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# Tackling TAMs for Cancer Immunotherapy: It's Nano Time!

**Yishun Yang**[a,b], **Jianfeng Guo**[a,c,*], **Leaf Huang**[a,*]

[a]Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

[b]Experiment Centre of Teaching and Learning, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

[c]School of Pharmaceutical Sciences, Jilin University, Changchun 130021, China

## Abstract

The tumor microenvironment (TME) is characterized as a highly complex environment surrounding tumors. The interactions between cancer cells/non-cancerous cells and cells/non-cell components within the TME create a supporting soil for tumor initiation, development, and metastasis. Among the cell types, tumor-associated macrophages (TAMs) have gained attention due to crucial roles in supporting tumors and conferring therapy resistance. Recent developments in nanotechnology raise opportunities for the application of nano targeted drug delivery systems (Nano-TDDS) in cancer therapy. Here we focus our discussion on the current knowledge of TAMs, and describe recent examples of Nano-TDDS-based TAM modulation, highlighting the formulation design for conquering *in vivo* delivery barriers associated with the TME and the potential of these strategies for clinical translation.

### Keywords

tumor microenvironment; tumor-associated macrophages; nanoparticles; drug delivery; translational research

## Tumor-associated macrophages as therapeutic targets in cancer

The components of tumor microenvironment (TME) mainly include [1]: 1) different cell types such as tumor cells, immune cells, and stromal cells [e.g. **fibroblasts** (see Glossary), **endothelial cells** (**ECs**), and **pericytes**]; 2) soluble signaling molecules (e.g. cytokines, chemokines, and growth factors); 3) the **extracellular matrix (ECM)**; 4) blood vessels; and 5) the hypoxic and acidic environments. Conventional therapies such as surgery, radiation therapy, chemotherapy and targeted therapy (e.g. small molecule inhibitors) aim to target

*Correspondence: leafh@email.unc.edu (L. Huang), jguo@jlu.edu.cn (J. Guo).



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tumor cells, whereas immunotherapy as the fifth pillar of cancer treatment modulates immune responses against tumors [2] Although these therapeutic strategies have generated significant progress, clinical outcomes are still far from satisfactory, which mainly result from the structural and physiological hurdles caused by the TME [3]. For example, the dense ECM and tortuous blood vessels hinder the trafficking of therapeutic agents to tumor cells [4]. Although therapeutic agents penetrate into tumors, they are often inactivated inside the hypoxic/acidic environments [5]. Additionally, the crosstalk between different cell types via the production of soluble signaling mediators leads to tumor **immune escape**; as a consequence, the immunosuppressive TME causes dramatic resistance to currently available immune-based therapies [6]. Therefore, understanding the complexity and diversity of TME is critically important as the knowledge will inspire the development of therapeutic modalities with enhanced therapeutic efficacy and reduced side effects.

Increased knowledge of cancer biology and the TME has revealed the role of tumor-associated macrophages (TAMs, a population of immune cells within the TME) in contributing to tumor initiation, development, and metastasis [7]. TAMs are generally considered “pro-tumoral macrophages”, and a plethora of such macrophages correlates with the adverse prognosis in patients with melanoma, renal cell carcinoma, and breast, ovarian, bladder, gastrointestinal, or prostate cancers [8]. Therapeutic strategies that deplete and/or modulate TAMs have demonstrated great promises in relieving the harsh TME and complementing current immunotherapies [9]. Recent developments in the fields of biotechnology and nanomedicine have advanced nano targeted drug delivery systems (Nano-TDDS) that work in accordance with the physical, biochemical and physiological environments of disease sites [10]. Indeed, Nano-TDDS designed for targeting the TME are anticipated to revolutionize cancer treatment [11]. In this review, the roles of TAMs in regulating the TME are described, and recent examples of Nano-TDDS-mediated TAM modulation are discussed, with particular focus on those developed to overcome *in vivo* delivery barriers associated with the TME and to achieve cancer immunotherapy for clinical benefits.

## TAMs are a key regulator of tumor microenvironment

Granulocytes and monocytes are derived in the bone marrow (BM) from hematopoietic stem cells (HSCs) via granulocyte-macrophage progenitors (GMPs) and monocyte/macrophage and dendritic cell precursors (MDPs). Monocytes are terminally differentiated into macrophages and dendritic cells (DCs) [12]. Macrophages as a heterogeneous cell population are generally categorized into two subtypes namely M1 (classically activated macrophages) and M2 (alternatively activated macrophages) [13]. In addition to significant roles in the innate immunity against invading pathogens, M1 (or M1-like) macrophages are in general considered “anti-tumorigenic” [14]. They produce pro-inflammatory cytokines such as interferon gamma (IFN-γ), interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) and chemokines such as C-X-C motif chemokine ligand 9 (CXCL9) and 10 (CXCL10) for directly killing tumor cells and for indirectly augmenting T-cell mediated antitumor activities [15]. In contrast, M2 (or M2-like) macrophages exert anti-inflammatory and tissue regeneration/wound healing functions. As tumors are known as “wounds that do not heal”, the number of M2 subtype is increased during tumor progression and becomes

dominant in TAMs. As “pro-tumoral macrophages”, M2 cells are accompanied with the production of 1) cytokines (e.g. IL-6 and IL-10), 2) chemokines [ CXCL8 (also known as IL-8) and C-C motif chemokine ligand 2 (CCL2) and 5 (CCL5)], 3) growth factors [transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF)], and 4) signaling mediators [e.g. indoleamine 2,3-dioxygenase 1 (IDO-1), cyclooxygenase type 2 (COX- 2), programmed death-ligand 1 (PD-L1), and arginase-1] [15]. These immunosuppressive products cause resistance to current immunotherapeutics [9]. The modulatory networks associated with the conversion between M1 and M2 (termed macrophage polarization) have been previously summarized [13] [14] [15]. Below we discuss the roles of TAMs as promoters of tumor progression and inhibitors of antitumor immunity in terms of the interactions between TAMs and the other key cell types of TME.

## TAMs and tumor cells

The initiation, proliferation and metastasis of tumor cells are highly associated with TAMs [16] [17]. Cancer is viewed as a disease involving dynamic changes in the genome, and increased mutability is required for initiation of cancer cells [18]. At the beginning of tumor development, TAMs can enhance genetic instability of pre-malignant cells by generating reactive oxygen/nitrogen species (ROS/RNS) (Figure 1) [16]. Aberrant mutations are therefore accumulated for the onset of tumorigenesis, and the elevated mutations also cause the failure of chemotherapy and targeted therapy [16]. When tumor is progressing, TAMs further produce cytokines, growth factors and signaling mediators to stimulate cancer cells that have acquired sufficient mutations, which promote tumor growth and metastasis (Figure 1) [17]. For example, TAMs release IL6/IL-10 for the induction of signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) pathways in cancer cells, causing the inhibition of apoptosis and the increase of cell cycle progression [17]. Moreover, TAMs secrete IL-1β, CXCL8 and TGF-β to drive **epithelial-mesenchymal transition (EMT)** of tumor cells, leading to tumor invasion and metastasis [19].

