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Review Article

Nanomaterial-Based Modulation of Tumor Microenvironments for Enhancing Chemo/Immunotherapy

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Abstract. The tumor microenvironment (TME) has drawn considerable research attention as an alternative target for nanomedicine-based cancer therapy. Various nanomaterials that carry active substances have been designed to alter the features or composition of the TME and thereby improve the delivery and efficacy of anticancer chemotherapeutics. These alterations include disruption of the extracellular matrix and tumor vascular systems to promote perfusion or modulate hypoxia. Nanomaterials have also been used to modulate the immunological microenvironment of tumors. In this context, nanomaterials have been shown to alter populations of cancer-associated fibroblasts, tumor-associated macrophages, regulatory T cells, and myeloid-derived suppressor cells. Despite considerable progress, nanomaterial-based TME modulation must overcome several limitations before this strategy can be translated to clinical trials, including issues related to limited tumor tissue penetration, tumor heterogeneity, and immune toxicity. In this review, we summarize recent progress and challenges of nanomaterials used to modulate the TME to enhance the efficacy of anticancer chemotherapy and immunotherapy.

KEY WORDS: chemotherapy; immunotherapy; modulation of microenvironment; nanomaterials; tumor microenvironment.

INTRODUCTION

To date, there have been numerous efforts to develop strategies for targeted delivery of anticancer chemotherapeutics to tumor cells. Receptors overexpressed on the surface of tumor cells have been utilized to design nanomaterials for direct delivery of anticancer drugs to tumor tissues. However, there has been little progress in translating tumor cell-targeted nanomaterials to clinical trial and receiving regulatory approval. Drawbacks in tumor cell-targeted delivery include the heterogeneity of tumor cells within a given tumor tissue and the lack of tumor cell-specific receptors.

The tumor microenvironment (TME) has drawn increasing recent attention as an alternative target for cancer therapy. There is increasing evidence that the TME plays important roles in controlling tumorigenesis, metastasis, and drug resistance (1). The TME is known to share complex features that rival the heterogeneity of tumor cells in the tumor tissues. The TME comprises diverse cell types, including cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T (Treg) cells. The extracellular matrix (ECM) of the TME is richly endowed with collagen and hyaluronan networks, tumor growth factors, anti-inflammatory cytokines, and tumor vascular systems (2). The TME is also characterized by upregulation of reactive oxygen species (ROS), hypoxia, and lower pH values (3–5). Because of its complexity, the TME has been widely studied as a potential locus of new pathways or novel molecular targets in the treatment of cancer (6).

Interactions among tumor cells and surrounding cells in the TME have been extensively investigated. Instead of attacking tumor cells, TME-resident CAFs, TAM cells are "tamed" by tumors to support tumor progression (7,8). Moreover, surface immune signaling by tumor cells in the TME blocks the recognition of tumor cells by nearby immune cells. The chronic inflammation of the TME is known to support the development of an immune-suppressive microenvironment, in which Treg cells and MDSCs negatively regulate the antitumor immune response (9–11). Immune checkpoint blockade has been proposed as a promising approach for restoring the ability of immune cells to attack tumor cells. Immune checkpoint-blocking agents, such as antibodies that target programmed cell death protein 1 (PD-1), programed death ligand-1 (PD-L1), or cytotoxic T lymphocyte-associated antigen-4 (CTLA4), have been

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commercialized. However, immune checkpoint blockade has generally been combined with other conventional therapies, such as anticancer chemotherapeutics (12). The use of such combination therapies in the clinic highlights the importance of actively modulating immunological features of the TME.

Nanomaterials have been extensively studied for the delivery of anticancer drugs directly to cancer cells. Nanomaterials have been reported to passively accumulate in the TME through the enhanced permeability and retention (EPR) effect, reflecting the leakiness of the disordered tumor vasculature. However, the EPR effect is known to depend on the tumor type and is too inefficient to sufficiently improve the therapeutic outcomes of therapeutic strategies based on nanomaterials that carry anticancer chemotherapeutic drugs (13,14). On the other hand, the dense matrix, high interstitial pressure, and poor vascular permeability characteristic of various solid tumor types, such as pancreatic cancer, gastric cancer, and breast cancer (15–17), might prevent the deposition of conventional nanotherapeutics and lead to restricted responses of these solid tumors. Nanomaterials have also been modified through conjugation of ligand molecules that directly target cancer cells rather than other cells of the TME. However, the heterogeneity of cancer cells within a given tumor tissue can limit the ability of such ligands to exclusively target anticancer drug delivery to the desired cells.

To improve the efficacy of anticancer chemotherapy and immunotherapy, researchers have recently employed nanomaterials that modulate biological and/or immunological features of the TME. Among the nanomaterials that have been investigated are those that target cells in the TME or alter the characteristics of the TME so as to overcome existing barriers against effective delivery. By modulating the physiological features of the TME, such as the ECM, vasculature systems, and/or hypoxia, advanced nanomaterials have improved the responses of tumors to chemotherapy. Other nanomaterial-based approaches that modulate immunological features of the TME can be used to deplete or reprogram CAFs, TAMs, Treg cells, or MDSCs. This review will cover the various nanomaterial-based strategies for altering the TME to enhance the efficacy of cancer chemotherapy or immunotherapy.

NANOMATERIALS THAT MODULATE THE TME TO IMPROVE THE EFFICACY OF CHEMOTHERAPY

The TME serves as an impediment to the therapeutic efficacy of nanomaterials that carry anticancer drugs. The ECM component of the TME in particular can serve as a barrier against deep tissue penetration of nanomaterials. Important features of the TME also include high interstitial fluid pressure and hypoxia; the increased interstitial fluid pressure of the TME, which is attributable to the dense ECM and diminished blood perfusion, is known to be a major obstacle in the delivery of anticancer chemotherapeutics (18,19). Various nanomaterials that modulate the ECM (20–23), disrupt the vascular system (24–27), or promote the perfusion of TME (28–32) have been designed to overcome this obstacle. Other efforts to enhance the efficacy of chemotherapy have focused on converting hypoxia to normoxia (33–37) (Fig. 1). Examples of nanomaterials modulating the TME for chemotherapy are listed in Table 1.

To increase the penetration of anticancer drugs in the TME, researchers have designed nanomaterials that disrupt the ECM or tumor vasculature, or increase blood perfusion.

Nanomaterials for Modulating the ECM

The ECM of the TME is a major barrier to the diffusion and penetration of nanomaterials carrying active substances. Hyaluronic acid and fibronectin, together with proteoglycans and collagen, are the major components of the ECM in the TME (38,39). One strategy that has been employed to enhance the diffusion of nanomaterials is depleting major components of the ECM using hyaluronidase (20,21), fibronectin inhibitors (22), or ROS (23).

In initial studies, nanomaterials (photosensitizer-conjugated micelles) were co-administered with hyaluronidase to increase tumor tissue penetration (20). In this study, 4T1 breast tumor-bearing mice were intratumorally treated with 3000 U of hyaluronidase, followed by intravenous administration of polymeric micelles composed of poly(maleic anhydride-alt-1-octadecene) coated with poly(-ethylene glycol) (PEG) and conjugated with the photosensitizer chlorin e6 (Ce6). Upon combination with hyaluronidase, these PEGylated, Ce6-conjugated micelles showed more than a 2-fold increase in accumulation in mice compared with that observed following treatment with polymeric micelles alone. Consistent with the increased tumor tissue delivery of micellar photosensitizers, the combination of hyaluronidase and irradiation with 660-nm light also produced greater photodynamic efficacy.

To diminish the ECM barriers of the TME, researchers have used a fibronectin inhibitor in conjunction with gold nanorods (22). In this study, Capan-2 tumor-bearing mice were orally administered cyclopamine, a natural alkaloid that inhibits the hedgehog signaling pathway and fibronectin production (40), prior to the intravenous injection of gold nanorods. This treatment regimen was shown to reduce the amount of fibronectin in the ECM and as well as increase blood perfusion at tumor sites. Gold nanorods in combination with oral cyclopamine treatment also improved photothermal efficacy compared to treatment with cyclopamine alone. The increased accumulation of gold nanorods in tumor tissues observed with oral cyclopamine co-treatment provided evidence supporting the higher photothermal efficacy of 808-nm NIR irradiation.

Although these studies have reported that therapeutic efficacy is enhanced by combining nanomaterials with ECM-depleting agents, the nonspecific and uncontrolled degradation of ECM caused by physically combining these features raises potential concerns about side effects, including opening new routes of tumor cell migration. A recent study sought to minimize the nonspecific degradation of ECM caused by physical combination by linking hyaluronidase to the surfaces of nanomaterials (21). In this study, polymeric nanoparticles composed of poly(lactic-co-glycolic acid)-b-polyethylene glycol (PEG-PLGA) were fabricated and loaded with doxorubicin (Dox). The surfaces of polymeric nanoparticles were then modified with maleimide functional groups and covalently conjugated with thiolated hyaluronidase. Hyaluronidase-conjugated nanoparticles showed 15.7-fold greater diffusion through a collagen gel matrix than plain nanoparticles and

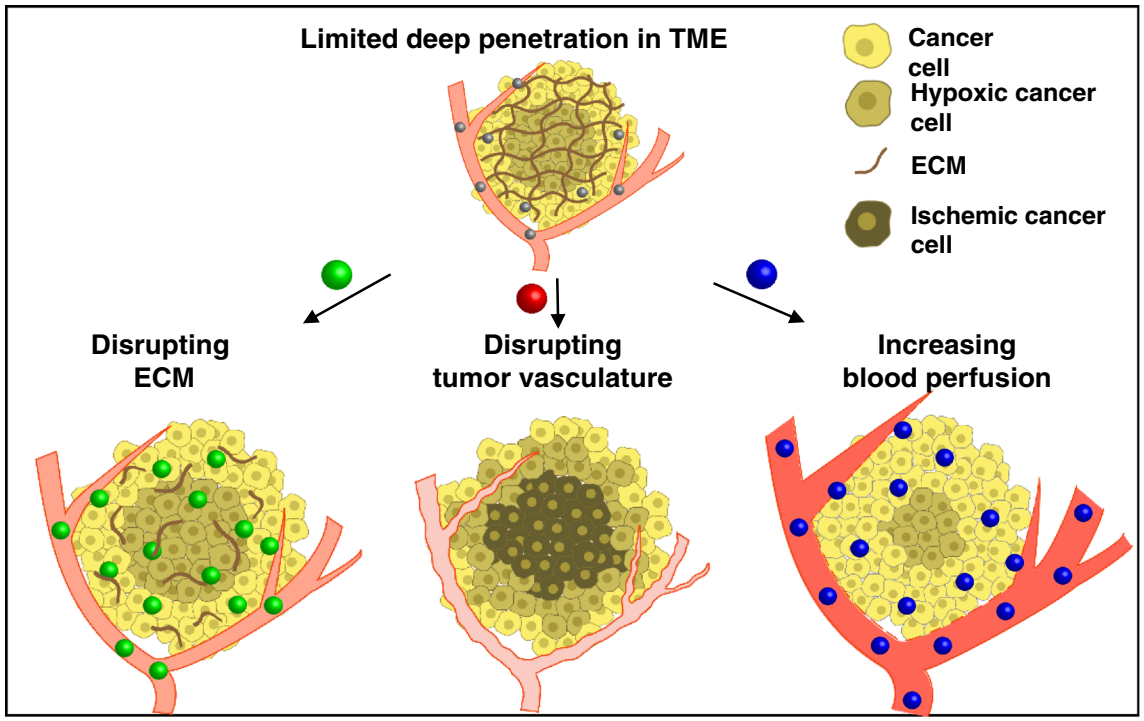

Fig. 1. Nanomaterials for modulating the TME so as to improve chemotherapeutic efficacy

2.4-fold greater diffusion than a physical mixture of nanoparticles with hyaluronidase. In a syngeneic 4T1 breast tumor model, tumor-bearing mice treated intravenously with hyaluronidase-conjugated polymeric nanoparticles showed greater tumor accumulation and anticancer effects than mice treated with unmodified nanoparticles or a physical mixture of nanoparticles and hyaluronidase.

