Macrophages associated with tumors as potential targets and therapeutic intermediates

Tumor-associated macrophages (TAMs) form approximately 50% of tumor mass. TAMs were shown to promote tumor growth by suppressing immunocompetent cells, inducing neovascularization and supporting cancer stem cells. TAMs retain mobility in tumor mass, which can potentially be employed for better intratumoral biodistribution of nanocarriers and effective tumor growth inhibition. Due to the importance of TAMs, they are increasingly becoming principal targets of novel therapeutic approaches. In this review, we compare features of macrophages and TAMs that are essential for TAM-directed therapies, and illustrate the advantages of nanomedicine that are related to the preferential capture of nanocarriers by M ϕ in the process of drug delivery. We discuss recent efforts in reprogramming or inhibiting tumor-protecting properties of TAMs, and potential strategies to increase efficacy of conventional chemotherapy by combining with macrophage-associated delivery of nanodrugs.

Keywords: biodistribution of nanoparticles • cancer stem cell • eradication of cancer cells • macrophage • nanocarrier • phagocytosis • reprogramming of macrophages • 'Trojan horses' • tumor-associated macrophage • tumor-supporting function

Role of macrophages in tumor development

Tumor-associated macrophages are too numerous to be ignored

Tumors are undoubtedly quasi-organs, where different cell types, including neoplastic cells, stem-like cells, fibroblasts, endothelial cells and immunocompetent cells, interact with each other. The population of immune cells in tumors is also complex, and these cells play numerous roles in tumorigenesis. Inflammation and infiltration of the tumor tissue by tumor-associated macrophages (TAMs), myeloid-derived suppressor cells and regulatory T cells have been shown to support tumor growth, invasion and metastasis [1]. Blood monocytes originated from bone marrow (BM) and spleen differentiate into two distinct types of macrophage (M ϕ), classically activated or M1 M ϕ ('killing' phenotype), and alternatively activated or M2 M ϕ ('healing' phenotype). TAMs may represent

up to 50% of the tumor mass and, as now generally accepted, most TAMs have the M2 phenotype [2]. In the vast majority of human cancers, high density of TAMs correlates with poor prognosis [3]. TAMs express immunosuppressive mediators and promote tumor growth through release of trophic factors and invasion-facilitating enzymes (Figure 1) [4]. Increased expression of IL-10 and TGF- β by TAMs contributed to local immunosuppression and greater lymph node involvement, and correlated with the late stage of lung cancer [5]. An important role of TAMs in angiogenesis was also determined as an important mechanism facilitating tumor progression [6].

The main function of M1 M ϕ is phagocytosis in response to bacterial stimuli and/or Th1 cytokine release, while the main function of M2 M ϕ is immunosuppression and trophic activity in response to Th2 cytokines [7]. TAMs produce various chemokines (e.g., CCL17 and CCL22) that preferentially

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Figure 1. Principle functions of tumor-associated macrophages and major factors released during tumor development.

MMP: Matrix metalloproteinase; NO: Nitrogen oxide; PGE: Prostaglandin; ROS: Reactive oxygen species; TAM: Tumor-associated macrophage.

attract T-cell subsets devoid of cytotoxic functions. In tumors, TAMs can be detected by the presence of a pan-macrophage marker CD68 that is common for both M1 and M2 M ϕ , hemoglobin scavenger receptor CD163, and by overexpression of mannose receptor CD206 [8]. Downregulation of CD11c was observed too, for example, in pancreatic cancer, as it progressed from chronic pancreatitis to ductal adenocarcinoma, and M ϕ switched to the M2 phenotype, supporting metastasis, angiogenesis and tumor development [9]. Enhanced tumor progression in mice was closely related to the transition of TAMs from high to low MHC class II phenotype [10]. Thus, the TAM population in tumors can also be heterogeneous, and many important details about it remain to be clarified in future research.

TAMs in tumorigenesis & metastasis

TAMs are originated from monocytes activated in the tumor microenvironment. Tumors actively recruit monocytes and M ϕ , and the M1-to-M2 conversion is a local tumor-associated event that becomes more frequent with tumor development. This process includes spleen M ϕ , which have been recently identified as TAM precursors [11]. Conversion of spleen M ϕ and activation

of blood-circulating monocytes evidently occur on the tumor periphery. Recent data suggest that the BM-derived mesenchymal stem cells (MSCs), pluripotent progenitor cells that contribute to the maintenance and regeneration of a variety of connective tissues, are recruited in large number into the stroma of developing tumors [12]. Although MSCs reside predominantly in BM, they are distributed throughout many other tissues, where they are thought to serve as a local source of dormant stem cells (SCs) [13]. Furthermore, the potential link between monocyte activation or M ϕ conversion into TAMs and cancer stem cells (CSCs) should not be ignored too. Although the origin of the very small population of CSCs currently remains unknown, tissue-specific SCs are considered as potential candidates. The mutual support of BM-derived SCs and tumor-associated immune cells was recently highlighted in tumor metastasis [14]. It seems that active CSCs should be able to promote the M1-to-M2 conversion, induce neovasculation formation via VEGF release, and build CSC-protective niches via tissue-repair pathways [15]. Recently, some of TAMs–CSCs interrelations were affirmed experimentally. TAMs were found to release a milk-fat globule EGF-VIII, which activated the CSC-specific pathways: STAT3, Hedgehog and Sonic, and