## TAMs and immune cells

As discussed earlier, the nature of TAMs is shifted during tumor development from M1 to M2, contributing to the immunosuppressive TME via the maintenance of a vicious cycle, in which anti-inflammatory cells such as **myeloid-derived suppressor cells (MDSCs)** and **regulatory T cells (Tregs)** are upregulated, but pro-inflammatory cells such as **cytotoxic T lymphocytes (CTLs)** are downregulated. As one of the primary TAM precursors, the subpopulation of MDSCs namely M-MDSCs differentiates into M2 macrophages at the tumor site under the stimulation of cytokines (e.g. IL-4, IL-13, IL-21, and IL-33) [20]. Reciprocally, TAMs produce chemokines (e.g. CCL2 and CCL5) to recruit the infiltration of MDSCs to tumors [20]. In addition, the chemokines derived from TAMs can recruit Tregs into tumor sites, in which TAMs exert the immunoinhibitory activity of Tregs via the release of IL-10, TGF-β, and prostaglandin E2 (PGE2), consequently suppressing CTL-mediated antitumor immunity (Figure 1) [21]. It is known that CTLs are the central effectors for antitumor immunity, but their expansion and action are significantly prohibited within the TME. CTLs are turned into the “exhausted” state (e.g. decreased proliferation and reduced cytotoxic mediators) under the stimulation of immunosuppressive factors (e.g. IDO-1, COX-

2, PD-L1, and arginase-1), and the upregulation of these immunosuppressive factors is highly associated with TAMs [22]. The roles of TAMs as immunosuppressive regulators in tumor immunity and immunotherapy have been further described elsewhere [7] [8].

## TAMs and tumor-associated fibroblasts (TAFs)

As the primary source of ECM components, fibroblasts play crucial roles in wound healing and scar formation [23]. At the site of wounds, fibroblasts become “activated”, leading to a faster proliferation and the secretion of ECM components at higher levels than the “resting” counterparts of healthy tissues [23]. Fibroblasts remain constantly activated in tumors where “wounds do not heal”, and such “activated” fibroblasts have been termed tumor-associated fibroblasts (TAFs). As the predominant stromal cell type within the TME, TAFs impart strong influences on tumor progression, immunosuppression and resistance to therapy, which have been recently reviewed elsewhere [24]. Increasing evidence indicates that TAMs and TAFs interact mutually as the conspirators in facilitating tumor progression [24] [25]. For example, TAMs produce growth factors (e.g. TGF-β, PDGF, and FGF) to stimulate the activation of TAFs [23]. Reciprocally, TAFs promote the infiltration of monocytes to tumors and their differentiation towards M2 phenotype by the secretion of signaling mediators [e.g. TGF-β, VEGF, colony-stimulating factor 1 (CSF-1), IL-6, CXCL8, and IL-1β] [26].

The changes in tumor ECM are accompanied with the transformation of normal tissues to tumors, which can be regulated by bi-directional TAM-TAF signaling pathways (Figure 1). It has been reported that tumor ECM is generally stiffer than the counterparts in healthy tissues, which cause chemoresistance to tumor cells [25]. The tumor ECM stiffness is often correlated with the amount of TAMs and TAFs, and the formation of stiffer tumor ECM are mainly due to the fact that TAMs maintain the activated status of TAFs for the induction of desmoplastic reaction (e.g. high levels of collagen Type I) and the generation of a dense fibrotic stroma wrapping the tumors [24]. When the tumor is developing, TAMs further produce proteases to remodel ECM for tumor angiogenesis (see discussion below).

## TAMs and vascular cells

As tumors grow and become metastatic, malignant cells require adequate oxygen supply, which is mainly achieved through **angiogenesis** [27]. TAMs can modulate vascular cells [e.g. ECs and pericytes] for the activation of quiescent vasculature [28]. For example, TAMs generate pro-angiogenic mediators including growth factors (e.g. VEGF, FGF, and the members of the WNT family) and cytokines (e.g. IL-1β, IL-6, and TNF-α), which can support the survival, proliferation and activation of ECs for promoting angiogenesis in favor of tumor growth (Figure 1) [29]. It has also been reported that angiogenic ECs stimulated by TAMs can upregulate the autocrine angiopoietin 2 (ANGPT2) signaling pathway, which in turn loosens the association of pericytes with blood vessels, leading to the vascular leakage in tumors [30]. Consequently, tumor blood vessels become more tortuous and leakier, facilitating the intravasation of tumor cells from the primary site into the blood circulation and eventually the metastatic spread [31]. In addition, TAMs account for the development of lymphatic vessels (lymphangiogenesis) that is another important route for tumor cells to disseminate into regional lymph nodes and distant metastasis [32].

Hypoxia is a hallmark feature of solid tumors and caused by rapidly growing malignant cells and poorly organized vasculature [32]. Tumor hypoxia is associated with the development of heterogeneous TME characterized by variable oxygen concentrations, reduced pH, and increased ROS. This hypoxic heterogeneity furthers tumor progression using a variety of pathways, such as the induction of immune escape, promotion of glycolysis, suppression of apoptosis, and resistance to therapy [33]. Hypoxia also functions critically in the regulation of TAMs. For example, TAMs migrate into hypoxic regions of the tumor following the stimulation of hypoxia-inducible factors such as CXCL12, endothelial cell monocyte-activating polypeptide-II (EMAP-II), endothelin 2, VEGF-A, and Semaphorin 3A [34]. Hypoxic TAMs upregulate pro-angiogenic and immunosuppressive cytokines to promote tumor angiogenesis, metastasis, and immune evasion [35]. Besides, hypoxia may regulate the polarization of TAMs towards pro-tumoral (M2) phenotype [36]. For example, lactate, one of the key inducers of M2 phenotype, is extensively secreted by anaerobic glycolysis of tumor cells in the hypoxic area [37]. However, opposite evidence indicates that hypoxia cannot significantly affect the expression of M2 markers or the amount of M2 macrophages [38], suggesting that hypoxia may not be the major driver of TAM polarization.

## Opportunities and challenges associated with targeting TAMs

As described above, TAMs govern the TME by cell-cell contact, and produce immunoinhibitory and pro-tumorigenic cytokines, chemokines, growth factors and proteases for the promotion of tumor growth, angiogenesis, and metastasis (Figure 1). Emerging evidence has also indicated that a high TAM prevalence is associated with poor prognosis in many solid tumors [8]. Therefore, TAMs have been identified as promising targets in oncology, and therapeutic modalities that aim to eliminate TAMs, inhibit infiltration of TAMs and/or activate the polarization of TAMs towards M1 phenotype have demonstrated great potential for clinical application (the active clinical trials of TAM-targeting agents for solid tumors has been summarized in [9]).