In another recent application, components of the ECM were destroyed by ROS-mediated, laser-assisted disruption, using purpurin-loaded liposomes (23). Upon illumination with 710-nm light, purpurin-loaded liposomes released ROS and disrupted the collagen matrix. Notably, despite disruption of the collagen matrix by ROS, migration of A549 lung cancer cells was not increased. In fact, a study using a three-dimensional (3D) organoid cell culture model revealed that the migration of cancer cells in the matrix-disrupted group was actually inhibited ~ 6.7-fold compared to the group with an intact matrix. Moreover, in A549 tumor-bearing mice, treatment with purpurin-loaded liposomes and light irradiation was shown to inhibit the growth of tumors more effectively than treatment with plain liposomes or purpurin-loaded, but unirradiated, liposomes.

The strategy of modulating the ECM may be advantageous for effectively enhancing the deep penetration of nanomaterials into solid tumor tissues. Such enhanced penetration can increase the possibility of nanomaterial uptake by tumor cells in the solid tumor mass. Despite this possibility, direct proof of the extent of nanomaterial penetration has been rarely demonstrated. Molecular imaging using probes that are activated in the TME may enable the selective visualization of nanomaterial locations in tumor tissues.

Despite these advantages, this strategy may raise concerns about whether enhancing deep penetration of nanomaterials could also promote tumor metastasis. Indeed, remodeling of the ECM is known to play a critical role in promoting invasion or migration of tumor cells (41). One promising strategy would be covalent conjugation of an ECM-modulating agent on nanomaterials to localize the destructive effect. Even in this case, the possibility that destruction of TME components might cause metastasis should be carefully monitored throughout the development period.

Nanomaterials for Disrupting the TME Vasculature

Rapidly proliferating cancer cells in solid tumors are crucially dependent on the development of new vasculature to maintain their supply of oxygen and nutrients. Cancer cells in hypoxic or poorly perfused regions are known to secrete vascular endothelial growth factor (VEGF), which stimulates the proliferation of blood vessel endothelial cells by binding to VEGF receptor-2 (42,43). Accordingly, nanomaterials that carry angiogenesis inhibitors (24) or small interfering RNAs (siRNAs) targeting VEGF (26,27) have been studied as a strategy for inhibiting the formation of new blood vessels in the TME.

Polymeric micelles loaded with a vascular-disrupting agent have been investigated for disrupting angiogenesis in the TME. One such agent, 5,6 dimethylxanthenone-4-acetic acid (DMXAA; also known as vadimezan), is known to selectively disrupt tumor vessels by inducing apoptosis of tumor endothelial cells and causing hemorrhagic necrosis and ischemia in tumor tissue through the STING (stimulator of interferon gene) pathway (44). Applying this concept, Lv and colleagues conjugated DMXAA to a derivative of PEG and formulated polymeric nanoparticles (24). These polymeric micelles, composed of DMXAA-conjugated PEG-b-poly-

Table 1. Examples of Nanomaterials that Enhance Anticancer Therapy by Modulating the TME

TME factors	Strategy	Nanocarrier	Active substance	Route	Combination (route)	Tumor	Ref.
Tumor ECM	Degradation of hyaluronic acid matrix	Polymeric micelles (PEGylated poly(maleic anhydridealt-1-octadecene))	Ce6	I.V.	Hyaluronidase (I.T.), 660 nm irradiation	4T1	(20)
		Hyaluronidase-conjugated polymeric micelles (PEG-PLGA)	Dox	I.V.		4T1	(21)
	Disruption of fibronectin matrix	Gold nanorods		I.V.	Cyclopamine (oral), 880 nm irradiation	Capan-2	(22)
	Degradation by ROS	Liposomes	Purpurin	I.V.	710-nm irradiation	A549	(23)
Tumor vasculature	Disrupting tumor vessels	Polymeric micelles (poly (ethylene glycol)-b-poly-[(N-2-hydroxyethyl)-aspartamide])	Vadimezan Dox	I.V.		MCF-7	(24)
		RGD modified-PEGylated-gold nanoparticles		I.V.	X-ray irradiation	Panc-1	(25)
	Anti-angiogenesis	Chitosan nanoparticles	Polymerized anti-VEGF siRNA	I.V.	Dox	PC3	(26)
		Chitosan lactate nanoparticles	Anti-CD73 siRNA	I.V.		4T1	(27)
Blood perfusion	Dilating tumor blood vessels	Polymeric nanoparticles (polyethylene glycol-polylactic acid)	Paclitaxel	I.V.	Captopril (I.P.)	U87	(28)
		Polymeric micelles (D-α-tocopherol polyethylene 1000 glycol succinate)	Nitric oxide, Paclitaxel	I.V.		S180	(29)
		RGD modified-polymeric micelle (PEG-disulfide-polylactic acid)	Porphyrin-copper chelate, Dox	I.V.		4T1	(30)
		Erythrocyte membrane-coated polypyrrole nanoparticles		I.V.	BQ123 (endothelin A receptor antagonist) (I.P.), 808 nm irradiation	hct116	(31)
	Normalize tumor vasculature	PEGylated gold nanoparticles	Endostatin	I.V.	5-fluorouracil	H22	(32)
Hypoxia	In situ oxygen generation	Polymer-lipid hybrid MnO_2 nanoparticles	Dox	I.V.		EMT6	(59)
		Terpolymer/albumin hybrid MnO_2 nanoparticles, PEG/lipid hybrid MnO_2 nanoparticles		I.T., I.V.		EMT6	(33)
		Albumin-MnO_2 nanoparticles	Ce6 or cisplatin prodrug	I.V.	660 nm irradiation	4T1	(34)
		Mesoporous tantalum oxide nanoshells	Catalase	I.V.	X-ray irradiation	4T1	(60)
		Mesoporous silica nanoparticles	Catalase		High intensity focused ultrasound	MDA-MB231	(36)

Intravenous (I.V.), intratumor (I.T.), intraperitoneal (I.P.), or oral routes were used for in vivo studies

[(N-2-hydroxyethyl)-aspartamide], were designed to liberate the anti-vascular agent in the TME in a sustained manner. To impart anticancer activity, these authors further loaded the hydrophobic core of DMXAA-conjugated micelles with Dox. Polymeric micelles loaded with Dox and conjugated with the vascular-disrupting agent exhibited greater antitumor efficacy than polymeric micelles loaded with Dox alone or modified only with the vascular disrupting agent.

Chitosan nanoparticles complexed with siRNAs have been studied as a strategy for blocking angiogenesis (26). In this study, thiolated anti-VEGF siRNA was complexed with a thiolated antisense strand, and the double-stranded siRNA molecules were polymerized through disulfide linkages. The polymerized VEGF-targeting siRNA was then complexed to thiolated glycol chitosan by electrostatic interaction, forming nanoparticles. These anti-VEGF siRNA-complexed chitosan

nanoparticles induced downregulation of VEGF expression and hampered the growth of new blood vessels. Intravenously administered anti-VEGF-siRNA-complexed nanoparticles exhibited synergistic effects with a low dose of metronomic Dox (1.2 mg/kg, once every 2 days) in PC3-xenografted mice.

Anti-CD73 siRNA-loaded chitosan lactate nanoparticles have also been studied as an angiogenesis-inhibiting strategy (27). CD73, a cell surface enzyme that facilitates the production of adenosine from AMP, is known to play a role in tumor growth, immune evasion, and resistance to anticancer drugs (45). It has previously been shown that CD73 promotes the expression of VEGF and enhances tumor angiogenesis, and that inhibition of CD73 with an antibody impedes angiogenesis and tumor growth (46). In a 4T1 breast cancer model, treatment with anti-CD73 siRNA-loaded chitosan lactate nanoparticles was shown to reduce CD73 mRNA levels and blood vessel density in the TME.

In a recent study, peptide-modified gold nanoparticles were combined with X-ray irradiation to disrupt tumor vascular system (25). In this study, the Arg-Gly-Asp (RGD) peptide, used as a tumor neovascular targeting ligand (47), was conjugated to gold nanoparticles. Image-guided X-ray radiation was used to selectively disrupt tumor vessels after treatment of mice bearing Panc-1-expressing pancreatic tumors with RGD-modified gold nanoparticles. Treatment of mice with these nanoparticles followed by X-ray radiation induced 3.0-fold and 10.0-fold greater DNA damage in tumor tissues compared with X-ray and nanoparticles alone, respectively.

Normalization of the tumor vasculature has been reported to increase the tumor accumulation and penetration of polymeric nanoparticles (48). Proangiogenic placental growth factor (PGF) is known to bind to VEGF receptor-1 and suppress angiogenesis (49,50). In a study by Lammers and colleagues (48), knockout mice lacking histidine-rich glycoprotein and wild-type mice were implanted with t241 fibrosarcoma tumor cells overexpressing histidine-rich glycoprotein. Compared with tumor-bearing knockout mice, wild-type mice bearing t241 tumors showed downregulated expression of PGF, increase of vascular normalization, and polarization of TAMs to the M1-like phenotype in the TME. Upon intravenous administration of polymeric nanoparticles based on poly(N-[2-hydroxypropyl]methacrylamide), 2.0% and 1.2% of the injected dose accumulated in t241 tumors in wild-type and knockout mice, respectively. Using a genetic mouse model, this study demonstrated the potential of tumor vessel normalization, not only in the delivery of polymeric nanoparticles but also in altering the phenotype of TAMs.

The strategy of disrupting the tumor vasculature may be advantageous when the effect of nanomaterials is localized only to the tumor vasculature. However, the strategy of disrupting the tumor vasculature may suffer from nonspecific alteration of vasculatures in normal tissues. To minimize such undesirable effect to the normal tissues, more elaborate nanomaterials selectively recognizing and disrupting the vasculatures in the tumor tissues should be developed. With the progresses on the tumor biology, more markers of tumor vasculature will be identified. The future direction of nanomaterials disrupting the tumor vasculature should be based on the interdisciplinary studies between tumor biology, biomaterials, and nanotechnologies.

