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Review article

Macrophage based drug delivery: Key challenges and strategies

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ABSTRACT

As a natural immune cell and antigen presenting cell, macrophages have been studied and engineered to treat human diseases. Macrophages are well-suited for use as drug carriers because of their biological characteristics, such as excellent biocompatibility, long circulation, intrinsic inflammatory homing and phagocytosis. Meanwhile, macrophages' uniquely high plasticity and easy re-education polarization facilitates their use as part of efficacious therapeutics for the treatment of inflammatory diseases or tumors. Although recent studies have demonstrated promising advances in macrophage-based drug delivery, several challenges currently hinder further improvement of therapeutic effect and clinical application. This article focuses on the main challenges of utilizing macrophage-based drug delivery, from the selection of macrophage sources, drug loading, and maintenance of macrophage phenotypes, to drug migration and release at target sites. In addition, corresponding strategies and insights related to these challenges are described. Finally, we also provide perspective on shortcomings on the road to clinical translation and production.

1. Introduction

Macrophages are natural immune cells derived from circulating monocytes and are widely distributed in all tissues [1,2]. They patrol for potential pathogens, respond to physiological changes and external challenges, and keep individuals healthy by phagocytosis and digestion of cellular debris, viruses, bacteria, senescent cells, cancer cells, and any other foreign matter [3,4]. These cells also play a vital role in homeostasis, antigen presentation, inflammation resolution, wound healing, tumor progression, and tissue repair [5–7]. During tissue injury, infection, or inflammation, monocytes preserved in the blood, spleen, and bone marrow migrate to tissues and differentiate into tissue-resident macrophages through sensing and recruitment of signaling molecules such as inflammatory cytokines and chemokines [8,9]. In addition, resident tissue progenitor cells or proliferation of local macrophages themselves may also supplement tissue-resident macrophage populations [2]. Because macrophages are a significant plastic cell that can switch reversibly from one phenotype to another, they play a dual role in the progression of many infectious and inflammatory diseases, including cancer, atherosclerosis, and rheumatoid arthritis. Some bacterial products and cytokines can activate and enhance macrophages, effectively clear infections, remove debris and tumor cells, promote tissue repair, and stop tumor development. On the other hand, they are driven toward inflammatory cells and immunosuppressive tumor-associated macrophages, leading to tissue damage and pathology [10,11].

Recently, macrophages have attracted more and more attention as therapeutic agents and drug delivery carriers due to their unique characteristics of stealth in blood circulation, non-immunogenicity, capacity for phagocytosis re-educated polarization, immune response and inherent inflammation homing [12–14]. Compared with other leukocytes and mesenchymal stem cells (MSCs), macrophages are more abundant and versatile cells. Not only can they extend the blood circulation time of drugs like red blood cells, but they also can cross impermeable barriers and specifically reach many tissues that are difficult for nanomedicines to reach, like neutrophils and mesenchymal stem cells, including (1) migrating to inflammation sites, such as tumors, acute or chronic inflammation, or in autoimmune disease; (2) crossing the blood-brain barrier (BBB) to target inflamed brain tissue; and (3) deep infiltration into the hypoxic areas of tumors that lack blood flow [15,16]. Meanwhile, compared to neutrophils which are most abundant

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during acute inflammation, macrophages are usually present in the late stages of acute inflammation or in chronic inflammation, which makes them suitable for drug delivery under both acute and chronic inflammation conditions [17,18]. As drug carriers, taking advantage of their phagocytic ability and large cell surface size, drugs can be loaded into macrophages or conjugated onto cell membranes. While neutrophils are not suitable for the cell backpack strategy due to the difficulty of evading the effects of membrane functions [19], mesenchymal stem cells have poorer drug loading capacity [20]. Moreover, unlike other cells, macrophages exhibit restrictive phenotypes or enhance therapeutic function using adoptively engineered macrophages [21,22].

Macrophage-based therapy can be dated back to an early study that used macrophages to intervene in tumor metastasis, conducted by the laboratory of Dr. Fidler at University of Pennsylvania in the 1970s [23]. Macrophages were isolated from the peritoneal cavity of C57B16 mice with B16 subcutaneous tumors. After stimulation with extracts of rat lymphocytes sensitized to mouse B16 tumors, the activated macrophages were injected intravenously into tumor-carrying C57B16 mice. The results showed a significant reduction in lung metastases [23]. Since the mid-1980s, macrophages have been explored in clinical trials for treating a variety of human tumors, providing modest therapeutic benefits in some patients but no lasting remission [24]. Several clinical trials have also begun using macrophages as therapeutic agents to enhance therapeutic effects, however, the results of these trials have not yet been reported (Table 1).