However, despite the progress, to date none of them have reached the clinic for patients. The identification and characterization of TAMs remain one of main hurdles for these TAM-targeting agents. Different macrophages (e.g. M1 subtype vs. M2 subtype, and locally proliferating macrophages vs. systemically recruited macrophages) coexist inside tumors at various stages of tumor progression [39], which significantly limit the specificity of therapeutic agents (particularly for those developed to deplete M2 subpopulation). In addition, *in vivo* delivery barriers (e.g. low drug solubility, short half-life, nonspecific biodistribution and poor cellular uptake) and the immunosuppressive TME dampen the efficacy of TAM-targeting agents [10]. Recently, drug formulations designed by exploiting the properties and functions of nanoparticles (NPs) have significantly ameliorated *in vivo* delivery hurdles (see discussion below) [40]. Nanoformulations have also been utilized for the modulation of TME, relieving harsh niches associated with the failure of drug delivery [41]. With increased knowledge of cell surface antigens/biomarkers for TAMs [42], “off-target” issue may be addressed using nanoformulations that have the specific targeting ligand for cell-specific transport. Below we highlight the advances made in the last decade on the NP designs used in Nano-TDDS with representative examples, give an overview of

Nano-TDDS designs geared towards overcoming *in vivo* delivery barriers, and review recent advances of these systems for delivery of therapeutic agents specifically to TAMs.

## Nano-TDDS to target TAMs for cancer immunotherapy

### Evolution of NPs for use in TAM-targeting Nano-TDDS

In the past decade, the development of Nano-TDDS based on a variety of nanomaterials (e.g. lipids/liposomes, polymeric NPs, inorganic NPs and biological/natural carrier mimics) has profoundly revolutionized the field of TAM-associated cancer immunotherapy (Figure 2A). Below we highlight representative examples of these NPs from the last decade, that have been developed to be incorporated in to Nano-TDDS. In 2008, it was reported that a polyethylene glycol (PEG)-modified liposomal NP was prepared to encapsulate dichloromethylene bisphosphonate (clodronate, a drug used to treat hypercalcemia associated with cancer) (Figure 2A) [43]. The liposome-clodronate could be recognized as foreign particles by phagocytic cells (e.g. macrophages); following cellular uptake, clodronate was released from NPs, causing the apoptosis of macrophages [44]. The intravenous (i.v.) administration of liposome-clodronate significantly suppressed TAMs in subcutaneous melanoma mouse model, achieving strong inhibitory effect of tumor growth [43]. In 2012, Zhang and colleagues produced a galactosylate-conjugated positively charged dextran (Gal-dextran) that could electrostatically bind anti-IL10 and anti-IL10RA oligodeoxynucleotides (ODNs), forming Gal-dextran-ODN (Figure 2A) [45]. Subsequently, Gal-dextran-ODN was packaged within pH-triggered PEG-histidine-aliginate (PHA) to form PHA-Gal-dextran-ODN. Following i.v. injection in **orthotopic** hepatocellular carcinoma (HCC) mouse model, PHA-Gal-dextran-ODN was delivered into the tumor site in which Gal-dextran-ODN was released in response to acidic TME. Gal-dextran-ODN was able to target macrophage galactose-type lectin (MGL) overexpressed on TAMs, which facilitated delivery of ODNs into TAMs, altering the phenotype of TAMs for tumor inhibition [45]. In 2016, a mannan (Man)-targeted, hyaluronic acid (HA)-coated manganese dioxide ($MnO_2$) NP was developed for targeting TAMs inside hypoxic area of tumors (Figure 2A) [46]. Man-HA-$MnO_2$ NPs specifically bind to the mannose receptor expressed on TAMs. Following cellular uptake, HA was utilized for reprogramming M2 TAMs to M1 macrophages. $MnO_2$ also reacted with $H_2O_2$ to produce $O_2$ for tumor hypoxia relief [46]. Consequently, i.v. administration of Man-HA-$MnO_2$ NPs achieved significant inhibition of tumor growth in subcutaneous breast cancer mouse model. In 2019, a cancer cell membrane-derived microparticle (MP) was developed to co-deliver $Fe_3O_4$ NPs and liposome-CpG NPs (Figure 2A) [47]. Following phagocytosis of MP-$Fe_3O_4$-Lipo/CpG by DCs, tumor cell antigens on the MP significantly induced strong antigen-specific immunological response along with CpG [**toll-like receptor** 9 (TLR9) agonist, is used to improve the vaccine immunity]. In addition, $Fe_3O_4$ was able to reprogram M2 TAMs to M1 phenotype [47]. The injection of MP-$Fe_3O_4$-Lipo/CpG into tail base of melanoma mice significantly altered the immunosuppressive TME for robust antitumor efficacy.

However, despite substantial advances in developing the NPs, the *in vivo* delivery barriers such as low drug solubility, short half-life, nonspecific biodistribution and poor cellular uptake that are associated with TAM-targeting agents still dampen therapeutic outcome.

Recently, an improved understanding of the environments inside the bloodstream and at the tumor site have further advanced the design of “ideal” Nano-TDDS to achieve stable, effective and safe delivery of TAM-targeting agents for cancer immunotherapy.

## Design of Nano-TDDS for overcoming in vivo delivery barriers

TAM-targeting agents mainly include chemotherapeutics, nucleic acids, and proteins/peptides, which have distinct physicochemical features such as molecular weight, solubility, ionization, and surface activity [48]. The delivery efficacy of these therapeutic agents using conventional drug delivery systems (mostly **liposomes**) is severely impeded by low loading capacity of drugs with dissimilar physicochemical properties, short half-life, nonspecific tissue distribution, and poor cellular uptake and trafficking [10]. Nanomaterials including polymeric NPs, inorganic NPs and natural (biomimetic) carriers have been used in Nano-TDDS as these may increase the loading capacity of drugs with distinct physicochemical features (see [40] for more details) (Figure 2B). Furthermore, Nano-TDDS based on certain nanomaterials (e.g. gold and iron) have highly sensitive optical/electronic properties therefore, they can achieve drug release in a spatiotemporal manner following the stimulation of the light with specific wavelengths [49]. Moreover, it is known that the particle size of NPs is critical for physiological stability following systemic administration [49]. Generally, NPs with a particle size of < 10 nm are subject to renal infiltration; while NPs with a particle size of > 10 nm may reduce the clearance from the kidneys, larger NPs (e.g. > 100 nm) can provoke the reticuloendothelial system (RES, also known as the mononuclear phagocyte system, a part of the immune system) towards rapid clearance by the immune system [49]. In addition, NPs with particle size of < 200 nm may passively penetrate into tumor area via the **enhanced permeability and retention (EPR) effect** [50]. Therefore, an “ideal” particle size is generally considered ~ 100 nm (Figure 2B).