Additional concerns in the development of tumor vasculature-modulating nanomaterials are the species dependence of in vivo tumor vasculature-disrupting effects. For example, the vascular disrupting agent, DMXAA, was shown to be effective in a mouse model, but not in humans, a difference that was interpreted as indicating that human STING, unlike mouse STING, is not activated by DMXAA (44,51). Patient-derived tumor models, genetic mouse model, or three dimensionally constructed human tumor organoids which mimic human vasculature systems need to be considered to test the effect of the vasculature disrupting nanomaterials.

Nanomaterials for Promoting Perfusion of the TME

Although inhibition of angiogenesis has been used to decrease the supply of nutrients to tumor tissues, recent studies have revealed that the poorly perfused state of the TME can limit the efficient delivery of chemotherapeutic agents (28,52). One strategy proposed for enhancing drug delivery to tumor tissues is to promote perfusion using angiogenesis-promoting chemical agents (52). In some studies, blood perfusion and deep penetration were increased by co-treating with vessel-dilating agents and nanoparticles (28,31); in others, the vasodilators were generated in situ or encapsulated in nanocarriers (29,30,32,53).

Enhancing blood perfusion in the TME has been studied as a means for improving the outcome of nanoparticle-based chemotherapy. A recent study used co-treatment with captopril, an angiotensin-converting enzyme inhibitor, and anticancer-drug-loaded polymeric nanoparticles (28). Laser-Doppler imaging revealed that intraperitoneal administration of 100 mg/kg captopril increased blood perfusion in the TME by more than 2.0-fold. The co-administration of captopril enhanced accumulation of paclitaxel-loaded, polymeric PEG-polylactic acid nanoparticles. In U87 glioma tumor-bearing mice, the co-administration of captopril and paclitaxel-loaded nanoparticles exerted greater anticancer efficacy than treatment with either captopril- or paclitaxel-loaded nanoparticles alone. These results were interpreted as indicating that captopril treatment induced bradykinin and inflammatory mediators, increased endothelial gaps, and subsequently enhanced perfusion.

Nitric oxide (NO) loaded in polymeric micelles has also been used to promote blood perfusion and increase drug delivery (29). In this application, a polymeric micelle composed of D-α-tocopherol polyethylene 1000 glycol succinate derivatives was designed to co-deliver NO and paclitaxel. Administration of three intravenous injections of these polymeric micelles into S180 tumor-bearing mice was shown to increase blood perfusion and blood vessel density in tumor tissues. The co-delivery of NO and paclitaxel in polymeric micelles also inhibited the growth of drug-resistant MCF-7/ADR tumors more effectively than treatment with either NO-carrying micelles or paclitaxel-loaded micelles alone.

Difficulties in entrapping gaseous NO in nanoparticles motivated a recent nanomaterials-based strategy for generating NO in situ (30), which would dilate tumor vessels and increase the permeability of therapeutic drugs. In this application, copper ion was used to catalyze NO production from endogenous NO donors such as S-nitrosoglutathione in

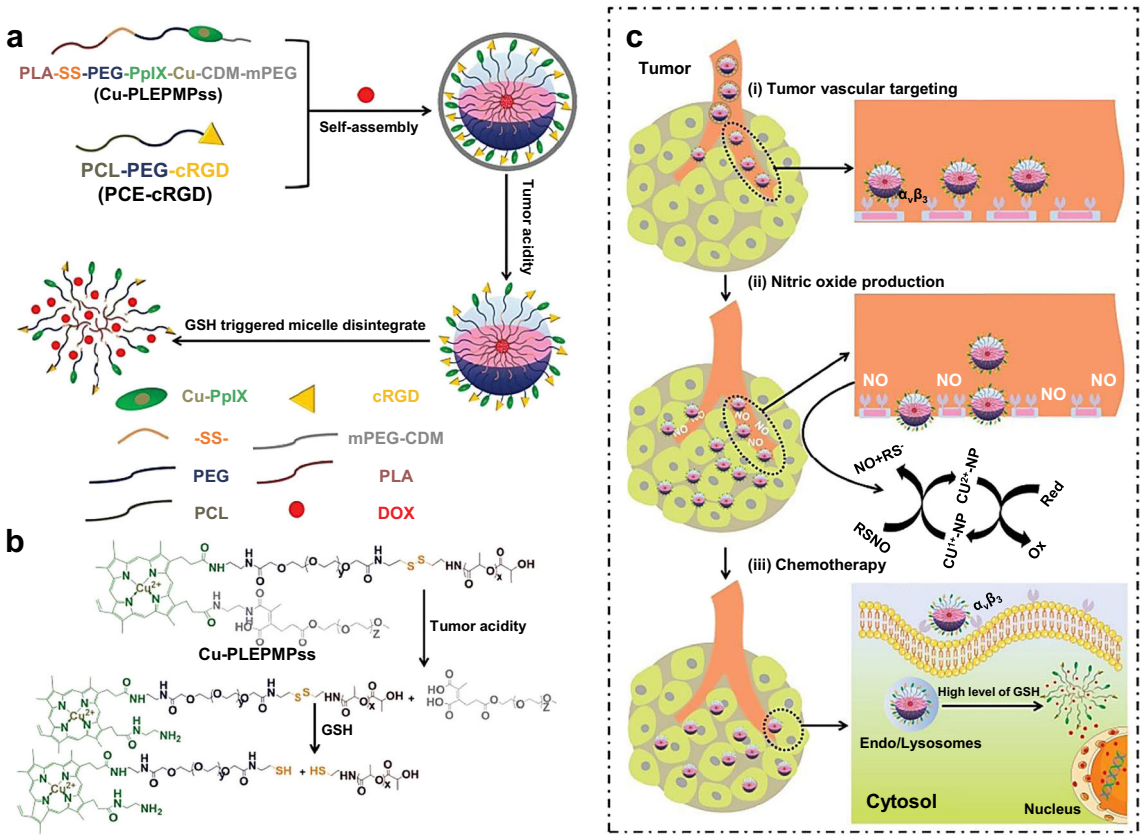

Fig. 2. Increasing blood perfusion in the TME using nanomaterials. **a** Polymeric micelles containing copper ion were triggered to disassemble in the TME. **b** Polymeric micelles were subsequently disintegrated by acidic pH and glutathione (GSH) in the TME. **c** Chelation of the polymer backbone by copper ion catalyzed the in situ production of NO from endogenous S-nitrosoglutathione. NO increased blood perfusion and penetration of the anticancer drug, Dox. Reprinted from (30) by permission

the tumor tissue. The copper ion-chelated porphyrin was derivatized with a pH-responsive PEG shield (2-propionic-3-methylmaleic anhydride-modified methoxy PEG) and introduced onto the surfaces of self-assembled polymeric micelles (Fig. 2). In addition, the outer shell of micelles was coated with poly(d,l-lactide) conjugated with PEG via disulfide bonds, conferring redox sensitivity on the micelle. The reduction in tumor volume in the group treated with the polymeric micelles was 14.5-fold greater than that in the group treated with free Dox. This enhanced anticancer effect was interpreted as resulting from in situ NO-mediated dilation of tumor vessels.

Other vasodilators apart from NO have been used to increase the delivery of therapeutic nanoparticles to tumor tissues. In one such study (31), BQ123, an endothelin A receptor antagonist, was intraperitoneally administered as a tumor vasculature-selective vasodilator. Treatment of mice with BQ123 was shown to promote higher accumulation of co-administered erythrocyte membrane-coated polypyrrole nanoparticles into tumor tissues compared to treatment with nanoparticles only. Because of this higher tumor tissue accumulation of nanoparticles, NIR irradiation of mice co-treated with BQ123 resulted in more effective tumor ablation compared to irradiation without BQ123 treatment.

Endostatin-loaded gold nanoparticles have been studied for normalizing tumor vascularization in the TME (32). In fast-growing tumors, blood vessels in the TME are formed through abnormal alignment of endothelial cells, which, together with compression of blood vessels, can result in resistance to blood flow and perfusion, limiting the distribution of anticancer drugs into the tumor (54). Li and colleagues adsorbed endostatin, a C-terminal non-collagenous fragment of type XVIII collagen, onto gold nanoparticles, and then further modified endostatin-adsorbed gold nanoparticles with PEG for increased stability. Intravenous administration of PEGylated, endostatin-loaded gold nanoparticles into hepatocarcinomatous H22 tumor-bearing mice did not affect the density of blood vessels in the TME but did increase blood perfusion by ~ 2.0-fold and pericyte coverage by ~ 2.0-fold; it also decreased vessel permeability to ~ 1/3 of that in controls. These changes indicate maturation and normalization of blood vessels. By combining 5-fluorouracil with endostatin-modified gold nanoparticles, these authors demonstrated significantly increased concentrations of 5-fluorouracil in tumor tissues compared to monotherapy with 5-fluorouracil, leading to greater suppression of tumor growth and extended survival of treated animals.

Promoting perfusion in the TME would be advantageous in that the increased perfusion could increase the amount of nanomaterial delivered to poorly perfused tumor tissues. However, to date, few studies have directly compared the effect of vascular normalization with that of angiogenesis in

modulating the efficacy of nanomaterials carrying anticancer chemotherapeutics. The effects of disrupting versus normalizing the tumor vasculature need to be compared in well-controlled studies. Moreover, in addition to the increased blood perfusion in the TME, the changes in pericyte coverage and vessel permeability should be studied in a quantitative manner.

One caveat of treating with blood perfusion-enhancing agents would be possible toxicity towards cardiovascular systems. In the case of endostatin, one of the major adverse effects in clinical trials was found to be cardiotoxicity (55). Thus, safety in cardiovascular systems should be carefully studied in evaluating approaches for modulating perfusion in the TME. Another disadvantage of the strategy of promoting perfusion in the TME is the short-term duration of the effect. Most current strategies for enhancing tumor perfusion and facilitating deposition of nanomaterials are based on the use of vasodilators, which transiently affect the vasculature. The limited duration of vasodilation may not sufficiently increase the accumulation of nanomaterials. One possible future strategy for overcoming this limitation would be to design implantable hydrogel systems, which might provide sustained and localized delivery of perfusion enhancers at tumor tissues.

Nanomaterials for Mitigating Hypoxia

Hypoxia is a prominent feature of the TME that is associated with poor tumor prognosis, metastasis, and drug resistance (56–58). Cancer cells adapt to the hypoxic TME through overexpression of hypoxia-inducible factor-1 (HIF-1), composed of HIF-1α and HIF-1β subunits. In hypoxic environments, HIF-1α is stabilized and forms a heterodimer with HIF-1β inside the nucleus, interacting with downstream co-activators to facilitate the transcription of genes involved in angiogenesis, metabolism, and survival (59). Various nanomaterials have been studied for their potential to mitigate hypoxia and overcome hypoxia-related drug resistance (33–37).