Macrophages are easily separated or isolated, loaded with drugs, and re-entered the circulation [25]. Professor Gendelman and co-workers at the University of Nebraska were the first to use macrophages as drug delivery carriers. They developed a bone-marrow-derived macrophage (BMDM) system to deliver nanoparticle indinavir (NP-IDV) formulation in HIV-1-infected humanized mice [26] and to deliver catalase to Parkinson's disease-affected brain regions in an animal model [27]. Shortly thereafter, a research team lead by Professor Clare of Indiana University School of Medicine reported a cellular Trojan horse for delivery of therapeutic nanoparticles into hypoxic regions of tumors by using monocytes and macrophages [28]. These pioneering works have greatly progressed the study of macrophages as a delivery carrier for different drugs. To date, macrophages have been used as a carrier for the delivery of; nanoformulated antiretroviral drug to the brain in a murine model of NeuroAIDS [29]; therapeutic and imaging contrast agents to tumors [30]; nanoparticle for glioma treatment [31] and for targeting rheumatoid arthritis (RA) [32]; light activated nitric oxide prodrugs into NIH-3T3/4T1 tumor spheroid models [33]; anticancer drug for efficient glioma therapy [34] and siRNA lipoplexes to cancer cells [35].

The basic procedures for macrophage-based drug delivery include candidate cell isolation, drug loading, macrophage polarization, reinfusion and homing to diseased tissue (Fig. 1). While promising, unavoidable challenges almost certainly remain in preclinical research that hinders the success of clinical translation. It is necessary to understand and address the technical aspects of macrophage-based drug delivery at an early stage to avoid problems in later development. This review will highlight the major challenges present in the four basic procedures described above when designing and utilizing macrophages for drug delivery, including selection of macrophage sources, limited amount of drug that can be loaded in macrophages, rapid conversion of macrophages from therapeutic phenotypes to detrimental states, ineffectiveness of disease targeting ability, and uncontrolled drug release at targeted sites. Also, corresponding strategies and insights related to these challenges are described. In addition, macrophage-related biomimetics drug delivery is discussed. Finally, we also provide perspective on shortcomings on the road to clinical translation and production.

2. Source and selection of macrophages

2.1. The origins of tissue resident macrophages

Tissue resident macrophages play a wide range of roles in homeostasis, immune surveillance, tissue repair and regeneration. In 1968, Dr. van Furth and Dr. Cohn of Rockefeller University concluded that macrophages were derived exclusively from the differentiation of circulating monocytes [36], a view that dominated for many years. However, in recent years, a growing number of pedigree tracing studies have refuted this hypothesis. Adult tissue macrophages of the skin, spleen, pancreas, liver, brain and lung are mostly produced by yolk sacs during embryogenesis, have the ability to self-renew and are maintained independently of monocytes [9,37,38]. In a few tissues, such as the kidneys and lungs, macrophages have chimeric origins, deriving from the yolk sac and bone marrow [2]. In many tissues, macrophages are maintained by self-renewal, but the extent to which self-renewing resident cell populations are maintained remains an open question. Studies have found that in certain tissues with high metabolic activity and high antigenicity, such as the liver, spleen, intestine and even certain cancerous tissues, macrophage self-renewal is unlikely to be sufficient to maintain its abundance [39,40]. This suggests that the recruitment and replenishment of bone marrow-derived monocytes is necessary to maintain a tissue macrophage population. These sites are replaced in a stable state by circulating monocytes, which differentiate into tissue-specific macrophages after entering the tissue [41,42]. This may also occur in certain inflammatory states [37,43]. During injury, infection, or tumor, inflammatory signals cause an influx of bone marrow-derived monocytes to the disease site and partially replace resident macrophages of embryonic origin in the event of depletion of tissue macrophages [44]. Bone marrow monocyte-derived macrophages can then establish a new population of cells with locally-specific macrophage functions that

Table 1
Clinical trials using macrophages.

Cell types	Route	Clinical phase (status)	Disease	NCT identifier
Autologous M2 macrophage	Intrathecal injection	Phase I (completed)	Non-acute stroke	NCT01845350
Autologous, bone marrow cluster of differentiation 34+ ($CD34^{+}$) cell-derived, lentiviral CSF2RA gene-corrected macrophages	Lung instillation	Phase I (ongoing)	Hereditary pulmonary alveolar proteinosis (hPAP)	NCT05761899
Autologous Incubated Macrophages (ProCord)	Spinal injection	Phase II (suspended)	Spinal cord injury (SCI)	NCT00073853
CT-0508 (adenovirus-transduced macrophages with anti-HER2-CAR)	N/A	Phase I (ongoing)	HER2 overexpressing solid tumors	NCT04660929
MON002 (autologous anti-fibrotic monocyte)	Intravenous injection	Phase I/II (ongoing)	Post-COVID-19 lung fibrosis	NCT04805086
MT-201-GBM monocyte vaccine (pp65CMV antigen monocytes)	N/A	Phase I (withdrawn)	Glioblastoma (GBM)	NCT04741984
Autologous Monocytes with Sylatron (Peginterferon Alfa-2b) and Actimmune (Interferon Gamma-1b)	Intraperitoneal Infusion	Phase I (terminated)	Ovarian cancer	NCT02948426
Temferon (autologous HSPC-derived myeloid cells expressing IFNα2)	N/A	Phase I/II (ongoing)	Glioblastoma (GBM)	NCT03866109