amplified a prominent drug resistance and tumorigenicity of CSCs [16]. In experiments with murine mammary CSCs, Yang *et al.* reported that drug resistance of CSCs was associated with new EGFR/STAT3/Sox-2 paracrine signaling pathway activity that was realized via complex interplay between CSCs and TAMs [17]. Microglia and brain M ϕ also served as mediators of glioma stem-like cell (GSLC) properties, producing high levels of TGF- β , which made GSLCs more invasive. CD133⁺ phenotype of GSLCs was also associated with the high expression of the TGF- β receptor. Release of TGF- β was accompanied with the elevated production of matrix metalloproteinase-9, a serine protease that increases invasiveness of GSLCs [18].

However, there are not enough current data to identify the nature of all tumor-associated factors engaged in M ϕ conversion to TAMs. Participation of M2 M ϕ in tumor development is similar to the process of wound healing. Wound healing advances through several precisely programmed phases: hemostasis, inflammation, proliferation and remodeling, which must occur in the correct sequence and time [19]. Wound triggers mobilization of BM-derived MSCs and endothelial progenitor cells involved in the neovascularization. These steps are very similar to tumorigenesis, when active CSCs initiate formation of primary tumor or metastatic nodes and, perhaps, play an important role in the M1-to-M2 conversion. Therefore, tumorigenesis could be considered as a deviated natural healing process with a participation of transformed SCs such as CSCs and M ϕ such as TAMs. Active CSCs located on the growing tumor periphery can release factors attracting M ϕ and converting them into TAMs. One of these factors, colony stimulating factor-1, a major growth factor of the mononuclear phagocytic lineage, as well as chemoattractant for these cells, was identified [20]. It helps to recruit M ϕ to the tumor site where they promote tumor progression to malignancy. Inhibition of TAM recruitment by a colony stimulating factor-1 neutralizing antibody in mammary tumors led to more efficient treatment by paclitaxel, reducing tumor progression and metastasis. Dormant CSCs residing in hypoxic area of tumors may be activated after chemotherapy when most peripheral cancer cells are eliminated and CSCs become exposed. Removing debris M ϕ recruited to the site may uncover dormant CSCs and activate them. It was shown that TAMs participate in reparative mechanisms after radiotherapy or antiangiogenic treatment. Depending on many factors, they either enhance or antagonize the efficacy of radio- or chemo-therapies, and immunotherapeutic agents such as tumor-targeting antibodies [21].

There is a strong correlation between amount of TAMs and metastasis [43]. In animal models with

inhibited metastatic development, TAM infiltration was diminished. Subcutaneous administration of liposomal clodronate markedly reduced the number of monocytes in peripheral blood and resulted in efficient suppression of both bone and muscle metastasis in a lung cancer HARA-B mouse model [44]. One of the existing metastasis hypotheses implied that metastasizing cells travel to peripheral niches occupied by BM-derived MSCs [45]. The phenomenon of cell fusion that is crucial in a variety of physiological processes, including wound healing and tissue regeneration, was also suggested as a source of metastases. According to the fusion hypothesis, TAMs form hybrids with tumor cells and travel to distant sites to initiate metastases [46]. Authors observed the formation of hybrid cells between M2 M ϕ , MCF-7 and MDA-MB-231 cancer cells in the presence of 50% polyethylene glycol (PEG) with the efficacy of 1.8–6.5%. These hybrids gained a CD44⁺/CD24⁻ phenotype that is characteristic to CSCs, and they furthermore demonstrated increased migration, invasion and tumorigenicity. The hybrids, however, had a reduced proliferative ability compared with parental cell lines. They exhibited chemotactic migration *in vitro* toward fibronectin and high frequency of metastasis when implanted in mice. Alternatively, according to the CSC hypothesis, mobile CSC-containing spheroids migrate out of the primary tumor site and start metastatic growth in the niches with favorable conditions (Figure 2). CSCs are required to initiate new tumor growth in the distant sites. When a CSC becomes active, it forms spheroid, which is capable of entering the bloodstream or lymphatic circulation and traveling until it finds a convenient niche. M ϕ associated with the repair of inflamed lesions may serve as niche-forming cells attracting CSCs. Metastatic foci can be further supported by mutual interaction of these two cell types and the conversion of M2 M ϕ to TAMs required for promoting tumor growth. The potential ‘fusion’ of these two hypotheses was recently discussed in [47].