One such nanomaterial studied for reducing the hypoxic status of the TME is MnO_2 nanoparticles embedded in PEGylated myristic acid (33). In this study, hybrid MnO_2 particles were designed to produce oxygen by reacting with endogenous H_2O_2 and H^+ in the relatively acidic TME. In EMT6 tumor-bearing mice, intratumoral injection of hybrid PEGylated myristic acid-MnO_2 nanoparticles induced a 60% reduction in HIF-1α expression levels 4 h after administration compared with untreated mice. Upon intravenous administration, these hybrid nanoparticles showed greater accumulation in tumor tissues compared with polymeric albumin-hybrid MnO_2 nanoparticles. Although this increased tumor accumulation of PEGylated myristic acid-MnO_2 nanoparticles was presumed to be attributable to mitigation of in vivo hypoxia in the TME, the change from hypoxia to normoxia was not demonstrated directly and will require confirmation in a future study.

In another study, hybrid PEGylated lipid derivative-MnO_2 nanoparticles were shown to mitigate hypoxia and reverse drug resistance (34). An in vitro study showed that, under hypoxic conditions, co-treatment with MnO_2 and Dox-loaded polymeric lipid nanoparticles increased Dox uptake in EMT6 tumor cells ~ 2.9-fold compared to treatment with free Dox alone. Intravenous administration of these hybrid PEGylated lipid derivative-MnO_2 nanoparticles was shown to reduce the expression of the hypoxia marker, HIF-1α, and that of drug resistance-related p-glycoprotein.

Human serum albumin-based nanoparticles entrapping MnO_2 have also been investigated for their potential to mitigate hypoxia and increase the efficacy of photodynamic chemotherapy (35), which is known to be decreased by insufficient oxygen in the TME (60). Chen and colleagues prepared human serum albumin conjugated with the photosensitizer Ce6 or a prodrug of cisplatin, and used them to formulate nanoparticles entrapping MnO_2. After intravenous injection into 4T1 tumor-bearing mice, the MnO_2-loaded and drug-conjugated human serum albumin nanoparticles gradually disassembled into smaller (< 10 nm) albumin-drug conjugates in the acidic TME as MnO_2 reacted with H_2O_2 and H^+. Treatment of 4T1 tumor-bearing mice with MnO_2-loaded, drug-conjugated human serum albumin nanoparticles followed by irradiation with 660-nm light exerted a greater anticancer effect compared to treatment with nanoparticles lacking MnO_2. Although the authors of this study speculated that the higher oxygen levels induced by MnO_2 delivery may have contributed to overcoming the resistance to photodynamic therapy, specific oxygen levels in the TME after in vivo administration were not reported.

In another application, catalase-loaded mesoporous tantalum oxide nanoshells were investigated for their potential to mitigate hypoxia in the TME and overcome resistance to radiation therapy (36). Notably, oxygen is known to play a major role in radiation therapy by absorbing X-rays (61). To increase oxygen levels in the TME, these researchers constructed mesoporous tantalum oxide nanoshells encapsulating catalase, which breaks down endogenous H_2O_2 to oxygen. The mesoporous tantalum oxide shells protect the catalase from attack by proteases and increase catalase contact with endogenous H_2O_2. In vitro treatment of 4T1 breast tumor cells with catalase-loaded mesoporous tantalum oxide nanoshells followed by X-ray irradiation induced greater oxygen generation and DNA damage in cells compared to treatment with nanoshells lacking catalase or X-ray irradiation alone. In 4T1 tumor-bearing mice, hypoxia in the TME was alleviated by systemic administration of these catalase-loaded mesoporous nanoshells, and tumors were ablated after subsequent X-ray irradiation.

Catalase-loaded mesoporous silica nanoparticles have also been studied for their potential to mitigate hypoxia in the TME and enhance the imaging resolution and anticancer efficacy of high-intensity focused ultrasound (HIFU) treatment (37). In this study, the combination of HIFU with catalase-loaded mesoporous silica nanoparticles increased the generation of oxygen bubbles, and improved the ultrasonography imaging signal compared to treatment with free catalase. In mice bearing MDA-MB231 breast tumors, the combination of HIFU with catalase-loaded nanoparticles ablated tumors with volumes up to 135 mm^3, whereas HIFU monotherapy or HIFU combined with mesoporous silica nanoparticles alone ablated only small tumors (volumes < 20 mm^3). Although this study demonstrated that catalase delivered in nanoparticles could generate more oxygen bubbles, it did not directly show in vivo conversion of

hypoxic conditions to normoxia or demonstrate the biological effects of catalase-enhanced oxygen bubbles on the expression of the hypoxia marker, HIF-1α.

Nanomaterials that mitigate hypoxia would be advantageous for enhancing the therapeutic effects of drugs that show hypoxia-related drug resistance. In addition to overcoming resistance to chemotherapeutic agents, mitigation of tumor hypoxia might also enhance responses to immunotherapy, radiation therapy, or other anticancer modalities. Previous studies have reported that hypoxia can suppress immune cell activities in the TME (62,63). The mitigation of hypoxia may affect the activities of immune cells in TME, but this possibility should be demonstrated. Nanomaterials that mitigate hypoxia act by generating oxygen from H_2O_2 in the TME through MnO_2 or catalase. However, the concentration of H_2O_2 varies depending on the type, stage, and location of the tumor, leading to differences in oxygen generation. Thus, in designing nanomaterials for mitigating hypoxia, the physiological features of specific tumors should be carefully considered in advance.

NANOMATERIALS FOR MODULATION OF CELLS IN THE TME

In addition to studies centered on enhancing the delivery of chemotherapeutic drugs to tumor cells, another field of nanomaterials has focused on modulating cells in the TME to enhance the efficacy of chemotherapy or immunotherapy. Various types of nanomaterials have been studied for modulating CAFs (64–70), TAMs (71–75), Treg cells (76–78), and myeloid-derived suppressor cells (MDSCs) (79–81) (Fig. 3). Examples of nanomaterials modulating the cells in the TME are listed in Table 2.

Nanomaterials for Modulation of CAFs

A number of approaches have been investigated for modulating, reprogramming, or even ablating CAFs in the TME. CAFs are myoblast-like cells present in the tumor stroma that are considered to originate from multiple cell types and thus represent a heterogeneous cell population (82). CAFs secrete numerous cytokines and chemokines, including transforming growth factor beta (TGF-β), interleukin-6 (IL-6), IL-10, and VEGF, all of which facilitate cancer proliferation and metastasis (83,84). Among CAF markers are α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and tenascin-C (TNC) (82). Because CAFs are common resident cells in various types of solid tumors, targeting CAFs is considered to be an advantageous strategy for overcoming the heterogeneity of tumor types and tumor cells. Liposomes (64), lipid/protein nanoparticles (70), ferritin nanoparticles (66), inorganic material (68,69), and polysaccharide nanoparticles (67) have all been used as nanomaterials to target CAFs in the TME.

TNC, a glycoprotein component of the ECM expressed by CAFs, has been investigated as a target for the delivery of a liposomal drug (65). In this study, a small peptide (FHKHKSPALSPVGGG) that binds to TNC was incorporated onto the surface of liposomes, and navitoclax, an apoptosis-inducing drug, was loaded inside the hydrophobic lipid layers of the liposomes. Peptide-modified liposomal navitoclax showed concentration-dependent apoptotic effects in TNC-positive LX-2 cells, but not in TNC-negative Hep G2 tumor cells. In Hep G2-xenografted mice, treatment with peptide-modified liposomal navitoclax induced a greater reduction in CAFs in the TME than treatment with plain liposomal navitoclax or phosphate buffer, decreasing the CAF population in the TME to only 18.8% of that observed in the group treated with phosphate buffer. This reduction in the CAF population in the group intravenously treated with peptide-modified liposomal navitoclax was also associated the smallest tumor volumes. Another study conducted by the same group demonstrated a synergistic effect of the combination of TNC-binding peptide-modified liposomal navitoclax and liposomal Dox (64). This synergy was interpreted as enhanced penetration of liposomal Dox resulting from depletion of the tumor stroma in the TME, but penetration of liposomal Dox in tumor tissues was not specifically demonstrated in this study.

Reducing the CAF population in the TME has also been studied using CAF-recognizing ferritin nanocages (66). Ferritin is a naturally abundant, globular protein capable of forming nanocages in which small molecules can be encapsulated (85,86). For ablation of CAFs, ferritin nanocages were loaded with the photosensitizer, zinc hexadecafluorophthalocyanine, and modified with a single-chain variable fragment (scFv) that recognizes fibroblast activation protein (FAP), a unique biomarker of CAFs (82,87). Following intravenous injection into tumor-bearing mice, the FAP scFv-modified ferritin nanocages were found to accumulate in tumor tissues, an effect that was diminished by pretreatment of mice with free FAP scFv Local irradiation of tumor tissues with 671-nm light was shown to selectively ablate CAFs. Notably, the reduction in the CAF population was shown to increase the infiltration of $CD8^+$ T cells into the tumor tissues. The immunological mechanisms by which the ablation of CAFs resulted in the recruitment of $CD8^+$ T cells to tumor tissues remain to be elucidated.

Reprogramming of CAFs using gold nanoparticles has been studied as an alternative to destroying them (69). The reprogramming of CAFs to a more dormant phenotype was tested using a pancreatic ductal adenocarcinoma model, chosen because of its notoriously dense ECM and lack of tumor vasculature (88). Intraperitoneal administration of gold nanoparticles was proposed to distort crosstalk between pancreatic cancer cells and stellate cells, and the resulting deactivation of stellate cells was hypothesized to diminish the production of ECM in pancreatic adenocarcinoma. In human pancreatic stellate CAF19 tumor-xenografted mice co-implanted with AsPc1 human pancreatic cancer cells, administration of gold nanoparticles was found to decrease the synthesis of the pro-fibrotic components, fibronectin, collagen, and α-SMA, while simultaneously enhancing the vascular systems in tumor tissues. However, in this study, the gold nanoparticles were not modified to contain CAF-targeting ligands. The molecular mechanisms by which plain gold nanoparticles deactivated stellate cells should be investigated further.