NPs can be modified with stabilizing groups (e.g. PEG) to improve pharmacokinetic profiles [51]. Although PEGylated NPs enhances physiological stability and improves biocompatibility, PEG also reduces cellular uptake of NPs [52]. To address this issue, the targeting ligand(s), which have high affinity with antigens/biomarkers overexpressed on tumors, can be conjugated onto PEG to achieve ligand-receptor-mediated cellular uptake (endocytosis) [53]. The Nano-TDDS can also be modified with bioactive/bioresponsive moieties (e.g. ROS-, pH-, and matrix metalloproteinase-sensitive groups) that are in response to changes in the TME for controlled drug release into tumors while sparing healthy tissues (Figure 2B) [54].

Indeed, recently Nano-TDDS have been developed using emerging nanomaterials (e.g. polymeric NPs, inorganic NPs and biomimetic NPs) with the modification of stabilizing groups, targeting ligands and bioreactive/bioresponsive moieties. These multifunctional delivery NPs with increased drug loading capacity, prolonged blood circulation time, cell- and tissue-specific drug delivery, and controlled drug release within the tumor site, demonstrate great promise [55] [56].

## Emerging Nano-TDDS in delivery of TAM-targeting agents

Recent *in vivo* studies using emerging Nano-TDDS for delivery of TAM-targeting agents for depletion and/or modulation of TAMs can be classified into two broad mechanisms namely 1) inhibition of TAM survival and recruitment and 2) improvement of TAM polarization. These are described in Table 1 and selected examples are discussed below.

### Inhibition of TAM survival and recruitment

Due to “pro-tumoral” roles of M2 macrophages, therapeutic agents against the survival and recruitment of TAMs are promising. It has been reported that a M2 macrophage-targeting peptide (M2pep) demonstrates higher specificity to M2-like macrophages than other leukocytes [57]. In addition, apolipoprotein A1 (ApoA1) has the natural affinity of the scavenger receptor B type 1 (SR-B1) that is highly expressed on M2-like cells [58]. Recently, a novel liposome (termed M2NP) was formulated containing a dual targeting moiety which was achieved by the conjugation of M2pep to the C-terminus of ApoA1-mimetic α-helical peptide through an amino acid linker [59]. The i.v. injection of M2NP generated higher binding affinity to M2-like TAMs than tissue resident macrophages of healthy tissues. Consequently, the suppression of survival signals in M2-like TAMs and the reduction of this cell type were successfully achieved in mice with melanoma using this dual-targeted liposomal NP containing **short interfering RNA** (siRNA; siCD115) for blocking colony stimulating factor-1 receptor (CSF-1R) (Table 1). This approach also significantly evoked the immune responses to slow down tumor growth, which was accompanied with the increase of immunostimulatory cytokines (IL-12 and IFN-γ) and reduction of immunosuppressive cytokines (IL-10 and TGF-β) [59].

It is known that Bruton’s tyrosine kinase (BTK) is overexpressed in TAMs for promoting tumor progression, angiogenesis, and immunosuppression [60]. Moreover, BTK also promotes the recruitment of myeloid cells into tumors, subsequently skewing the polarization of TAMs towards M2 phenotype [61]. Therefore, the inhibition of BTK may prevent TAM infiltration and normalize TAM polarization. Recently, a lipid-based nanocomplex has been developed by self-assembling the egg phosphatidylglycerol (EPG, for amphiphilic structure formation), sialic acid (SA)-octadecanoic acid conjugate (OA, for amphiphilic structure formation), and Ibrutinib (IBR, a BTK inhibitor) (Table 1) [62].The SA is the N- or O-substituted derivative of neuraminic acid, and has a high affinity with Siglec-1, an endocytic receptor overexpressed on TAMs [62]. Following i.v. administration, the resultant nanocomplex effectively delivered IBR into tumor-infiltrating macrophages via ligand-receptor mediated pathway, which significantly inhibited immunosuppressive cytokine release, reduced angiogenesis, and suppressed tumor growth in animals with sarcoma, without showing significant toxicity [62].

Siglec-1 is not only expressed on TAMs, but also found on peripheral blood monocytes (PBMs) [61]. Therefore, an SA-targeted liposomal NP was produced for delivery of epirubicin (EPI, chemotherapeutic drug) into both TAMs and PBMs (Table 1) [63]. Following i.v. injection, the targeted NP containing EPI could significantly inhibit the survival of TAMs for downregulation of immunosuppressive factors and prevent the recruitment of PBMs into tumors for generation of new TAMs. Consequently, tumor growth

was remarkably suppressed in sarcoma-bearing animals by this therapeutic formulation, without eliciting toxic effects [63].

Recently, a lipoprotein NP (bLP) has been produced for delivery of $DiOC_{18}(7)$ (a photothermal agent) to induce photothermal therapy for reshaping the stromal TME [64]. Under the stimulation of 808 nm laser irradiation on the tumor site, the resultant nanoformulation (D-bLP) profoundly remodeled tumor stroma, which was accompanied with reduction of TAFs and TAMs. Consequently, the efficacy of second-wave treatment (bLP containing mertansine, a chemotherapeutic drug; M-bLP) was significantly enhanced, achieving remarkable suppression of tumor growth and metastasis in breast cancer mouse models [64].

### Improvement of TAM polarization

Therapeutic agents that inhibit M2 polarization and/or enhance M1 activity have also demonstrated potential for reprogramming of TAM polarization [9] [62]. Recently, a β-cyclodextrin NP (CDNP) for delivery of resiquimod (R848, an agonist of TLR7 and TLR8) has been demonstrated in mouse tumor models [65]. The resultant formulation significantly altered the TAMs towards the M1 phenotype, suppressed tumor growth, and protected animals against tumor re-challenge [65]. Furthermore, the antitumor immune responses were improved using the resultant formulation when combined with **anti-programmed cell death protein 1 (anti-PD-1) therapy**, demonstrating the potential of NP-based strategies to reprogram TAMs for cancer immunotherapy [65].

ROS is known as a key modulator in polarization and activation of macrophages, and the polarization from M2 to M1 is highly associated with a high level of ROS [66]. Recently, the galactose-targeted Zinc protoporphyrin IX (ZnPP, a ROS-inducing material) has been developed to increase ROS in cancer cells. ZnPP grafted poly(l-lysine)-b-PEG polypeptide micelles (ZnPP PM) were able to complex Poly I:C (PIC, a TLR3 agonist) via electrostatic interaction( Table 1). Consequently, TAM-targeted delivery of PIC within ZnPP PM increased ROS level, synergistically converting TAMs from M2 to M1 [66].