Role of macrophages in nanodrug delivery

One of the major limitations of systemic nanomedicine is the rapid elimination of nanocarriers from blood circulation by phagocytes of the reticuloendothelial system (RES). Phagocytosis is preceded by opsonization, or protein binding on the nanocarrier surface [48]. Over 70 different serum proteins have been found heterogeneously adsorbed on the surface of gold nanoparticles (NPs) after intravenous (iv.) injection [49]. Surface properties and opsonization strongly affect the nanodrug delivery; therefore, characterization of NPs using an *in vivo* uptake by M ϕ is currently required as an important quality test [50]. Surface PEGylation

Figure 2. Putative role of TAMs in metastasis and tumorigenesis and potential application of TAMs for drug delivery in tumor volume. (A,i) Mobilization of healing M2 M ϕ to lesion site, (A,ii) lesion seeding with cancer cell spheroid-containing CSCs, and (A,iii) conversion of M ϕ to TAMs; the latter assists in (A,iv) cancer cell proliferation and tissue penetration. (B) Release of angiogenic factors by TAMs induces growth of neovascularity. (C) Monocyte activation and M ϕ conversion into TAMs in growing tumor. (D) Accumulation of nanodrugs in tumor via neovascularity (enhanced permeability and retention effect) and the potential route of their intratumoral distribution by TAMs, which can capture nanodrugs to carry them into the hypoxic area. Dashed lines show release of modifying/activating factors. Solid lines show cell movement. Yellow cells are CSCs. Blue ovals are neovascular endothelial cells.

CSC: Cancer stem cell; M ϕ : Macrophage; TAM: Tumor-associated macrophage.

was able to significantly increase the plasma half-life of different nanocarriers. Although PEGylation reduced

RES uptake and extended the plasma half-life of nanocarriers, often it negatively affected the cellular uptake.

Multivalent modification of NPs by small vector molecules provides a practical technique for cell-specific targeting [51]. A significant amount of research aimed to reduce the RES uptake of nanocarriers and increase the percentage of the injected dose accumulated in tumors by using vectorized nanocarriers has produced no evident breakthrough. An extremely complex interplay of different factors affects the fate of NPs *in vivo* and poses a serious barrier to wide applications of therapeutic NPs [52]. **Table 1** summarizes the examples of various M ϕ -targeting approaches and applications of nanocarriers.

There are several factors that affect the capture of NPs by M ϕ . The size, surface charge and shape of NPs affect it in a nonspecific way. Many have studied the effect of surface charge of NPs on cellular uptake and concluded that neutral particles are generally less attractive to cells compared with the charged NPs. Cationic NPs are better captured by M ϕ compared with anionic NPs [53]. NPs with either a high positive or negative charge demonstrated high liver retention and low tumor accumulation compared with NPs with low negative charge [54]. The size and shape of NPs are also important for their fate *in vivo*. M ϕ are able to capture and engulf

Table 1. Macrophage-mediated drug delivery and treatment options.

Disease	Nanomaterials	Purpose	Models	Ref.
Cancer	Gold nanoshells	Killing tumor cells by NIR irradiation of loaded M ϕ	Spheroids/ <i>in vitro</i>	[22–24]
Cancer	Macrolide-conjugated gold nanorods	Killing cancer cells around TAMs by NIR irradiation	<i>In vitro</i>	[25]
Cancer	SN38-loaded iron oxide magnetic NPs	Using M ϕ for killing cancer cells by magnetic field	<i>In vitro</i>	[26]
Cancer	Gold NPs attached to CpG, anti-IL-10 and anti-IL10 receptor oligos	M ϕ -targeted delivery to inhibit TAMs in cancer therapy	<i>In vitro/in vivo</i>	[27]
Cancer	Oligomannose-coated liposomes/5-FU	M ϕ -targeted delivery to control metastasis	<i>In vivo</i>	[28]
Cancer	Mannose-polymeric micelles/siRNA	M ϕ -targeted delivery to inhibit cancer growth	<i>In vitro</i>	[29]
Cancer	LYP1-conjugated protein NPs	Visualization of M ϕ -rich areas for diagnostic purpose	<i>In vitro/in vivo</i>	[30]
Cancer	Hemoglobin-decorated liposomes	Targeting M ϕ via CD163 receptor	<i>In vitro</i>	[31]
Cancer	miR-143 entrapped in microvesicles	Using M ϕ for anticancer miR-143 delivery	<i>In vitro/in vivo</i>	[32]
Cancer	QDs/PAMAM linked to the surface of M ϕ	Imaging/drug delivery to hypoxic regions of tumors	<i>In vivo</i>	[33]
Cancer	QDs loaded in M ϕ	Imaging of brain tumor	<i>In vivo</i>	[34]
Cancer	HPMA-Gd NPs	MRI imaging by loaded M ϕ	<i>In vivo</i>	[35]
HIV	Nanogels loaded with NRTI triphosphates	Drug delivery to brain M ϕ to treat HIV infection in CNS	<i>In vitro/in vivo</i>	[36]
HIV	fMLF-NPs	M ϕ targeting for antiviral drug delivery	<i>In vivo</i>	[37]
HIV	Antiretroviral drugs or drug-loaded NPs	Nano-ART using M ϕ to treat HIV infection in the brain	<i>In vitro/in vivo</i>	[38,39]
Parkinson's disease	Catalase/pDNA in PEG-PLL complexes	CNS delivery of catalase using M ϕ	<i>In vitro/in vivo</i>	[40,41]
Tuberculosis	Rifampicin-PLGA NPs	M ϕ as carriers for NP delivery to the disease site	<i>In vivo</i> inhalation	[42]