Docetaxel-loaded, PEGylated polysaccharide nanoparticles designed to decrease stromal cells in the TME and enhance the anticancer efficacy of docetaxel have been reported (67). In this study, a three-component conjugate

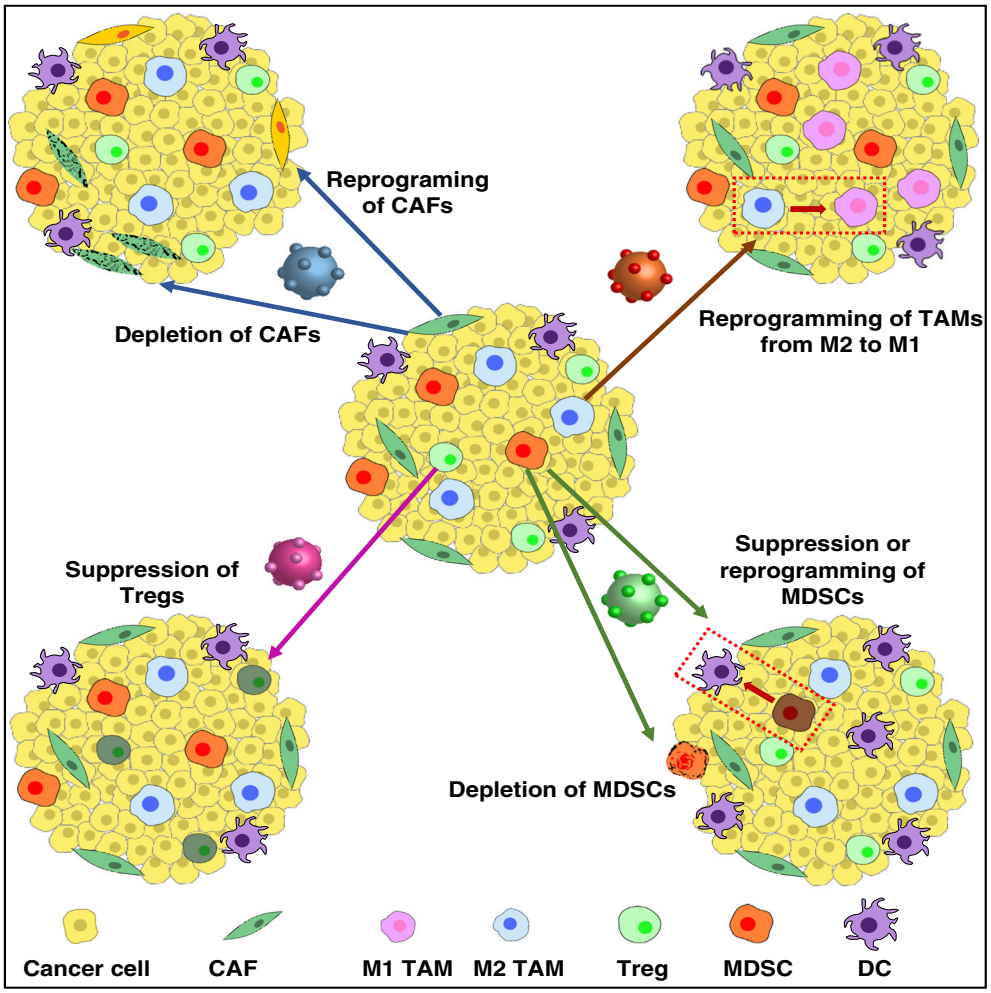

Fig. 3. Nanomaterial-based modulation of the TME to improve the efficacy of chemo/immunotherapy

composed of PEG, carboxymethylcellulose, and docetaxel was shown to self-assemble into nanoparticles containing docetaxel in the hydrophobic core. The resulting PEGylated carboxymethylcellulose nanoparticles were reported to bind predominantly to α-SMA-positive CAFs. Although it was speculated that this favorable CAF binding resulted from the interaction of serum albumin (adsorbed onto the nanoparticle during circulation in the blood) with SPARC (a secreted acidic protein rich in cysteine that is highly produced by CAFs), the exact mechanisms remain to be clarified. After intravenous administration, docetaxel-loaded, PEGylated polysaccharide nanoparticles showed 13.2-fold higher accumulation in the stromal region than in the tumor epithelial region. Together with enhanced accumulation in the tumor stroma, these nanoparticles were shown to decrease CAFs by 90% and increase blood perfusion by 15.2-fold compared with that observed in the untreated group. A notable aspect of this study is that it provided support for anticancer mechanisms through quantitative analyses of the in vivo CAF population and measurement of blood perfusion.

Graphene-based nanomaterials carrying Dox and promelittin have been investigated for selective activation of FAP on CAFs (68). FAP, which cleaves specific proline sites, is a serine protease that is rarely found in normal tissues but is highly expressed on the surface of CAFs (87). Melittin, a 26-amino-acid peptide component of honey bee venom, is capable of forming transient pores in the cell membrane. By tagging a nonfunctional, but FAP-cleavable, promelittin lipid derivative onto Dox-loaded, reduced graphene oxide (rGO) nanosheets, it was possible to confine the pore-forming activity of melittin to the TME (Fig. 4). These promelittin-tagged, Dox-loaded rGO nanosheets did not exert an anticancer effect on HT29 cancer cells but produced notable anticancer activity upon incubation with co-cultures of CAFs and HT29 cells, owing to the liberation of melittin by FAP expressed on CAFs and enhanced delivery of Dox-loaded nanosheets to tumor cells and CAFs. Intravenous injection of promelittin-tagged, Dox-loaded nanosheets into HT29 tumor-xenografted mice provided deeper tumor penetration of the nanosheets and a greater antitumor effect compared with treatment with Dox-loaded rGO nanosheets or the promelittin derivative alone.

Reprogramming of CAFs through delivery of the gene encoding the secreted form of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (sTRAIL) using a protamine-plasmid DNA complex coated with lipid has been reported (70). In this study, a DNA plasmid encoding sTRAIL was complexed with cationic arginine-rich protamine. For enhanced recognition of CAFs, the protamine–

Table 2. Examples of Nanomaterials for Modulating Cells in the TME

Cells at TME	Strategy	Nanocarrier	Active substance	Route	Combination	Tumor	Ref.
CAFs	Depletion	Tenascin C targeting peptide-modified liposome	Navitoclax	I.V.		HepG2	(65)
					Dox-loaded liposomes	HepG2	(64)
		Anti-FAP scFv-ferritin nanocage	Zinc hexadecafluorophthalocyanine	I.V.	671 nm irradiation	4T1	(66)
		PEGylated polysaccharide nanoparticles	Docetaxel	I.V.		PAN02, OCIP19, OCIP23	(67)
		Promelittin-PEG-rGO nanosheets	Dox	I.V.		HT-29	(68)
	Reprogram	Gold nanoparticles		I.P.		AsPc1 + CAF19	(69)
		Anisamide-derived lipid-coated protamine-DNA polyplex nanoparticles	sTRAIL encoding plasmid	I.V.		UMUC3 + NIH 3T3	(70)
TAMs	Reprogram	SR-B1/M2 targeting peptide-cationic lipid nanoparticles	Anti-CSF-1R siRNA	I.V.		B16F10	(71)
		Poly (β-amino ester) copolymer polymeric nanoparticles	IL-12	I.V.		B16F10	(72)
		Hyaluronic acid-polyethylenimine complex nanoparticles	miRNA-125b	I.P.		KRAS G12D/P53 GEM	(73)
		PEGylated cyclodextrin nanoparticles	Resiquimod	I.V.		MC38	(74)
		Iron oxide nanoparticles		I.V.		KP1-GFP	(75)
Treg cells	Suppression	GITR antibody-modified layer-by-layer polymeric nanoparticle (PEG-pHis-pGlu-PLGA)	Imatinib	I.V.	808 nm irradiation	B16/BL6	(77)
		Polymeric oxaliplatin-based micelles	NLG-919, Oxaliplatin	I.V.		4T1	(77)
		Polymer/lipid hybrid nanoparticles (PEG-PLGA/N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryoxycarbonyl-aminoethyl) ammonium bromide)	CTLA-4 siRNA	I.V.		B16	(78)
MDSCs	Suppression	Lipid-coated ApoA-gold nanoparticles		I.V.		B16	(79)
		Lipid-coated mesoporous silica nanoparticles	Interleukin-2, Dox, All-trans retinoic acid	I.V.		B16F10	(80)
		Lipid-coated calcium phosphate nanoparticles	Gemcitabine	I.V.		B16F10	(81)

plasmid DNA complexes were coated with a lipid derivative of anisamide, which is known to bind to sigma receptors. To mimic the in vivo condition, these researchers co-cultured 3T3 fibroblasts cells with UMUC3 tumor cells in a Transwell system. In vitro transfection of fibroblasts with the anisamide-modified nanocomplexes of plasmid DNA encoding sTRAIL decreased the viability of co-cultured tumor cells (45.6%) compared to treatment with a nanocomplex of plasmid DNA encoding the non-secreted form of TRAIL, suggesting that sTRAIL secreted by sTRAIL-transfected fibroblasts diffused into tumor cells and induced apoptosis. In both stroma-vessel desmoplastic bladder cancer (UMUC3/3 T3) and desmoplastic pancreatic cancer (BXPC3) mouse models, administration of anisamide-tagged nanocomplexes encoding sTRAIL as four repeated intravenous injections was shown to increase sTRAIL mRNA expression levels in tumor tissues and enhance anticancer effects compared with administration of a nanocomplex encoding control GFP protein or a non-secreted form of TRAIL.

Nanomaterials that modulate CAFs would be advantageous compared with those that target cancer cells per se from the standpoint of overcoming tumor heterogeneity. Compared with cancer cells, which undergo mutations and differ in their expression of specific target receptors, CAFs may acquire fewer mutations and exhibit lower heterogeneity. Thus, CAF-modulating approaches may be applicable to diverse solid tumors, which generally contain CAFs within the TME. In addition to overcoming the heterogeneity of tumors, modulating CAFs could reduce stromal density, resulting in deeper penetration. However, killing and loosening of stromal regions of solid tumors might increase the chances of tumor metastasis. One way to confront the potential for metastasis would be to activate immune systems that can recognize and attach to metastasized tumor cells. The design of nanomaterials that simultaneously modulate CAFs and induce immunotherapeutic features may be one research direction in this field.