The polarization of TAMs from M2 to M1 may be achieved by the modulation of TME using Nano-TDDS. For example, tumor cells produce macrophage colony stimulating factor (MCSF) for deforming TAM polarization from M1 to M2 [67]. Tumor cells also express CD47, a “don’t eat me” signal that ligates with signal regulatory protein alpha (SIRPα) receptor on macrophages to suppress phagocytosis [68] [69]. Therefore, Kulkarni and colleagues produced liposomal NPs containing dual inhibitors for blocking CD47-SIRPα and MCSF-CSF-1R pathways [70], which induced effective remodeling of M2 macrophages and superior phagocytic activities, achieving antitumor efficacy in tumor-bearing mice without significant toxicities (Table 1) [70].

Moreover, the elevated interstitial fluid pressure, high density of ECM and disorganized blood vessels significantly limit tumor penetration of Nano-TDDS. Therefore, a liposomal NP containing hydralazine (HDZ, a medication for high blood pressure and heart failure) has been achieved to normalize tumor blood vasculature in advanced desmoplastic melanoma (Table 1) [71]. Following i.v. injection, the HDZ-liposome favorably modulated the vascular

dilation, tumor hypoxia, and tumor permeability, which were accompanied with the TME modulation including the TAM polarization from M2 to M1. Consequently, this strategy significantly improved the therapeutic efficacy of liposomal doxorubicin as the second-wave treatment in mice with tumor over 400 $mm^3$ [71].

It has been reported that the liver metastasis is highly associated with activated hepatic stellate cell (aHSC)-mediated liver fibrosis, and relaxin (RLN, an anti-fibrotic peptide) is capable of deactivating aHSCs and thus resolving liver fibrosis [72]. Recently, an amino ethylanisamide (AEAA)-targeted PEGylated lipid NP (termed LCP) has been produced to specifically deliver the RLN plasmid into cancer cells and aHSCs within the metastatic lesion, for the production of RLN protein (Table 1) [72]. Consequently, the stromal niche in liver metastases was successfully reversed by LCP-mediated expression of RLN, which normalized the polarization of TAMs and improved antitumor immune responses, significantly inhibiting metastatic progression and prolonging animal survival [72].

## Concluding Remarks and Future Perspectives

An improved understanding of cancer biology reveals the role of TAMs in contributing to tumor initiation, development, and metastasis (Figure 1). Therapeutic agents that eliminate TAMs, inhibit infiltration of TAMs and/or activate the polarization of TAMs towards M1 phenotype have demonstrated great potential for clinical application [9]. Recent advances in biotechnology and pharmaceutics have facilitated the development of Nano-TDDS for delivery of TAM-targeting agents (Table 1). However, it is worth noting that none of them have till date been successfully applied for patients (see Outstanding Questions).

The complexity associated with the origin and nature of TAMs tremendously impedes the application of Nano-TDDS. Although certain TAM receptors (e.g. mannose receptor and Siglec-1 receptor) have been reported for development of Nano-TDDS [46] [62], they are also found in other cell types such as the DCs, which can significantly dampen the delivery efficacy of Nano-TDDS. Recent advances in analytical technologies such as high-resolution imaging [73], flow cytometry [74] and next-generation sequencing [75] are anticipated to achieve a comprehensive view of TAMs, which may potentially identify unique TAM receptors (or antigens), allowing the design of Nano-TDDS with novel targeting ligands for enhanced delivery efficacy and reduced side effects. In addition, due to the crosstalk between TAMs and other components inside the TME, Nano-TDDS may be designed for targeting these components (e.g. cancer cells [76], DCs [47], TAFs [64], stroma [72] and vasculature [71]) to indirectly modulate TAMs.

Although the progress of multifunctional Nano-TDDS has been tremendous compared to conventional drug delivery strategies such as liposomes, the modification of nanomaterials with stabilizing groups, targeting ligands and bioactive/bioresponsive moieties may complicate the large-scale and reproducible production of Nano-TDDS, and such extensive modifications may also cause unwanted toxic issues [77] [78]. Therefore, the balance between the therapeutic efficacy, the preparation/scale-up and the toxicity of Nano-TDDS must be taken into account to ensure NP-based TAM-targeting strategies can be successfully applied for patients (see [40] [77] [78] for more details).

Furthermore, it is worth noting that while a range of Nano-TDDS have demonstrated great potential for drug delivery in TAMs in different tumor-bearing models (Table 1), the comparative efficacy has not always translated into the clinic. One of the reasons would be in the choice of the preclinical model. The potential to achieve successful clinical translation can only be evaluated using appropriate preclinical systems. Most of the studies have been performed with subcutaneous (S.C.) tumor models (tumor cells are normally inoculated into the flank of mice, Table 1). However, orthotopic (O.T.) and metastatic (M.T.) tumor models are more clinically-relevant systems since they have similar TME as the original tumors and are considered to more closely resemble the natural tumorigenesis in human [79]. Besides, spontaneous tumour models that arise in genetically engineered mouse models or are established in response to carcinogenic, radiation, or viral stimulation may also closely mimic the clinical situation [80]. Therefore, these clinically-relevant preclinical models are highly recommended for the assessment of Nano-TDDS-based cancer immunotherapy.

Despite these challenges, profound opportunities exist to further advance the concept of Nano-TDDS which provide high loading efficiency, favorable pharmacokinetic profiles, TAM-specific delivery, and TME-controlled drug release. Thus, it can be expected that with rapidly increasing knowledge in cancer immunology, nanomedicine, and animal models of cancer , NP-based TAM therapeutics for either TAM elimination, repolarization or both will revolutionize the field of cancer immunotherapy in the coming years.

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## GLOSSARY

**Angiogenesis**
The development of new blood vessels from a pre-existing vascular network

**Anti-programmed cell death protein 1 (Anti-PD-1) therapy**
Programmed cell death protein 1 (PD-1) is a cell surface receptor expressed on activated T cells. PD-L1, which is overexpressed on tumor cells, binds to PD-1 on activated T cells suppressing T cell-mediated tumor killing. PD-1/PD-L1 inhibitors (anti-PD-1/PD-L1) are immune checkpoint inhibitors targeting PD-1 or PD-L1. They are used to block the PD-1/PD-L1 pathway to treat human cancers

**Cytotoxic T lymphocytes (CTLs)**
Immune cells that play significant roles in host defense against infection by viruses and other pathogens and are capable of directly killing the infected cells. In tumors, CTLs release cytotoxic mediators such as IFN-γ, granzymes and/or perforin to destroy cancer cells

**Endothelial cells (ECs)**
Endothelial cells form the linings of the blood vessels, control the flow of substances and fluid into and out of a tissue

**Enhanced permeability and retention (EPR) effect**

Increased accumulation of macromolecules, such as liposomes, macromolecular drugs, and nanoparticles in tumors much more than they do in normal tissues

