPE	OVA	E.G7-thymoma OVA	[46,47]
PC/DOTAP/DOPE	Trp2 peptide/α-GalCer	Murine B16F10 melanoma	[48,49]
Lipid nanoparticles			
Lipid-calcium-phosphate (LCP)	Trp2 peptide/CpG	Murine B16F10 melanoma	[50,51]
Liposome-protamine-DNA (LPD)	E7 peptide/CpG	Murine TC-1 lung endothelial	[52,53]
Liposome-KALA-DNA (MEND)	OVA	E.G7-thymoma OVA	[54,55]
Lipid-zinc phosphate	MPLA/Trp2	Murine B16F10 melanoma	[56]
Lipid-67Ga-magnetite	OVA/CpG	Murine B16F10-OVA melanoma	[57]
Polymeric nanoparticles			
PLGA	Hgp100/Trp2	Murine B16 melanoma	[58]
PLGA	mSTEAP peptide	Murine TRAMP-C2 prostate	[59]
PLGA	OVA/STAT3 siRNA	EG7-OVA thymoma	[60]
Erythrocyte membrane-enveloped PLGA	MPLA	Murine B16F10 melanoma	[61]
PLGA/PEG-b-PLGA/EG-b-PCLi	Melan-A/Hgp100	Murine B16F10 melanoma	[62]
Micelle			
PEG-b-P[Asp(DET)] + P[Asp(DET)]	pDNA encoding SART3/CD40L/GM-CSF	Murine CT-26 colorectal carcinoma	[63,64]
PEG-PLL-PLLeu	PIC/SART3 siRNA/OVA	OVA-transfected B16 melanoma	[65,66]

sulation. After demulsification with ethanol and purification by centrifugation in chloroform, additional lipids such as 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl]-3-trimethylammonium propane DOTAP), 1,2-dioleoyl-(sn-glycero-3-phosphocholine (DOPC), Cholesterol and 1,2-distearoyl-sn-glycero-3-phospho ethanolamine-*N*-[amino (polyethylene glycol)-2000] (DSPE-PEG2000) in chloroform were added to form an asymmetric bilayer around CaP cores. The synthesis route of LCP NPs is shown in Fig. 1.

3.2 Design strategy of nanovaccine for cancer immunotherapy

A variety of strategies have been reported that can be used to rebuild the tumor microenvironment to render it less immunosuppressive and enable improved vaccine potency (Table 2).

Previous studies in our lab used a potent mannose-modified LCP nanoparticle-based vaccine containing both tumor-specific antigen and adjuvant, to activate the DCs (Fig. 2). It also generated a strong *in vivo* CTL response against the poorly immunogenic self-antigen tyrosinase-related protein 2 (Trp2) through an *N*-terminal diphosphoserine-modified peptide (p-Trp2), co-delivered with CpG oligo as adjuvant. This ultimately resulted in a potent antitumor immune response against the Trp2 expressing melanoma [50]. Previous studies also indicated that Trp2 LCP vaccination results in potent growth inhibition of a B16F10 melanoma model in both early and late stages [51].

While such a vaccine still could stimulate a strong

Table 2 Various combination strategies for improving cancer immunotherapy

Strategies	TME remodeler	Vaccine	Ref.
Blocking immune checkpoints	Anti-CTLA-4/Anti-PD-1	Flt3L peptide	[70]
Blocking CCL2 recruitment	Anti-murine CCL2/CCL12	HPV-E7 peptide	[71]
Blocking MDSC differentiation	All-trans retinoic acids	C3 tumor-specific peptide	[72]
Inhibiting IDO	2,3-Dioxygenase 1-methyl tryptophan	Ret peptide	[73]
Blocking immunosuppressive cytokine	STAT-3 inhibitor	HER-2 DNA vaccine	[74]