5-FU: 5-fluorouracil; ART: Antiretroviral therapy; fMLF: *N*-formyl-methionyl-leucyl-phenylalanine; Gd: Gadolinium; HPMA: *N*-(2-hydroxypropyl)-methacrylamide; M ϕ : Macrophage; NIR: Near infrared; NP: Nanoparticle; NRTI: Nucleoside reverse transcriptase inhibitor; PAMAM: Polyamidoamine; PEG-PLL: Polyethylene glycol-poly(L-lysine); PLGA: Poly(lactic-co-glycolic acid); QD: Quantum dot; TAM: Tumor-associated macrophage.

particles within a diameter range of tenths of nanometers to micrometers. Larger particles are internalized by Mφ more efficiently [55]. Gold nanorods, similarly to rod-like bacteria, are more efficiently captured by Mφ than spherical gold NPs [56]. A recent study of more than 20 antiretroviral nanodrugs showed that formulations with elongated shape and sharp edges are more attractive for Mφ [57]. On the other hand, new promising drug carriers, carbon nanotubes with a high length-to-width ratio, tend to reduce phagocytic ability and induce a proinflammatory response (TNF- α , reactive oxygen species release) of Mφ [43]. Mechanical properties such as elasticity of nanocarriers could also influence cellular uptake in murine RAW 264.7 Mφ. The uptake of solid NPs was mediated by a clathrin-dependent mechanism, while soft nanocarriers were internalized preferentially via macropinocytosis [58].

Poor penetration of small anticancer drug molecules into solid tumors significantly limits their efficacy. This phenomenon is even more pronounced for nanodrugs. Penetration of NPs in tumor mass and size dependence for biodistribution of metal NPs was recently analyzed in [59]. In application to tumor therapy, it is commonly accepted that enhanced permeability and retention effect is responsible for the accumulation of injected polymeric anticancer drugs and nanocarriers in tumors via leaking neovasculature. However, it may be only a part of tumor-associated events. Evidence suggests that NPs do not migrate inside tumors from neovasculature in xenograft animal models [60]. In real, immunocompetent conditions, evidently, TAMs will be able to capture selected NPs and promote their distribution into hypoxic areas of tumor, enhancing the therapeutic effect of nanodrugs. Many liposomal and micellar nanocarriers take advantage of the passive targeting by enhanced permeability and retention effect in solid tumors. This effect was translated to the clinic, where liposomal drug formulations reportedly exhibited better efficacy and lower toxicity [61]. It was shown that Mφ often serve as intermediates in the biodistribution of therapeutic NPs. Inhalation of rifampicin-poly(lactic-co-glycolic acid) microspheres led to the accumulation in alveolar Mφ, initiating a potent bactericidal effect against a *Mycobacterium* infection in the tuberculosis rat model, while no toxicity to Mφ was observed [42]. Iv.-injected quantum dots (QDs) found application during brain tumor therapy. QDs could be efficiently sequestered by Mφ and delivered across the blood–brain barrier (BBB) into brain tumors. This type of optical imaging provides a real-time feedback during surgery or biopsies of brain tumors [34]. Similarly, Mφ were able to carry iron oxide NPs for MRI in transgenic murine mammary tumor models [62]. *N*-(2-hydroxypropyl)-methacrylamide-conjugated gadolinium (Gd) chelate was recently applied to MRI [35].

This *N*-(2-hydroxypropyl)-methacrylamide–Gd conjugate that also carried a mannose moiety for Mφ targeting demonstrated up to sevenfold enhancement of imaging compared with another contrast agent, Gd-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, without Mφ targeting. Several attempts of Mφ bioengineering have been made for nanodrug delivery. Holden *et al.* modified the surface of RAW 264.7 Mφ with PEG-coated QDs or fluorescein-labeled polyamidoamine dendrimer (generation 4.5). The engineered cells could serve as cellular vehicles to hypoxic areas of tumors, overcoming some limitations of Mφ-loading strategies, such as cytotoxicity and low loading capacity [33].