**Epithelial-mesenchymal transition (EMT)**
A cellular process whereby cells lose the epithelial features (e.g. cell polarity and cell-cell adhesion) and obtain mesenchymal characteristics (e.g. migration and invasion). It is a "one-way" process taking place through different cellular states. The EMT is related with tumor initiation/invasion/metastasis and drug resistance

**Extracellular matrix (ECM)**
A three-dimensional network of extracellular components (e.g. collagens, enzymes, and glycoproteins) that can structurally and physiologically support neighboring cells

**Fibroblasts**
The predominant cell type of the connective tissue. Fibroblasts regulate the ECM turnover by the production of ECM components (e.g. type I, III and IV collagens and fibronectin) and ECM-degrading proteases [e.g. matrix metalloproteinases (MMPs)]. They also maintain the homeostasis of neighboring epithelial cells by the production of growth factors (e.g. VEGF). During wound repair, they produce ECM to create a scaffold for other effector cells, and generate cytoskeletal components for the contraction of wounds

**Immune escape**
The process by which cancer cells can avoid the immune system by disturbing any of the key T-cell activities (e.g. T-cell generation, T-cell infiltration, and cell killing) that are indispensable to initiate and exert an anticancer immune response

**Liposomes**
Liposomes generally form a water core enwrapped by lipid bilayer(s). In this amphiphilic structure, the core may encapsulate the water-soluble (hydrophilic) drugs, and the water-insoluble (hydrophobic) drugs may be entrapped inside lipid bilayer(s). Cationic liposomes that are formed using lipids with positively charged groups may also bind nucleic acids via electrostatic interaction

**Myeloid-derived suppressor cells (MDSCs)**
The nature of monocytes and granulocytes (mostly neutrophils) is deformed under cancerous conditions, demonstrating immature phenotype and morphology, ineffective phagocytic activity, and high expression of anti-inflammatory cytokines. As a consequence, these aberrant cells are proliferated and converted to MDSCs. There are two main subpopulations namely monocytic (M-) and polymorphonuclear (PMN-) MDSCs. M-MDSCs are phenotypically and morphologically similar to monocytes and may differentiate into M2 macrophages in tumors

**Orthotopic**
Orthotopic tumor models involve the seeding of tumor cells into the relevant organ of tumor origin in animal models

**Pericytes**

Pericytes are cells that enwrap the endothelial cells. They interact externally with the wall of capillaries, and promote the survival of ECs but limit their proliferation at quiescent vessels. They also stabilize the EC junction to limit vascular permeability under normal conditions

**Regulatory T cells (Tregs)**
Immune cells that regulate the proliferation and activation of T and B cells for the maintenance of periphery tolerance (the second branch of immunological tolerance after central tolerance, preventing autoimmune diseases). In tumors, these cells function as the inhibitor of anticancer immune responses

**Short interfering RNA (siRNA)**
19–25 base pairs long double-stranded non-coding RNA that plays a key role in the RNA interference pathway

**Toll-like receptors (TLRs)**
Transmembrane receptors that play a crucial role in the activation of innate immunity due to their ability to recognize pathogen-associated molecular patterns. TLRs have been recognized as key factors involved in tumor pathogenesis, regulating both tumor cells and tumor-infiltrating immune cells

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### Outstanding Questions

- How can we identify the diversity of TAMs in or between primary sites/metastases and in or between patients?
- How do we design Nano-TDDS to facilitate targeted delivery of TAM-targeting agents?
- Is it possible to develop Nano-TDDS for remodeling the TME as an alternative strategy to facilitate depletion and/or polarization of TAMs?
- Is excessive modifications to achieve multifunctional Nano-TDDS always necessary for the development of NP-based TAM-targeting strategies?
- How do we choose the appropriate preclinical models for Nano-TDDS-based cancer immunotherapy?

## Highlights

- Tumor-associated macrophages (TAMs) have been identified as promising targets in oncology due to their roles within the tumor microenvironment (TME) as promoters of tumor progression and inhibitors of antitumor immunity.
- Although therapeutic agents that eliminate TAMs, inhibit recruitment of TAMs and/or normalize the polarization of TAMs demonstrate great potential for clinical application, the efficacy is still limited.
- One of the major barriers to clinical translation is the paucity of efficient and safe approaches to exert the delivery of TAM-targeting agents into tumors following systemic administration.
- Recent advances in nanotechnology and biomedical engineering provide opportunities for the development of nano targeted drug delivery systems (Nano-TDDS) in the treatment of cancer, which is hoped to overcome *in vivo* delivery barriers associated with TAM-targeting agents.