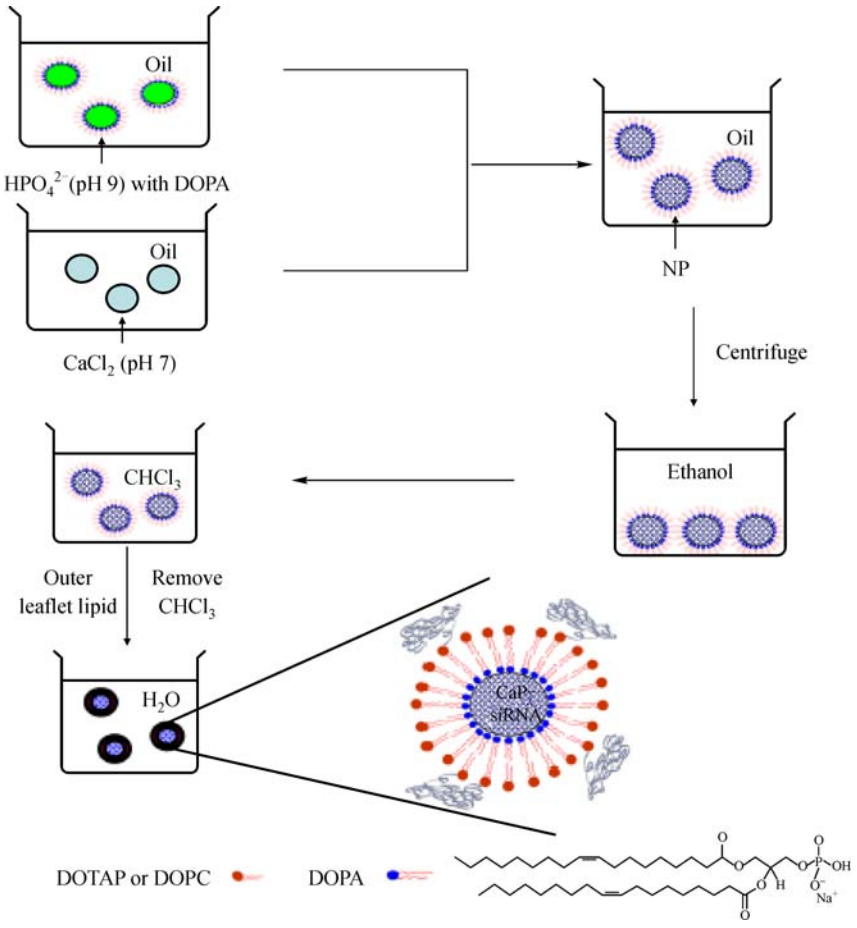

Fig. 1 Synthesis route of LCP NPs for antigen peptide loading. Reproduced with permission from Li et al. [68]. Copyright 2012 Elsevier

cytotoxic T-cell response once the tumor progressed to a late stage, it was not able to inhibit such a large tumor's growth effectively, in large part due to the suppressive TME present in late stage tumors. Thus, to remodel the microenvironment, it is also necessary to get help from a drug that could play pivotal roles not only in killing tumor cells but also in dealing with the immune environment such as reducing immunosuppressive cytokines in the TME.

As discussed earlier, TGF-β represents an influential and multifaceted signal within the TME, whose down-regulation can support enhanced effector cell function. To target

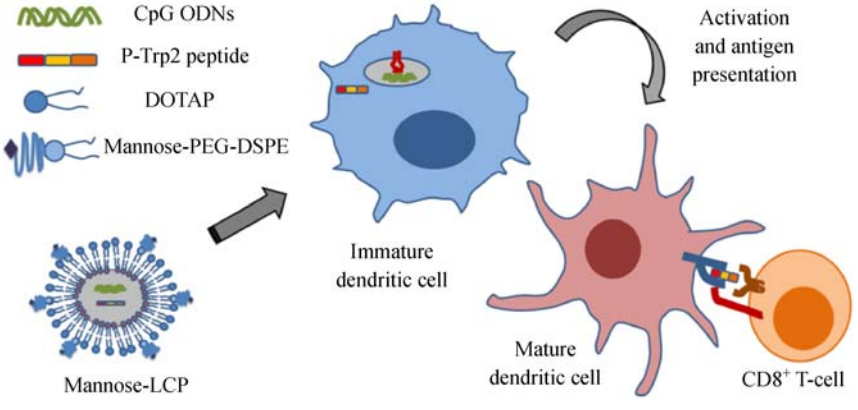

Fig. 2 Design strategy and characterization of mannose-LCP NP-based vaccine. LCP encapsulating the modified melanoma specific antigen (p-Trp2 peptide) and a potent adjuvant (CpG ODN) is designed for an efficient antigen-loading and activation of DCs. Reproduced with permission from Xu et al. [50]. Copyright 2013 Elsevier

the TME, another core-membrane type NP, i.e., lipid-protamine-hyaluronic acid (LPH) or its equivalent lipid-protamine-DNA (LPD), was often used [75]. The delivery of siRNA using LPH NP resulted in about 50% knock-down of TGF-β in the late stage TME. TGF-β down-regulation boosted the vaccine efficacy and inhibited tumor growth by 52% compared with vaccine treatment alone, as a result of increased levels of tumor infiltrating $CD8^+$ T-cells and decreased level of Tregs [51]. This combination therapy strategy is illustrated in Fig. 3.