In order to enhance the capture of nanocarriers by Mφ, a surface modification with tumor lymphatic-specific and TAM-targeting peptide LyP1, selected by phage display approach, was proposed in several reports. For example, LyP1 was introduced onto the exterior surface of a fluorescently labeled heat shock protein (Hsp) from *Methanocaldococcus jannaschii*. The LyP1–Hsp hybrid demonstrated an enhanced affinity to Mφ *in vitro*. *In vivo* injection of LyP1–Hsp allowed *in situ* visualization of Mφ-rich murine carotid lesions and *ex vivo* fluorescent imaging [30]. Another strategy was based on hemoglobin-scavenging activity of TAM through CD163 receptor-mediated endocytosis [31]. Hemoglobin-decorated liposomes encapsulating a fluorescent calcein showed high specificity towards Mφ. Another versatile platform was developed that could enhance selectivity of chemotherapy against specific types of cells [63]. Cells of the monocyte–Mφ lineage predominantly express carboxylesterase (hCE-1). Various prodrugs have been synthesized using a linker with the esterase-sensitive motif. They demonstrated up to 1000-fold higher potency in hCE-1⁺ monocytes than in other cell types. Chemical conjugates of this type with nanocarriers could find widespread therapeutic applicability as well. Recently, many of the Mφ-targeting approaches have been summarized in [64].

'Trojan horses' of drug delivery

Cell-mediated drug delivery is based on the *ex-vivo* loading of patient's cells (e.g., monocytes, Mφ, SCs and erythrocytes) with drugs or nanodrugs that do not affect the cell viability and mobility. After injection, these cells are able to migrate to the sites of inflammation, target tumors or cross biological barriers such as the BBB [65]. Particular attention was directed towards immunocytes and SCs due to their intrinsic homing affinity towards neoangiogenic sites, and inflammatory and tumor lesions. After resolving some evident pitfalls, cell-based therapies are expected to evolve into a more personalized medicine as opposed to conventional chemotherapy or nanomedicine. Phagocytic properties of Mφ can be employed for nanodrug loading. Recently,

the accepted therapy of neurogenerative diseases was enhanced by the innovative approach allowing delivery of bioactive proteins in Mφ as cell carriers [40]. In the designed enzyme formulations (nanozymes) for therapeutic brain delivery, catalase was packed using cationic diblock copolymers. These nanozymes have been rapidly internalized by monocyte-derived Mφ and released in active form within 24 h. Loaded Mφ iv. injected into a Parkinson's disease mouse model were soon found in the brain; the treatment ameliorated oxidative stress in mice. Haney *et al.* also reported an application of Mφ loaded with a similar ionic complex of the plasmid DNA encoding catalase [41]. Loaded Mφ secreted extracellular vesicles (exosomes) packed with catalase, thus attenuating oxidative stress in contiguous neurons. *In vitro* studies demonstrated a significant neuroprotective effect of this strategy, and Parkinson's disease mice showed improved motor functions following the treatment. Important data on the requirements to nanocarriers obtained in the research demonstrate that nanocarrier design for cell-mediated drug delivery may differ from conventional drug-delivery systems.

The ability of Mφ to cross the BBB was recently exercised for the delivery of antiviral drugs in the nano-antiretroviral therapy approach [38]. Therapeutic antiretroviral drugs against HIV, encapsulated in biodegradable NPs, were loaded *ex vivo* into the monocyte-derived Mφ. Injected Mφ were able to deliver drugs across the BBB and inhibit HIV infection in the brain. Recently, this approach was extended to crystalline antiviral drugs obtained by high-pressure wet milling technology, which is a simple and inexpensive method of obtaining nanodrugs (ritonavir, indinavir and efavirenz) within a defined size range [39]. Polymer-stabilized drugs loaded in Mφ could reduce the virus titer by more than two orders of magnitude after systemic administration in humanized animal models of HIV infection in the brain. Recently, Gerson *et al.* applied nanogels loaded with activated nucleoside reverse transcriptase inhibitors (NRTIs) for the treatment of HIV infection in the brain [36]. These nanogels have been vectorized by apoE receptor-specific peptide in order to increase penetration across the BBB and demonstrated an increased uptake by monocyte-derived Mφ. In a humanized HIV mouse model, the vectorized nanogels delivered therapeutic doses of NRTIs to the brain and were able to reduce viral activity tenfold compared with free NRTI. Similarly, a significant part of the NRTI-loaded nanogels could be captured by peripheral Mφ fighting the HIV infection systemically. Mφ targeting is important for systemic anti-HIV therapy in order to suppress latent HIV reservoirs. Surface modification of nanocarriers for effective delivery of anti-HIV drugs was comprehensively reviewed in [66]. Wan *et al.* reported a novel