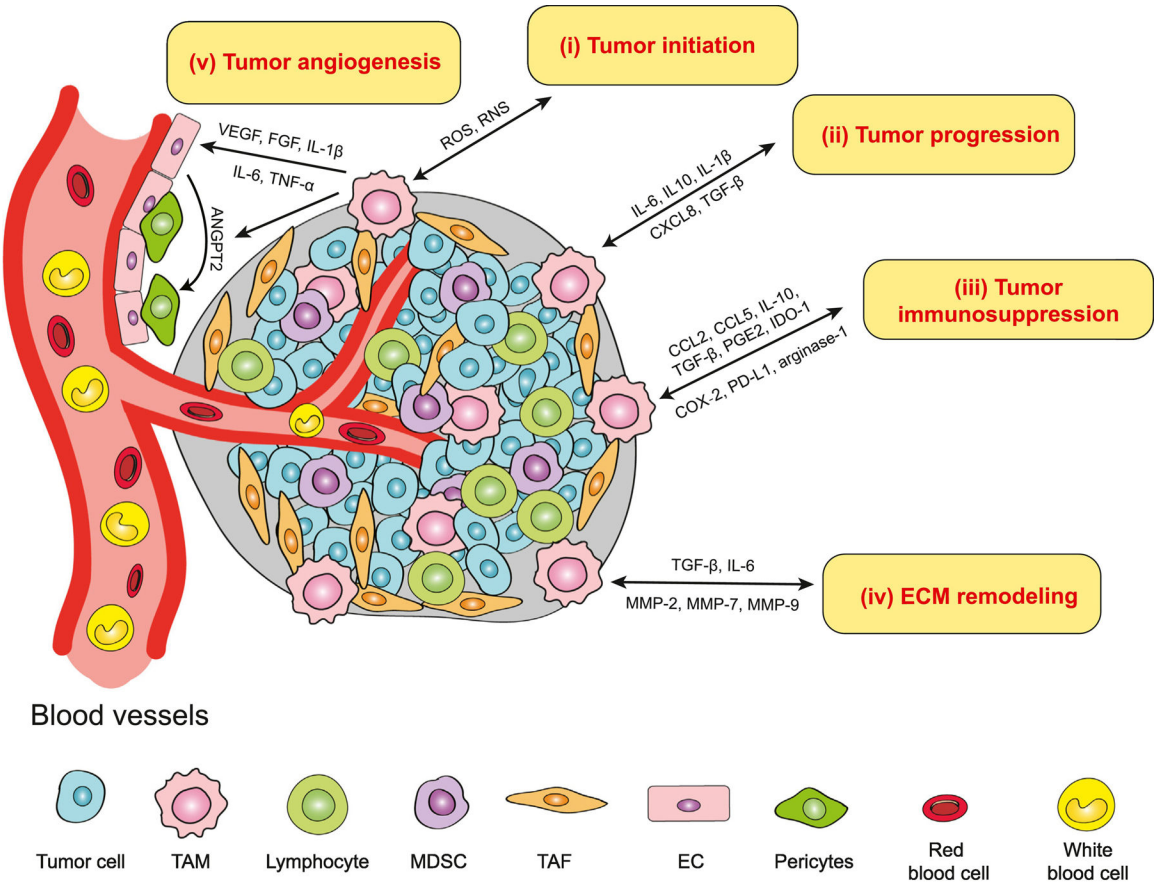


**Figure 1. Tumor-associated macrophages (TAMs) are a key regulator of the tumor microenviroment (TME).**

TAMs regulate the TME in mutiple ways such as 1) tumor initiation, 2) tumor progression, 3) tumor immunosuppression, 4) extracellular matrix (ECM) remodeling, and, 5) tumor angiogenesis. The crosstalk between TAMs and different cells of tumor microenvironment (TME) during these processes is associated with a number of pro-tumoral and immunosuppressive cytokines, chemokines, and growth factors

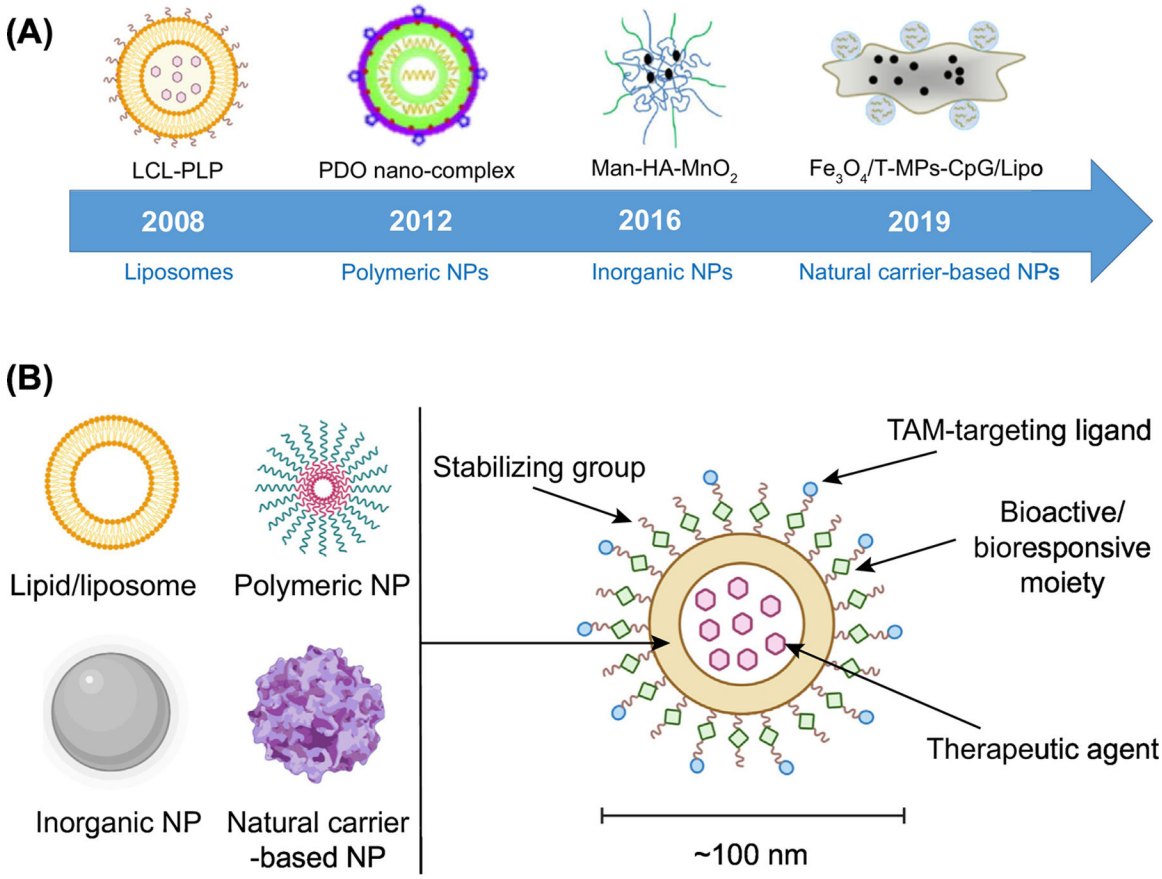


**Figure 2. Design of nano targeted drug delivery systems (Nano-TDDS) for TAMs.**

(A) Schematic shows representative examples of nanomaterials such as lipids/liposomes, polymeric NPs, inorganic NPs and biological/natural carrier mimics that have been developed in the last decade for TAM-targeting Nano-TDDS. (B) A multifunctional Nano-TDDS is depicted that can be achieved by keeping the size of the nanomaterial to ~100 nm, and with the modification of the aforementioned nanomaterials with stabilizing groups, targeting ligands and bioactive/bioresponsive moiety. Such “ideal” Nano-TDDS potentially provide high loading efficiency, favorable pharmacokinetic profiles, TAM-specific delivery, and TME-controlled drug release. **Abbreviations:** LCL-PLP, prednisolone phosphate encapsulated in long-circulating liposomes; PDO, PEG-histidine-modified alginate-galactosylated cationic dextran-CpG oligodeoxynucleotide; Man-HA-$MnO_2$, HA-coated mannan-conjugated $MnO_2$ particles; $Fe_3O_4$/T-MPs-CpG/Lipo, Nano-$Fe_3O_4$-carried tumor-derived antigenic microparticles surface-decorated with CpG-loaded liposomes.