Nanomaterials for Modulation of TAMs

Macrophages are a type of antigen-presenting cell (APC) derived from monocytes whose major function is to remove cell debris and pathogens through phagocytosis. Macrophages

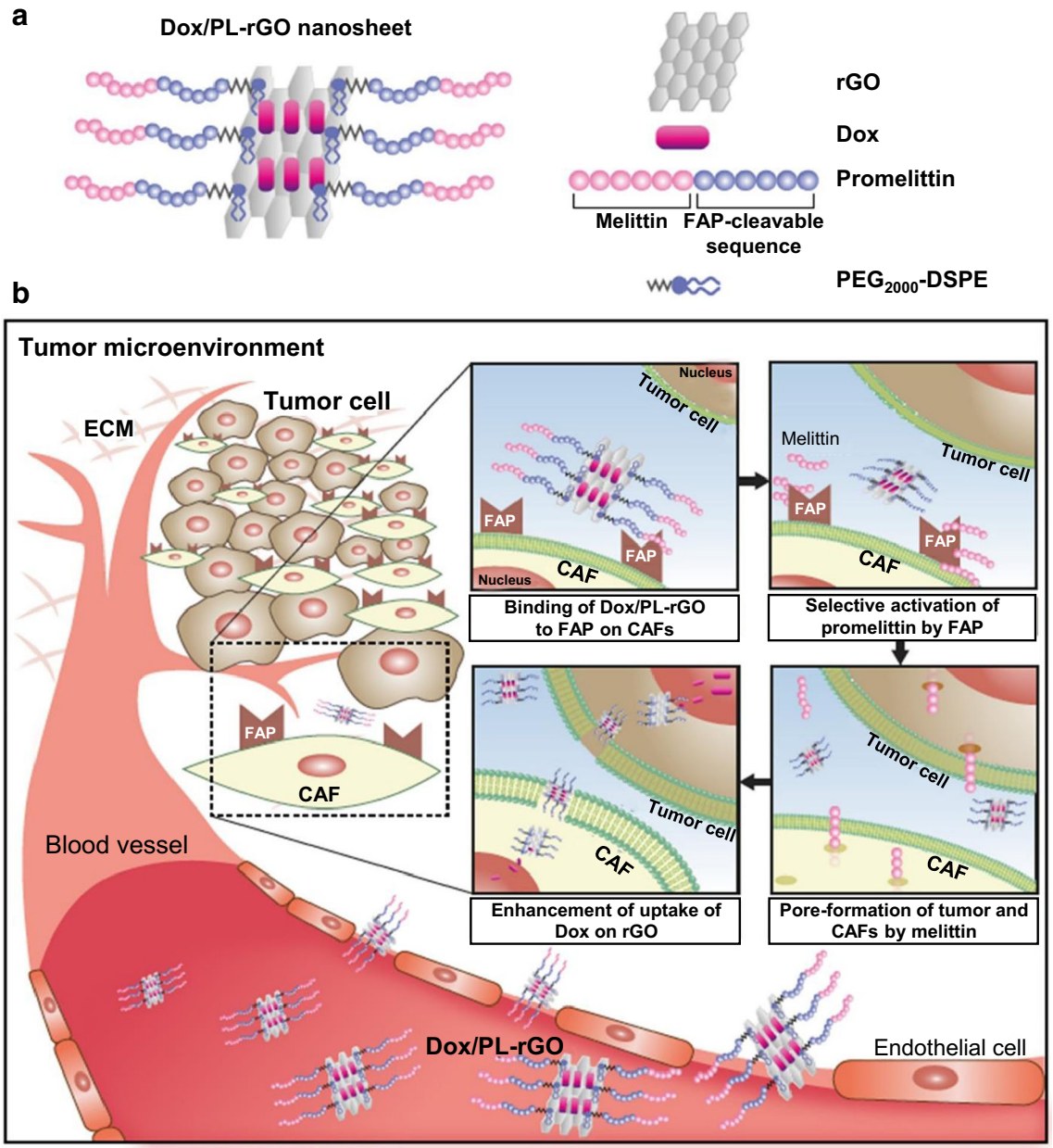

Fig. 4. Nanomaterials activated by CAFs to improve chemotherapy. Selective activation of promelittin- and Dox-loaded rGO nanosheets by CAFs promotes the delivery and therapeutic efficacy of anticancer drug. **a** Design of promellitin- and Dox-loaded rGO nanosheets. **b** Illustration of selective activation of rGO nanosheets at the TME. Reprinted from (68) with permission

can be divided into the M1 phenotype, which produces pro-inflammatory cytokines, and the M2 phenotype, which inhibits immune reactions. TAMs in the TME are known to be of the M2-like phenotype. TAMs support tumor progression by facilitating angiogenesis, metastasis, and suppression of T cell and natural killer (NK) cell activities (89–91). As such, TAMs have been studied as important targets in cancer immunotherapy.

Approaches for selectively eliminating M2-like TAM have been investigated. In the TME, colony-stimulating factor 1 (CSF-1) is a cytokine involved in the differentiation and proliferation of TAMs. Targeting the CSF-1 receptor has been studied using lipid nanoparticles (71). In this study, researchers noticed that expression of the CSF-1 receptor was restricted to TAMs (71,92) and exploited this fact to deliver anti-CSF-1R siRNA to TAMs using lipid nanoparticles modified with dual CSF-1 receptor-targeting peptides. The dual targeting peptides were constructed by linking a scavenger receptor B type 1 (SR-B1) targeting peptide and an M2-macrophage-binding peptide. In a B16F10 melanoma tumor model, anti-CSF-1R siRNA complexed to the peptide-modified cationic nanoparticles showed selective delivery to M2-like TAMs in the tumor relative to macrophages in normal tissues. Treatment with the siRNA-loaded lipid nanoparticles decreased the population of M2-like TAMs in the TME (determined based on expression levels of the M2 macrophage marker CD206) to a greater extent than observed in the untreated group or the group treated with scrambled siRNA-loaded lipid nanoparticles. This

reduction in the population of M2-like TAMs in the TME resulted in infiltration and activation of $CD8^+$ T cells, increased interferon (IFN)-γ, and a reduction in the expression levels of PD-1 and T cell immunoglobulin and mucin-domain containing-3 proteins.

A polymeric nanoparticle encapsulating IL-12 has been studied for reprogramming M2-like TAMs into an M1-like phenotype (72). IL-12 is a pro-inflammatory cytokine mainly secreted by APCs that promotes differentiation of naïve $CD4^+$ T cells into Th1 cells (93). In addition, IL-12 is known to induce repolarization of the macrophage phenotype from tumor-supportive to tumor-suppressive (94). Taking advantage of these immunological features, Wang and colleagues entrapped IL-12 in poly(β-amino ester) copolymer nanoparticles. Intravenous administration of IL-12-entrapped polymeric nanoparticles into mice bearing tumors derived from B16F10 melanoma cells was shown to increase the expression of C–C chemokine receptor 7 and inducible nitric oxide synthase (NOS)—both known M1 macrophage markers—while decreasing expression of the M2 macrophage markers, arginase-1 and CD206. Intravenous or intratumoral injection of mice with IL-12-loaded polymeric nanoparticles resulted in a greater antitumor effect compared to treatment with free IL-12.

In an attempt to increase the population of M1-type macrophages in the TME, another study used hyaluronic acid-modified polyethylenimine complexed to microRNA (miRNA) (73). It has been reported that miRNA-125b is involved in increasing the expression of major histocompatibility complex II and the co-stimulatory molecules, CD40, CD86, and CD80, on macrophages and promoting macrophage responsiveness to IFN-γ (95). To increase binding to the CD44 receptor on macrophages, Parayath and colleagues (73) modified cationic polyethylenimine with hyaluronic acid and prepared a polyplex by electrostatic complexation with miRNA-125b. In KRAS G12D/P53 genetically engineered mice bearing non-small-cell lung cancer, intraperitoneal injection of hyaluronic acid-conjugated poly(ethylenimine) complexed with miRNA-125b increased uptake of the complexes by peritoneal macrophages and enhanced migration of macrophages to the lung. Notably, the ratio of CD80 to CD206—markers of M1 and M2 macrophages, respectively—in lung tissue was more than 3-fold higher in the group treated with polyplexed miRNA-125b compared to the group treated with scrambled miRNA-loaded nanoparticles.

Cyclodextrin-based nanoparticles entrapping an immune-modulating agent have also been investigated as a strategy for repolarizing TAMs towards the M1 phenotype (74). These nanoparticles, prepared with a derivative of beta cyclodextrin, were loaded with resiquimod in the hydrophobic core (74). Resiquimod, an agonist of both toll-like receptor 7 and 8, is in clinical trials for cancer therapy because of its immune-modulating properties (96,97). Upon intravenous administration of resiquimod-loaded cyclodextrin-based nanoparticles into MC38 colorectal cancer-bearing mice, the M1 macrophage population was increased and tumor growth was restricted. Additionally, the combination of resiquimod-loaded cyclodextrin-based nanoparticles and an anti-PD-1 antibody enhanced antitumor effects against MC38 tumors compared to treatment with anti-PD-1 antibody alone.

Ferumoxytol, an iron oxide nanoparticle approved by the FDA for treatment of iron deficiency anemia (98), has been used for reprograming of TAMs and increasing antitumor efficacy (75). The rationale for using iron oxide nanoparticles for TAM reprogramming is that cytotoxic hydroxyl radicals produced by iron oxide in response to macrophage-derived H_2O_2 can convert M2 macrophages to M1 phenotypes. Treatment of co-cultures of macrophages and MMTV-PyMT cancer cells with iron oxide nanoparticles in Transwell systems induced expression of caspase-3 and increased apoptosis in tumor cells compared with untreated controls or treatment without macrophages. Macrophages exposed to the iron oxide nanoparticles showed increased levels of the M1 phenotype-related markers, NOS, TNF-α, and CD86 compared with macrophages that were not treated with ferumoxytol. In addition to elevated expression of M1 phenotype markers, expression of the M2 phenotype markers, arginase-1, CD206, and IL-10, was reduced. Moreover, systemic administration of ferumoxytol suppressed lung and liver metastases of KP1-GFP-Luc cancer.

Nanomaterials that eliminate M2 type TAMs would be advantageous in activating natural immune systems that have been tamed in the TME. The activation of immune systems in the TME may eliminate the toxic side effects associated with the killing of normal cells and tumor cells by anticancer chemotherapeutics. Although the macrophage population in the TME is modulated by specific nanomaterials, the existence of immune checkpoint blockade can still hinder phagocytosis and tumor antigen presentation by macrophages. To maximize the anticancer effect of M1 macrophage populations by nanomaterials, it would be desirable to consider combining nanomaterials with immune checkpoint-blocking antibodies or other agents that promote the immunogenic cell death of tumor cells.

One additional potential problem of this TAM-modulating approach is the possibility of inflammatory disorders caused by an imbalance between M1 and M2 populations, given that the effects and distribution of nanomedicines are not completely specific to the tumor. An uncontrolled increase in M1 macrophages in normal tissues could potentially cause inflammation. Another hurdle in targeting TAMs is the diverse and indefinite phenotype of these cells: TAMs may express markers of both M1 and M2 states (99); thus, M1 and M2 may be two extremes of macrophage phenotype. A quantitative determination of M1/M2 ratios in tumor tissues and normal tissues would be crucial in evaluating TAM phenotype-modulating nanomaterials.

Nanomaterials for Modulation of Treg Cells

Treg cells are a group of cells with diverse immune-suppressive functions. Treg cells suppress immune responses by destroying B and effector T cells, and by producing granzyme, perforin, and anti-inflammatory cytokines, such as TGF-β and IL-10. Treg cells downregulate the functions of APCs by producing molecules such as CTLA4 (100–102). Although suppression of immune activity is a normal function of Treg cells, the same process hinders antitumor immune response and promotes cancer progression. Therefore, a higher number of Treg cells in tumor tissue are generally associated with poor prognosis (103). Nanomaterials such as polymeric nanoparticles (76,77) and polymeric/lipid hybrid nanoparticles (78) have been studied for their potential to deliver Treg cell-suppressing agents as adjuvants in cancer immunotherapy.