approach to enhance capture of nanocarrier by Mφ for anti-HIV therapy [37]. A known chemoattractant for Mφ, *N*-formyl-methionyl-leucyl-phenylalanine peptide was conjugated onto PEGylated NPs in order to obtain targeted nanocarriers that could be internalized by peritoneal Mφ, which are the primary HIV reservoirs. Optimized nanocarriers carrying PEG (MW: 20 kDa) and two copies of the *N*-formyl-methionyl-leucyl-phenylalanine ligand demonstrated the highest uptake by Mφ. Scavenger receptors in Mφ (e.g., CD204 that recognizes opsonins) are active in the capture of nanodrugs from the circulation. Dextran-coated superparamagnetic iron oxide NPs were studied in J774A.1 Mφ in order to evaluate what receptors participate in the NPs uptake [67]. These studies unambiguously demonstrated that NPs are captured via interaction with scavenger receptors, but not via dextran recognition by specific carbohydrate receptors. Capture of many NPs by Mφ occurs via exocytosis, while receptor-mediated endocytosis prevails for nanocarriers modified by ligands. As it will be shown below, mannose receptors in Mφ can be utilized in order to increase the uptake of mannose-modified nanocarriers via receptor-mediated endocytosis.

Targeting TAMs by nanodrugs

TAM targeting is one of the priorities in the development of novel nanodrugs. There are three potential therapeutic approaches involving TAMs: using TAMs for anticancer drug delivery (similarly to the 'Trojan horse' approach); killing TAMs by targeted delivery of cytotoxic drugs; and reprogramming or suppressing TAM activity by specific molecular inhibitors.

Mφ ability to recognize inflammatory lesions could be utilized for targeted delivery of anticancer therapeutics or imaging agents into tumors, especially inner hypoxic areas that are difficult for conventional chemotherapeutic drugs to reach. In anticancer therapy, gold nanoshells can be employed for hyperthermic ablation therapy by laser irradiation that induces death of surrounding cancer cells. Using two-photon fluorescence, Madsen *et al.* demonstrated efficient penetration of nanoshell-loaded Mφ inside of multicellular cancer spheroids [22]. Surprisingly, Mφ accumulated not only in the hypoxic area of spheroids, but distributed evenly in the spheroid volume. This property can also be valuable to enhance distribution of cytotoxic drugs in tumors. Choi *et al.* used monocytes to deliver gold nanoshells into hypoxic regions of tumors [23]. They demonstrated that gold nanoshells are effectively internalized by monocytes. As 'Trojan horses', the internalized gold nanoshells accumulated in cancer spheroids and induced cell death by photothermal treatment. In a similar study, gold nanoshells were loaded in peritoneal Mφ, which may efficiently serve as cellular vectors. In coculture of charged Mφ and cancer

cells, cancer cells were selectively killed following near-infrared irradiation [24]. Gold nanorods modified with a macrolide motif via the PEG linker also demonstrated enhanced affinity to M ϕ and induced fast cancer cell death around the gold nanorod-loaded M ϕ [25]. Thus, based on the fast progress of this research, M ϕ -mediated ablation therapy can be considered as one of the most promising cancer treatment strategies to date, especially when accompanied by diagnostic imaging.

Combination of ablation and chemotherapies was recently reported by Wang *et al.* [26]. They designed a nanoplatform based on the magnetic core/shell iron/iron oxide NPs. A topoisomerase I inhibitor, SN38, was bound to the surface of NPs via a cleavable carboxylesterase linker. For tumor homing, SN38-NPs were loaded into RAW 264.7 M ϕ , which expressed intracellular carboxylesterase upon addition of doxycycline. When delivered to the tumor site, SN38-NPs induced a strong combination anticancer effect by magnetic hyperthermia and the release of SN38. Ikehara *et al.* developed another M ϕ -based combination therapy in order to control breast cancer metastasis in milky spots, the initial places of metastatic development [28]. Oligomannose-coated liposomes (OMLs), loaded with 5-fluorouracil and magnetic NPs, were injected into the peritoneal cavity. Peritoneal M ϕ actively internalized OMLs and gradually accumulated in the omentum layer. Controlled release of 5-fluorouracil and tumor growth inhibition was observed when an electromagnetic field was applied in order to shear the coadministered OMLs in a mouse intraperitoneal metastatic model. By contrast, no apparent tumor reduction was observed for either treatment alone.

Killing TAMs

Therapeutic targeting of M ϕ may represent a crucial strategy in addition to conventional anticancer interventions [68]. Trabectedin, a commercially available anticancer drug of marine origin, was found to be selectively cytotoxic to TAMs and circulating monocyte precursors [69]. The TAM-depleting effect was determined as a key element of the trabectedin anticancer activity.