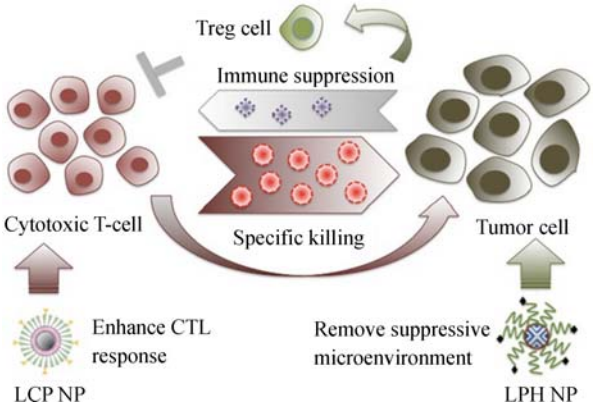

Fig. 3 Strategies to improve the efficacy of LCP vaccine by combination of systemic induction of antigen-specific immune response with LCP vaccine and targeted modification of TME with siRNA. Reproduced with permission from Xu et al. [51]. Copyright 2014 American Chemical Society

Another strategy involves inhibition of the STAT3 signaling pathway. Studies have shown that STAT3 (signal transducer and activator of transcription factor 3) is an important transcription factor that mediates immune suppression [76]. As a bifunctional cytoplasmic protein, STAT3 is a collection point of EGFR, IL-6/JAK, Src, and other oncogenic tyrosine kinase signaling pathways, showing persistent activation in most tumor cells [77]. In addition to the regulation of cell apoptosis-related gene expression, the transcription factor participates in the regulation of immune suppression in the TME in many aspects, such as inducing activation and amplification of immune-suppressive cells, up-regulating the expression of immune suppressive cytokines [76,78]. Therefore, blocking the STAT3 signaling pathway provides an important target to reverse the immune suppression effect of the TME [79].

Curcumin is a component of turmeric and one of the oldest natural polyphenols in traditional Asian medicine [80]. As a well-known JAK-STAT3 pathway inhibitor, curcumin is shown to inhibit MDSCs in the spleen and tumor tissue in breast cancer models and 3LL Lewis lung cancer models [81,82]. As such, curcumin-PEG conjugate (CUR-PEG), an amphiphilic curcumin-based micelle, was delivered intravenously to the tumor to support micro-environment remodulation in concert with the LCP vaccine. The combination therapy dramatically down regulated the STAT3 pathway and significantly boosted *in vivo* CTL response and IFN-γ production in the B16F10 tumor-bearing mice. CUR-PEG and LCP vaccine combination therapy resulted in an enhanced inhibition of MDSCs and Tregs, an elevation in the $CD8^+$ T-cell population, and a distinct M2 to M1 macrophage phenotype switch [83].

Anti-inflammatory triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) is another STAT3 blocker employed in our lab's work [84]. We investigated the delivery of CDDO-Me targeting the TME for an effective treatment against advanced stage tumors [85]. Intravenous delivery of CDDO-Me using a poly-lacticglycolic-acid NP combination with subcutaneous Trp2 vaccine resulted in an increased antitumor efficacy and apoptosis within tumor tissue than Trp2 vaccine alone in B16F10 melanoma. There was a significant decrease of both Tregs and MDSCs and a concomitant increase in the CTL infiltration in the TME of the vaccinated animals. Also, CDDO-Me remodeled the tumor associated fibroblasts, collagen and microvessels in TME, while enhancing the Fas signaling pathway which could sensitize the tumor cells for CTL-mediated killing.

4 Conclusions and outlook

Given the limited successes of cancer vaccines in clinical development and the potent synergies which can be exploited therein, remodeling the TME to better support antigen-specific CTL function represents a valuable avenue within tumor immunotherapy development. The LCP-based platform represents a strong mechanism for antigen delivery as well as combination therapy, rendering it a powerful tool for reengineering of the tumor immune microenvironment for boosting immune therapy.

At present, with the rapid advances in the study of the mechanism on immune tolerance, multiple clinical studies of malignant tumors by overcoming the various immune suppression mechanisms have been launched. It is believed that in the near future, tumor immunotherapy will prove to be a strategy which truly exhibits strong specificity, fewer side effects, and improved therapeutic efficacy.

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