Polymeric nanoparticles encapsulating imatinib, an FDA-approved tyrosine kinase inhibitor that is conventionally prescribed for leukemia and gastric cancer (104), have been investigated for their ability to suppress Treg cell function in the TME (76). Imatinib decreases the activation of signal transducer and activator of transcription (STAT) 3 and 5, and downregulates the expression of the transcription factor Foxp3, thereby suppressing the function of Treg cells (105,106). Ou and colleagues entrapped imatinib and IR-780 in PLGA nanoparticles, and modified their surfaces with anti-GITR (glucocorticoid-induced TNF receptor family-related protein) antibodies for enhanced Treg cell-targeting ability. The nanoparticles were further coated with polyhistidine and PEG-block-poly(L-glutamic acid) using a layer-by-layer method. The presence of PEG on the outermost surfaces of polymeric nanoparticles provides stability during circulation in the blood, whereas polyhistidine, which is protonated at the acidic pH in the tumor, serves as a pH-sensitivity-conferring layer that deshields and exposes the GITR antibody on the particle's surface. NIR irradiation enhanced the release of imatinib from the polymeric nanoparticle matrix and exerted a photothermal anticancer effect. Treatment with drug-loaded polymeric nanoparticles followed by NIR irradiation decreased the population of Treg cells in the tumor tissues compared to treatment with free imatinib or pretreatment with GITR alone.

Indoleamine 2,3-dioxygenase (IDO) inhibitors entrapped in polymeric oxaliplatin-based micelles have also been investigated for their potential to impair the functions of Treg cells (77). IDO converts tryptophan to kynurenine and thereby impairs the function and proliferation of cytotoxic lymphocytes. Upregulation of IDO in the TME thus facilitates the differentiation and proliferation of Treg cells and generates an immunosuppressive environment. Among IDO inhibitors is NLG-919, a potent IDO inhibitor that is undergoing several immunotherapy clinical trials. Feng and colleagues (77) synthesized a dimeric NLG-919 containing a disulfide bridge that acts as a bioreducible linker in the TME and then encapsulated it in micelles that self-assembled from a PEG amphiphilic derivative of oxaliplatin. This combination was predicted to synergize through the immunogenic cell death induced by oxaliplatin and IDO inhibition by NLG-919. The PEG moiety of the particles was designed such that, in the acidic TME, it would be removed by cleavage of the pH-sensitive linker. In 4T1 tumor-bearing mice, intravenous administration of polymeric micelles encapsulating IDO inhibitors caused the greatest decrease in the population of Treg cells and the highest $CD8^+$ T cell/Treg cell ratio in tumor tissue compared to treatment with saline, free NLG-919, oxaliplatin or a physical mixture of oxaliplatin and NLG-919.

Delivering CTLA4 siRNA with polymeric and lipid hybrid nanoparticles has been shown to decrease the number of tumor-infiltrating Treg cells (78). In a recent study by Li and colleagues, siRNA specific for CTLA4 (siCTLA4) was loaded into cationic nanoparticles composed of PEG-block-poly(D,L-lactide) and cationic lipid N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryoxycarbonyl-aminoethyl) ammonium bromide. Administration of three intravenous injections of nanoparticles carrying siCTLA4 altered the composition of T cells inside B16 melanoma tumor tissue, producing a decrease in Treg cells and an increase in $CD8^+$ T cells. Repeated intravenous administration of nanoparticles carrying siCTLA4 into B16 melanoma tumor-bearing mice was found to increase levels of the pro-inflammatory cytokines, IFN and TNF, and inhibit tumor growth to a greater extent than treatment with saline, plain nanoparticles or control-siRNA-loaded nanoparticles.

Compared with strategies for modulating CAFs and M2-type TAMs in the TME, the use of a nanomaterial-based approach for suppressing Treg cells in the TME has been less actively studied to date. More studies are needed to judge whether suppressing Treg cells offers any unique advantages over other types of immune cell modulation in the TME. One concern in modulating Treg cells in the TME is the efficiency of nanomaterial targeting of Treg cells. Treg cell populations in the TME are known to be low. If some Treg cells are not effectively modulated by the nanomaterials, anti-inflammatory cytokines secreted from the remaining Treg cells can still exert an immunosuppressive influence in the TME. Moreover, there are other physiological and cellular processes for suppressing immunological features of the TME. Ultimately, overcoming such limitations will require multifunctional nanomaterials that can suppress Treg cells and activate the secretion of inflammatory cytokines.

Nanomaterials for Modulation of MDSCs

MDSCs, which originate from myeloid progenitor cell that are unable to mature into dendritic cells (DCs), granulocytes, or macrophages, are innate immune cells that play an important role in cancer immunosuppression. In the immunosuppressive TME, IL1-β, IL-6, IFN-γ, and VEGF contribute to the differentiation of MDSCs. MDSCs promote the differentiation of Treg cells while inhibiting the activation and proliferation of cytotoxic T cells (10,11). Nanomaterials, such as high-density lipoprotein (HDL) derivative-coated gold nanoparticles (79) and lipid-coated mesoporous silica nanoparticles (80), have been used to suppress MDSCs, whereas gemcitabine-loaded liposomes (81) have been studied for depletion of MDSCs.

HDL is a natural carrier circulating in the bloodstream that scavenges cholesterol and delivers it to target cells. Cholesterol-carrying HDL has been reported to have a high affinity for scavenger receptor type B1 (SR-B1), which is highly expressed in innate immune cells (107–109). ApoA-1, a component of HDL, has demonstrated anticancer effects; consistent with this, high plasma concentrations of apoA-1 are correlated with a lower cancer risk. However, the mechanism underlying this anticancer activity is still unclear (110–112). In a recent pioneering study, a synthetic HDL nanoparticle, with a gold nanoparticle as the core material and surface-modified with human apoA-1 protein, was designed to suppress MDSCs in the TME (79). Biological HDL nanoparticles were mimicked by exploiting two types of lipid—1-2-dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3phosphoethanolamine-N-[3-(2-pyridyldithio) propionate]—which were coated secondarily on the surface of human apoA-1 protein-coated gold nanoparticle at a molar ratio of 1:4. The resulting synthetic HDL nanoparticles were targeted to SR-B1 protein expressed on MDSCs. In B16 melanoma model mice, intravenous administration of these synthetic HDL nanoparticles was shown to

reduce the MDSC population and metastatic nodules in the lung.

In another recent study, lipid-coated calcium phosphate nanoparticles containing gemcitabine were used to deplete MDSCs rather than inhibit their activity (81). Although gemcitabine is a synthetic analog of nucleotide used for systemic treatment of various types of cancer, it was recently reported to have a specific killing effect on MDSCs at a relatively low dose (113,114). In B16F10 melanoma mice, treatment with gemcitabine-loaded, lipid-coated calcium phosphate nanoparticles significantly decreased the number of MDSCs in peripheral blood and tumor tissue compared to treatment with free gemcitabine. Notably, depletion of MDSCs was associated with a reduction in Treg cells and maintenance of the $CD8^+$ T cell population.

Mesoporous silica nanoparticles entrapping all-trans retinoic acid (ATRA) have been investigated for reprogramming MDSCs (80) (Fig. 5). ATRA is a derivative of vitamin A that has been approved for the treatment of acute promyelocytic leukemia (115). It is also known to sensitize cancer cells to chemotherapy and promote the differentiation of MDSCs into antitumor phenotypes, such as mature DCs or macrophages (116). In a study by Kong and colleagues, the hollow core of mesoporous silica nanoparticles was loaded with ATRA and Dox. The drug-loaded nanoparticles were then coated with IL-2 through physical adsorption and further coated with dipalmitoyl phosphatidylcholine, cholesterol, and DSPE-PEG_{2000} for added stability in vivo. Intravenous injection of these mesoporous silica nanoparticles decreased the population of MDSCs by 2.7-fold compared with untreated controls, in association with an increase in mature DCs, NK cells, and cytotoxic T cells. In addition to altering the composition of MDSCs and other immune cells in the TME, this study is notable for its investigation of the pharmacokinetic patterns of nanoparticle-mediated drug delivery. Compared with the physical mixture of IL-2, Dox, and ATRA, administration of the three components in a nanomaterial form altered the pharmacokinetics of ATRA, increasing its half-life, mean residence time, and area under curve values by more than 2-fold compared to treatment with a physical mixture of the three components.

Reprogramming or suppressing MDSCs would be an advantageous approach for potent immunological modulation of the TME at the progenitor cell level, although its ability to rapidly modulate established immune cell populations in the TME could be limited. Experiments designed to develop nanomaterials carrying cytokines such as IL-2 for promoting immunological reshaping of the TME have been conducted. The uncontrolled release of cytokines in the bloodstream can result in severe immune-related adverse effects, such as capillary leak syndrome or secondary cytokine release (117). Thus, minimizing such side effects of MDSC-cytokine reprogramming will ultimately require the design of nanomaterials that selectively liberate cytokines at the TME.

CHALLENGES AND FUTURE PERSPECTIVES

Although nanomedicine has achieved great progress in developing multifunctional nanomaterials that both modulate the TME and support chemo/immunotherapy, this field still faces numerous challenges. The remaining challenges generally fall under the heading of systems for evaluating TME simulation and the design of nanomaterials. In addition to overcoming current challenges, it will be important to study combinations of TME-modulating nanomaterials with other agents, and application of TME-modulating nanomaterials to other types of cancers besides solid tumors.

One current challenge is to develop in vitro and in vivo evaluation systems that simulate the human TME and thus provide platforms for testing the efficacy of nanomaterials. In this context, co-cultures of tumor cells and tumor tissue-derived cells would better mimic in vivo TME conditions and thus provide a closer correlation between in vitro and in vivo studies. The TME is a complex combination of various factors—including the ECM, cytokines, and stromal cells—that are strongly related to tumor promotion and invasion. In evaluating TME-modulating nanomaterials in vitro, particular attention needs to be paid to the choice of cultured cells. It has been reported that the cells in the TME are tamed and thus are different from cells in normal tissues. For example, CAFs in the TME are known to promote tumor progression and metastasis, whereas fibroblasts in normal tissues inhibit tumor progression (118). Yet, despite such prominent differences between cells in the TME and their counterparts in normal tissues, some studies have used fibroblast cell lines, which may be quite different from CAFs.

In particular, important for in vitro models used to evaluate nanomaterials is the need to develop 3D culture systems that mimic the TME in vivo. To date, the most popular model for evaluating the efficacy of nanoparticles in vitro has been 2D monolayer-cultured cancer cells. However, these 2D systems cannot recapitulate the barriers of 3D networks, which will affect the penetration, diffusion, and contact of nanoparticles with cancer cells. Several 3D culture models that closely mimic features of the TME, including the ECM, blood vasculature, and supporting stromal cells, have recently been developed. Breast cancer cells co-cultured with fibroblasts using magnetic levitation were shown to form millimeter-size 3D spheroids containing ECM proteins (119). The application of 3D tumor models to screen the effects of nanoparticles would provide greater insight into the interactions of nanomaterials with the TME.