Mannose receptor CD206 can be exploited for M ϕ -specific targeting and potential delivery of nanodrugs. A single-chain peptide binding the CD206 receptor was attached to nanocarriers (nanobodies), which allowed them to selectively target CD206⁺ TAMs, especially in hypoxic regions [70]. In another example, pH-sensitive polymeric micelles functionalized with mannose moieties have been synthesized using 'click' chemistry for siRNA encapsulation [29]. M ϕ avidly recognized these NPs and captured 13-times more siRNA compared with breast cancer cells. This nanoplatform allowed gene silencing in M ϕ by

$87 \pm 10\%$. When exploiting the targeting of TAMs, the uptake by normal M ϕ remains an issue. An interesting variant of the targeting approach was reported by Zhu *et al.* [71]. This new platform used mannose-modified NPs that also contained an acid-sensitive PEG modification and allowed a significant reduction in uptake by the RES due to effective PEG shielding at neutral pH. In the acidic tumor microenvironment, PEG shedding resulted in the efficient targeting of TAMs. Another method to avoid the capture of NPs by phagocytosis was based on the CD47 receptor, a putative marker of body self-recognition expressed in tumors that was a poor prognostic factor [72]. Attachment of a minimal peptide corresponding to CD47 protein to the surface of NPs was suggested as a potential method to avoid capture of NPs by RES phagocytes. The 'self-peptide' delayed M ϕ -mediated clearance of NPs and promoted persistent circulation, which enhanced dye and drug accumulation in tumors [73]. In addition, CD47 down-regulation by inhibitory siRNA can potentially be used to make cancer cells more sensitive to cleansing by the immune system.

Legumain, a stress protein and a member of the asparagine endopeptidase family overexpressed in TAMs, can serve as an efficient therapeutic target [74]. Legumain-expressing DNA vaccine was able to induce strong CD8⁺ T-cell response against TAMs, dramatically reducing the TAM quantity in tumors. Proficiency of this TAM eradication strategy was demonstrated in murine models of metastatic breast, colon and lung cancers, where 75% of vaccinated mice survived lethal tumor cell challenges and 62% were completely free of metastases. Delivery of these or similar agents in nanocarriers is probably the simplest strategy of TAM cleansing, given the high specificity of uptake of drug-loaded NPs by M ϕ .

Reprogramming TAMs

The plasticity of TAMs can be used for reprogramming of the immunosuppressive M2 phenotype into the tumor-suppressing M1 phenotype. An interesting example of the targeted delivery of oligonucleotides into TAMs for cancer immunotherapy was described by Huang *et al.* (Figure 3) [75]. The CpG oligonucleotide, also known as an immunostimulant, was encapsulated in M ϕ -targeted composite NPs, which could be efficiently captured by TAMs. In contrast with the free CpG oligonucleotide, CpG-NPs stimulated infiltration of M1 M ϕ and dendritic cells into the tumor mass. Using a combination of CpG oligonucleotide, anti-IL-10 and the anti-IL-10 receptor antisense oligonucleotides, the authors demonstrated effective reprogramming of TAMs and produced a significant antitumor effect in a hepatoma murine model.

Figure 3. Effect of an immunostimulatory CpG oligonucleotide and antisense oligonucleotide against IL-10 and the IL-10 receptor encapsulated in specially designed nanoparticles on reprogramming of tumor-associated macrophages in murine tumor model. Galactosylated cationic dextran was used to pack these oligonucleotides (GDO), and it was then encapsulated in acid-sensitive PEG–histidine-modified alginate NPs (PDO). After injection in mouse and accumulation in tumors, NPs were stripped off PEG in the acidic tumor environment, which made them recognizable by TAMs via MGL. The release of active oligonucleotides initiated M2-to-M1 conversion and subsequent tumor growth inhibition. **(A)** Shows accumulation of PDO NPs in murine tumor by fluorescent (Cy5.5) bioimaging. **(B)** Shows confocal images of fluorescently labeled oligonucleotides (red) and TAMs (green F4/80 marker). Oligonucleotides were effectively colocalized with the TAMs.

GDO: Glycerol dioleate; iv.: Intravenous; MGL: Macrophage galactose-type lectin; NP: Nanoparticle; ODN: Oligodeoxynucleotide; PDO: Polyethylene glycol–histidine-modified alginate nanoparticles; PEG: Polyethylene glycol; PHA: Polyhydroxyalkanoate; TAM: Tumor-associated macrophage. Reproduced with permission from [75].

A key to successful immunotherapy is to overcome the local immunosuppression within the tumor microenvironment and activate immune defense that will result in tumor eradication. Thymosin- α is an immunomodulating hormone that could activate TAMs into dendritic cells, which participate in antitumor host response and produce high amounts of cytokines such as IL-1 and TNF- α [27]. Nanodelivery of thymosin- α can be a viable approach for increasing the activity of the immune system of cancer patients. Expression of proteins or factors regulating activity of TAMs or M2-to-M1 transition can potentially be induced or suppressed by gene therapy. Recently, an important role of miRNA in the regulation of TAM activation and functions by miR-155, miR-146 and miR-511 was discussed in a newly published review [76]. THP-1 M ϕ have been recently proposed as cellular carriers of therapeutic RNAs [32]. These M ϕ were loaded with microvesicles containing miR-143 in order to protect the therapeutic RNA from

degradation. M ϕ secreted miR-143 in the microvesicular form both *in vitro* and *in vivo*. The active miR-143 content in serum, tumor and kidney of host animals was significantly increased, suggesting this method can be utilized as a potential approach to anticancer miR delivery to tumors.