**Table 1.**

Recent *in vivo* studies on delivery of TAM-targeting agents using Nano-TDDS, including formulation strategy, therapeutic agent, tumor model, and therapeutic mechanism.

| Formulation strategy | Therapeutic agent | Tumor model | Therapeutic mechanism | Ref. |
|---|---|---|---|---|
| Lipid and Liposomes | | | | |
| Lipid-based NPs with α-peptide and M2pep dual targeting ligands | Anti-CSF-1R siRNA | S.C. B16-F10 melanoma mouse | Depletion of TAMs by CSF-1R blocking | [59] |
| Liposomes with sialic acid targeting ligand | Ibrutinib (a BTK inhibitor) | S.C. S180 sarcoma mouse | Depletion of TAMs by BTK inhibition | [62] |
| Liposomes with sialic acid targeting ligand | Epirubicin (chemotherapeutic drug) | S.C. S180 sarcoma mouse | Depletion of TAMs by targeting the MPS pathway and EPR effect | [63] |
| Lipid-based NPs | Anti-CD47 and anti-SIRPα antibody conjugate (CD47 and SIRPα inhibition) | S.C. 4T1 breast cancer mouse; S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by CD47 and SIRPα inhibition | [69] |
| Lipid-based supramolecular NPs | Conjugated BLZ945 (a CSF1R inhibiting amphiphile) and SHP099 (a SHP2 inhibitor) | S.C. 4T1 breast cancer mouse; S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by CSF-1R and SHP2 inhibition | [70] |
| Liposomes | Hydralazine (an antihypertension vasodilator) | S.C. BPD6 desmoplastic melanoma mouse | Reprogramming of TAMs by normalizing tumor blood vessels | [71] |
| Lipid-based NPs with amino ethylanisamide targeting ligand | Relaxin (an anti-fibrotic peptide) | M.T. CT26-FL3 colorectal cancer mouse | Reprogramming of TAMs by normalizing liver fibrosis | [72] |
| Lipid-based supramolecular NPs | BLZ945 (a CSF-1R inhibitor) and selumetinib (a MEK inhibitor) | S.C. 4T1 breast cancer mouse | Reprogramming of TAMs by inhibition of CSF-1R and MAPK pathways | [81] |
| Lipid-based supramolecular NPs | BLZ945 (CSF-1R inhibitor) and anti-SIRPα (SIRPα inhibition) | S.C. 4T1 breast cancer mouse; S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by CSF-1R and SIRPα inhibition | [82] |
| Polymeric NPs | | | | |
| β-cyclodextrin NP (CDNP) | Resiquimod (R848, an agonist of TLR7 and TLR8) | S.C. MC38 colon cancer mouse | Reprogramming of TAMs by TLR signaling stimulation. | [65] |
| Poly(l-lysine)-b-poly(ethylene glycol) polypeptide micelles with macrophage galactose-specific C-type lectin (MGL) and grafted galactose groups | Zinc protoporphyrin IX (ZnPP, ROS-inducing agent) and Poly (I:C) (a TLR3 agonist) | S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by induction of reactive oxygen species(ROS) and TLR signaling stimulation | [66] |
| Cationic poly(β-amino ester) NPs with Di-mannose targeting ligand | mRNAs encoding interferon regulatory factor 5 | M.T. ID8 ovarian cancer mouse; M.T. B16-F10 melanoma mouse; O.T. DF-1 glioblastoma mouse | Reprogramming of TAMs by gene therapy | [83] |
| 2,2-bis(acryloyloxymethyl)propionate NPs with amino ethylanisamide targeting ligand | Mitoxantrone (an antineoplastic) and Celastrol (an antineoplastic) | S.C. BPD6 desmoplastic melanoma mouse | Reprogramming of TAMs by chemotherapy | [84] |
| Poly(lactic-co-glycolic acid) NPs with mannose targeting ligand | Indocyanine green (a photosensitizer) and titanium dioxide (ROS photogeneration agent) | S.C. 4T1 breast cancer mouse; S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by phototherapy | [85] |
| Poly(lactic-co-glycolic acid) NPs | Poly (I:C) (a TLR3 agonist), resiquimod (R848, TLR7/8 agonist) and Macrophage Inflammatory Protein-3 alpha (MIP3α, a chemokine) | S.C. TC-1 lung carcinoma mouse | TAM recruitment by cancer vaccination and TLR signaling stimulation | [86] |

| Formulation strategy | Therapeutic agent | Tumor model | Therapeutic mechanism | Ref. |
|---|---|---|---|---|
| Cyclodextrin-lysine polymeric NPs | R848 (TLR7/8 agonist) | S.C. MC38 colon cancer mouse; S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by TLR signaling stimulation | [87] |
| Cyclodextrin-lysine tri-monomer NPs | R848 (TLR7/8 agonist) | S.C. MC38 colon cancer mouse; S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by TLR signaling stimulation | [88] |
| Cholesteryl pullulan nanogel | Long peptide antigen and CpG oligoDNA (a TLR9 agonist) | S.C. CT26 colon cancer mouse; S.C. CMS7 and CMS5a fibrosarcoma mice; S.C. B16 melanoma mouse | Reprogramming of TAMs by improving antigen presentation of TAMs | [89] |
| Inorganic materials | | | | |
| $MnO_2$ NPs with mannan targeting ligand | $MnO_2$ (production of $O_2$), Hyaluronic acid (HA, reprogramming TAMs from M2 to M1) | S.C. 4T1 breast cancer mouse | Reprogramming of TAMs by HA-mediated pathway and $MnO_2$-mediated anti-hypoxia effect | [46] |
| Hollow manganese dioxide (H-$MnO_2$) NPs | Chlorine e6 (a photodynamic agent) and doxorubicin (an antineoplastic) | S.C. 4T1 breast cancer mouse | Inhibition and reprogramming of TAMs by phototherapy and chemotherapy | [90] |
| Calcium carbonate NPs | CD47 antibody | S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by CD47 inhibition | [91] |
| Iron chelated melanin-like NPs | Iron chelate polydopamine (TAM polarization from M2 to M1) | S.C. CT26 colon cancer mouse; O.T. 4T1 breast cancer mouse | Reprogramming of TAMs by phototherapy | [92] |
| Iron oxide NPs | Ferumoxytol (iron supplement) | S.C. MMTV PyMT mammary cancer mouse | Reprogramming of TAMs by phototherapy | [93] |
| Biological/natural carrier mimics | | | | |
| Lipoprotein-based NPs | $DiOC_{18}(7)$ (a photothermal agent); Mertansine (a microtubulin inhibitor) | O.T. and M.T. 4T1 breast cancer | Depletion of TAM by photothermy | [64] |
| Tumor-derived antigenic microparticles containing lipid NPs and iron oxide NPs | CpG oligoDNA (a TLR9 agonist) | S.C. B16-F10 melanoma mouse | Reprogramming of TAMs by cancer vaccination | [47] |

**Abbreviations:** NPs, nanoparticles; siRNA, small interfering RNA; S.C., subcutaneous; CSF-1R, colony stimulating factor-1 receptor; BTK, Bruton's tyrosine kinase; MPS, mononuclear phagocyte system; EPR, enhanced permeability and retention; TAM, tumor-associated macrophage; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; SIRPα, signal regulatory protein α; SPH2, src homology 2-containing phosphotyrosine phosphatas; M.T., metastatic; ROS, reactive oxygen species; Poly (I:C), Polyinosinic-polycytidylic acid; O.T., orthotopic; TLR, Toll-like receptor; HA, Hyaluronic acid; MMTV-PyMT, mouse mammary tumor.