In evaluating TME-modulating nanomaterials, it is important to provide in vivo validation that the predicted changes in the TME actually occur, in addition to demonstrating tumor growth inhibition effects. Although 3D organoid cell systems have often been used to mimic the TME, demonstration of an anticancer effect alone may not provide in-depth insights into the working mechanisms of the TME-modulating nanomaterials. Thus, data obtained in vitro, even using 3D organoid systems, should be accompanied by in vivo data demonstrating changes in the TME, such as disruption of the ECM, mitigation of hypoxic conditions, disruption of vascular systems, and infiltration of immune cells.

In most in vivo studies, TME immune cell-modulating nanomaterials have been tested in non-orthotopic tumor models, in which tumor formation is generally established by subcutaneous implantation of cancer cells. Such subcutaneous tumors show considerable differences in their pattern of immune stromal cells compared with that of orthotopic

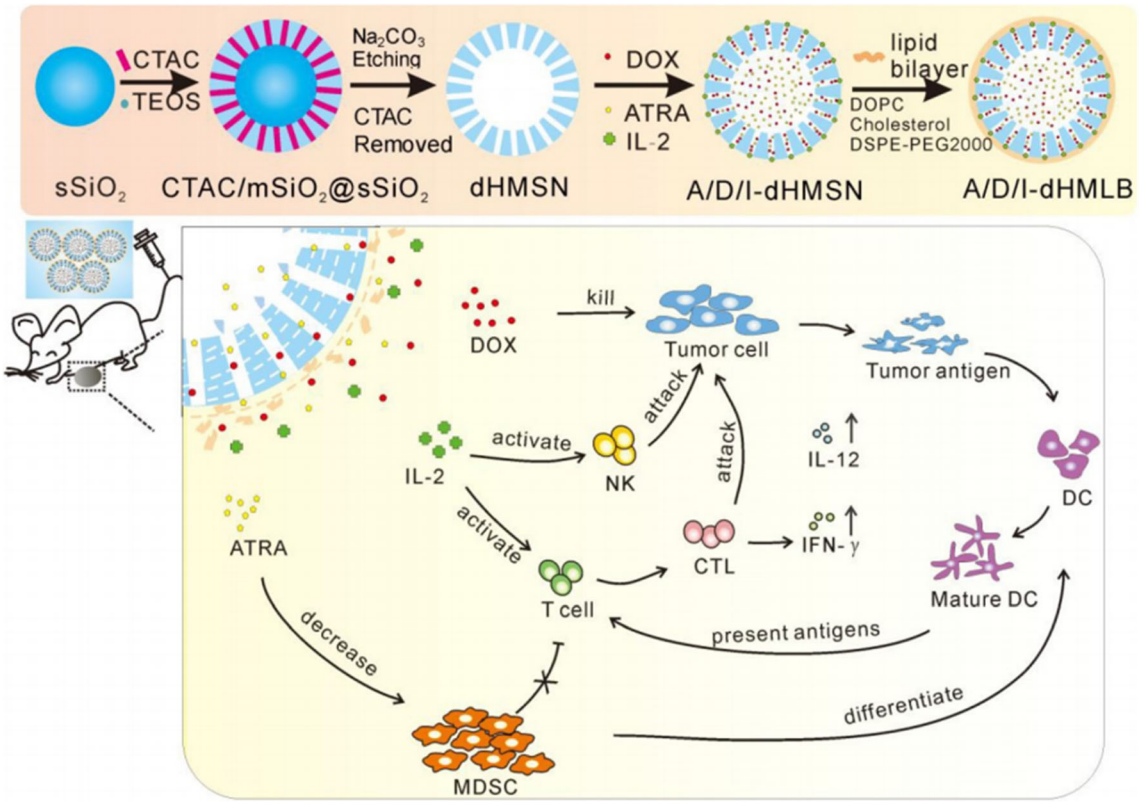

Fig. 5. Nanomaterials used for reprogramming MDSCs. All-trans retinoic acid (ATRA), delivered by mesoporous silica nanoparticles, induced the differentiation of MDSCs into mature DCs. Co-treatment with IL-2 and Dox, incorporated in nanoparticles, synergized in the reversion of the immunosuppressed TME. Reprinted from (80) by permission.

tumor models. In human colorectal cancer models, subcutaneous tumors exhibit less infiltration of immune cells, including T cells, B cells, and NK cells, but greater recruitment of immunosuppressing MDSCs (120). Thus, for studies of nanomaterials that modulate immunological features of the TME, it would be desirable to perform evaluations in orthotopic models. Several recent studies have evaluated the efficacy of nanoparticles in orthotopic breast cancer or colorectal cancer models (121–123).

Future studies will need to analyze the pharmacokinetics and pharmacodynamics of TME-modulating nanomaterials. Most TME-modulating nanomaterial development efforts have focused on their therapeutic anticancer efficacy. Although demonstrating an anticancer effect is crucial at initial phases of development, the pharmacokinetics of drugs and other immunomodulating agents should be investigated. Moreover, the biodistribution and retention of compounds delivered by nanomaterials should be studied in parallel. Notably, few studies have investigated the effects of TME-modulating nanomaterials in other non-target tissues. If nanomaterials containing TME-modulating drugs or other immunomodulating agents are distributed to the spleen or other immune organs, they could affect T cell composition in these tissues, a possibility that should be studied.

Combination ratios and pharmacodynamic relationships at target sites should be carefully considered in the design of nanomaterials co-loaded with TME-modulating agents and anticancer drugs. For multiple drug-carrying nanomaterials, combination ratios should be studied in advance to maximize their synergistic therapeutic effect. Basic studies of combination indexes might be useful for constructing nanomaterials that carry multiple components. Because ECM- or vasculature-modulating agents do not kill cancer cells, nanomaterials have usually been co-loaded with TME-modulating agents and anticancer drugs. However, for nanomaterials modified to contain ECM- or vascular-disrupting agents, their locations in the TME could be crucial for their ability to exert the desired pharmacological effects.

For the design of TME-modulating nanomaterials, the specificity of ligands that target the TME needs to be verified. In some studies, nanomaterials have been modified with a targeting ligand (e.g., RGD peptide) that can bind both the tumor vasculature and tumor cells (25). Although it might be possible for a higher fraction of TME-targeted, ligand-modified nanomaterials to associate with the tumor vasculature rather than tumor cells, the ability of such nanomaterials to bind and be taken up by tumor cells could reduce their selective delivery to the TME and thus limit their efficacy. With progress in understanding marker proteins of various cells in the TME, it will become possible to modify nanomaterials with new antibodies or peptide ligands that can recognize specific cells in the TME.

Combining imaging agents and TME-modulating nanomaterials would be one avenue for future studies. For intravenously administered TME-modulating nanomaterials, tumor tissues would be reached via EPR-based accumulation, which is known to occur because of the leaky vasculature of tumor tissues resulting from rapid tumorigenesis and

haphazard blood vessel formation. The ability of TME-modulating nanomaterials may thus depend on the extent of the EPR effect. In tumor tissues with extensive EPR effects, the accumulation of nanomaterials may be substantial. By contrast, in tumors with negligible EPR effects, such as pancreatic cancer, tumor tissue accumulation and TME modulation of nanomaterials would be minimal. Indeed, inconsistent EPR effects were shown to result in variable responses in patients treated with TME-modulating nanoparticles (124). Screening and selection of patients who would benefit from EPR-based accumulation of nanomedicines should precede translation of TME-modulating nanomaterials.

Because the goal of nanomaterial-based modulation of the TME is to alter physiological and/or immunological microenvironments of solid tumors, the effects of TME-modulating nanomaterials on metastasized or initial phases of undetectable tumors, which has received little research attention, still need to be demonstrated. To date, the selection of nanodrugs that modulate the TME has depended on the stage of tumor growth and type of tumor. The TME-modulating strategy may show clear effects only in large-size tumors where the TME has a greater impact on tumorigenesis; the distribution of TME-modulating nanomaterials to small-size metastasized tumors remains in question.

Future nanomaterials for degrading extracellular components or disrupting tumor vasculature will need to be designed and constructed for prolonged retention in the TME. One factor working against this is the uptake of nanomaterials by tumor cells, which can diminish the effects of TME-modulating agents. In many studies, the TME-modulating nanomaterials tested also entrap anticancer agents that act on targets inside tumor cells. Targeting tumor cells as well as the ECM using the same type of nanomaterial may not be effective. Sequential activation of nanomaterials that liberate TME-modulating agents at the ECM and deliver anticancer drugs to tumor cells might be one strategy for delivering pharmacologically different agents to their respective target sites.

One potential future approach would be to combine immune checkpoint blocking and TME-modulating nanomaterials. The clinical approval of several immune checkpoint-blocking antibodies has opened a new avenue for targeting the tumor immune microenvironment. Although positive results have been observed in clinical trials, responses were limited in 20–30% of treated patients (125). This modest success rate may be attributable to the strong integrity of stromal cells in the TME as well as the development of other pathways that exert negative regulatory effects. Various strategies that utilize advanced nanomaterials have been investigated for improving the efficacy of immunotherapy (126). Innate immune cells, which are recruited into the TME, also represent attractive targets for therapeutic nanocarrier development (127). Further research into the relationship between the ECM and tumor stromal immune cells could lead to more efficient strategies for fighting cancer.

CONCLUSIONS

Numerous studies have established the important role of the TME in tumor progression, invasion, and metastasis. Conventional nanomedicines that target tumor cells only are unable to provide sufficient efficacy to eliminate tumors owing to tumor heterogeneity, a lack of specific targeting ligands, and limited penetration into tumor tissues. Modulating the TME using various nanomaterials instead of directly targeting tumor cells has shown a certain degree of success. Nanomaterial-based modulation of the TME has been studied for its potential to enhance the efficacy of chemotherapy, immunotherapy, and chemoimmunotherapy. Nanomaterials that disrupt the ECM and/or tumor vasculature and increase blood perfusion have been developed to increase the penetration and intracellular delivery of anticancer chemotherapeutics. Nanomaterials that modulate CAFs, TAMs, Treg cells, or MDSCs have been shown to alter the activities and populations of immune cells in the TME. Despite considerable progress, nanomaterial-based physiological modulation of the TME must overcome several hurdles, including the possibility of tumor cell metastasis into the bloodstream nonspecific uptake by the cells in TME, and limited penetration depth. In the case of nanomaterial-based immunological modulation of the TME, the challenges include improvements in specific immune cell targeting, concurrent blocking of immune checkpoint blockade, and the possibility of immune toxicity. Moreover, the design of evaluation systems that effectively mimic clinical situations may increase the feasibility of successful translation of these nanomedicines. Although this field is still in its infancy and faces several challenges, modulating the TME may provide new targets and modalities that improve clinical outcomes of nanomaterial-based cancer therapy.

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