Some small drug molecules (e.g., a copper *N*-[2-hydroxy acetophenone] glycinate chelate), were found to be able to reprogram TAMs [77]. Administration of copper *N*-(2-hydroxy acetophenone) glycinate chelate induced reactive oxygen species formation and triggered p38MARK and ERK1/2 activation pathways, causing an upregulation of intracellular glutathione. The resulting downregulation of TGF- β production was specific to M1 M ϕ and enhanced the antitumor immune response. Similarly, the NF- κ B signaling pathway is a key factor in cancer-related inflammation where malignant progression can eventually reprogram TAMs [78]. When this pathway was inhibited in TAMs by NF- κ B

inhibitors, M ϕ became cytotoxic to tumor cells and expressed high levels of IL-12, promoting recruitment of natural killer cells, such as M1 M ϕ .

Bisphosphonates, such as zoledronic acid, evidently could trigger the reversal of the TAM phenotype [79]. Unfortunately, no definitive data outlining the mechanism of this transformation are available. These drugs encapsulated in tumor-targeted nanocarriers can affect TAMs very specifically. For example, liposome-encapsulated clodronate, a bisphosphonate drug, was used to target TAMs in mesothelioma tumors following intraperitoneal injection, which resulted in a 15–17-fold reduction in the relative tumor burden and a significant reduction in the number of invasive metastases [80]. Treatment with liposomal clodronate also efficiently eliminated TAMs in the murine F9 teratocarcinoma and human A673 rhabdomyosarcoma mouse tumor models. Combinational therapy using the liposomal clodronate and an anti-VEGF antibody resulted in reduced tumor blood vessel density and significant inhibition of tumor growth ranging from 75 to >92% [81]. The bone-targeting alendronate can potentially enhance accumulation of nanocarriers in specialized M ϕ including TAMs. Conversion of M2 M ϕ into M1 M ϕ can be potentially initiated by drugs suppressing the other major M2 M ϕ features (e.g., arginase activity or the production of polyamines). These inhibitors represent another potential class of drugs directed to the reprogramming of TAMs. In summary, TAM reprogramming as a part of combinational therapeutic approaches can be considered as a powerful future anticancer strategy.

Rapid accumulation of knowledge about features and functions of M ϕ and, specifically, TAMs, will accelerate the development of new drugs with more specific mode of action supporting or inhibiting the role playing by M ϕ in various disease states. Hitchhiking the natural ability of M ϕ to capture NPs is an extremely productive approach to the development of novel cell-associated nanocarriers, which are able to deliver drugs into the

areas of restricted access such as the CNS or hypoxic zones of tumors. Mobilization of different drugs and defense mechanisms in the treatment of cancer or CNS diseases, including drug delivery and nanomedicine approaches, is the only viable solution to eradication of many resistant to therapy illnesses.

Future perspective

Rapid progress in the understanding of TAM ontogenesis and functions will certainly bring new designs of targeted drugs and nanocarriers. The most promising approaches, in the authors' opinion, which could be advanced to the clinic in the near future, include therapies using TAM-killing or -transforming drugs or drug conjugates, tumor thermoablation in magnetic field using ferromagnetic NPs, and application of slow-releasing cancer drug conjugates using nanocarriers, which are actively accumulated in TAMs and distributed in the tumor mass. However, in order to be successful and eradicate metastatic cancers, the treatment paradigm should be shifted to combination therapies including, predominantly, interventions suppressing TAM-related tumor-supporting functions.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Executive summary

- Recent data confirm the importance of tumor-associated macrophage (TAM)-targeted therapy, because TAMs act as a significant component of the tumor microenvironment and execute tumor-protecting functions.
- Macrophages have a strong affinity to capture nanodrugs and can be harnessed to deliver nanodrugs to specific sites of their natural accumulation, such as inflamed lesions and tumors.
- Distribution of small drugs is not affected by macrophages. Accumulation and distribution of NPs and polymer conjugates are affected.
- TAMs can be used to deliver nanodrugs throughout the tumor mass, including hypoxic areas, and enhance therapeutic action against drug-resistant cells such as cancer stem cells, which are poorly treatable by conventional chemotherapies.
- Development of specific vectors recognizing TAMs would allow us to modulate or silence particular TAM functions, or reprogram them into immunocompetent macrophages.
- In addition, targeted nanodrugs can selectively kill TAMs by delivering cytotoxic compounds.

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