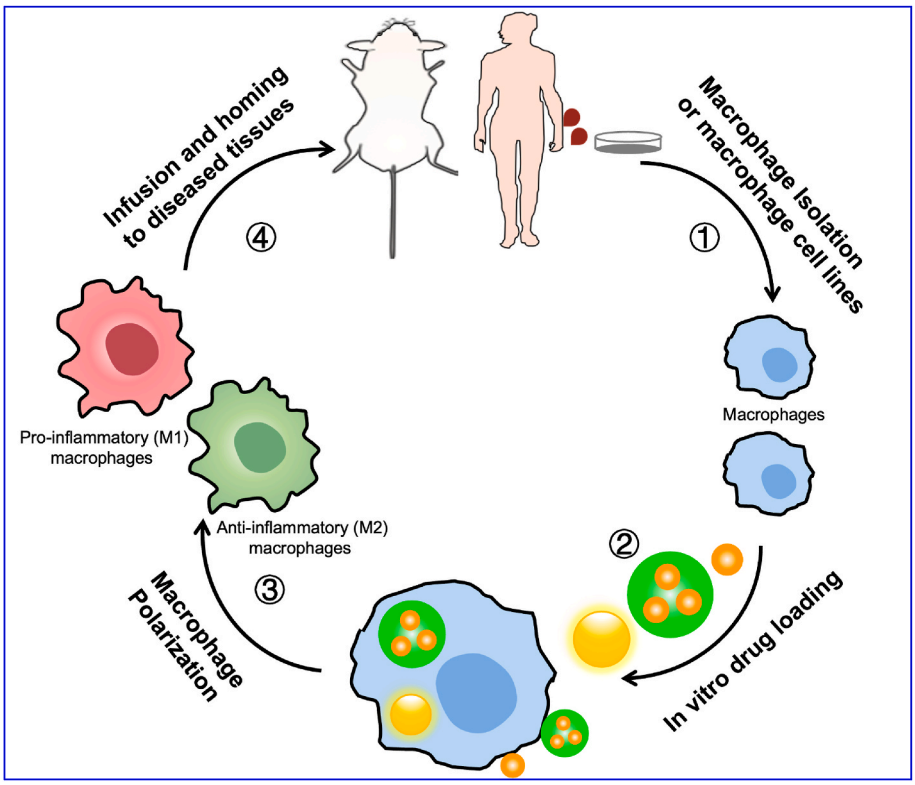

Fig. 1. Schematic diagrams of the basic procedure of fabricating macrophage-based drug delivery systems for disease treatment. The main process includes following: ① Macrophage selection, including primary macrophage isolation and macrophage cell lines; ② In vitro drug loading; ③ Macrophage polarization; ④ Infusion and homing to diseased tissues.

closely resemble the original tissue macrophage phenotype. This high plasticity gives monocyte-derived macrophages potential for cell therapy, aiming to replace local macrophage populations with engineered exogenous cells.

2.2. *Different sources of macrophages for therapeutic applications*

The first step in the application of macrophage-based cell therapy is to select the most suitable source of macrophages. In general, there are two sources of macrophages as therapeutic agents or transporters

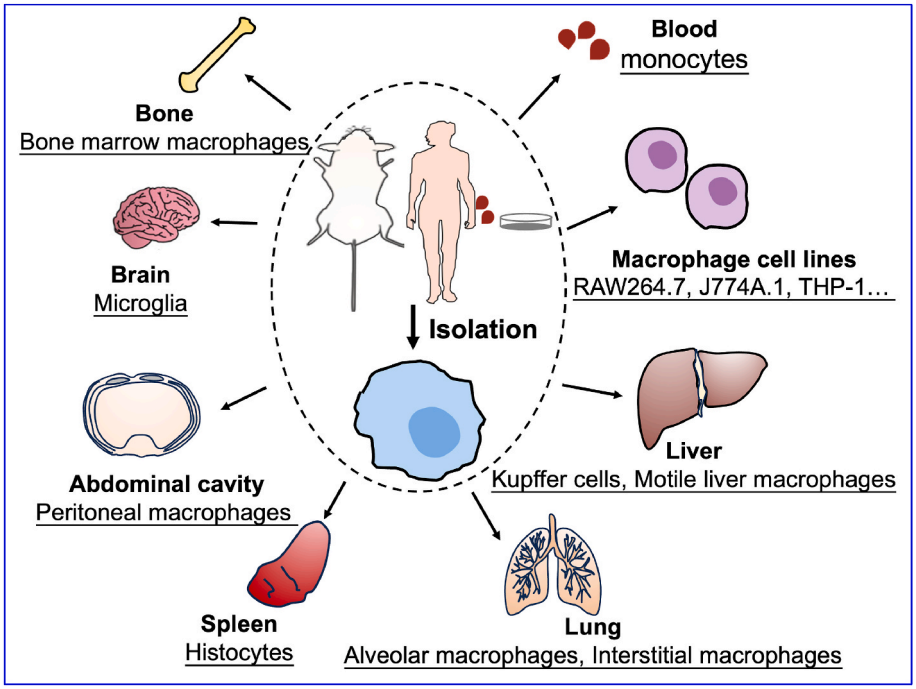

Fig. 2. Different sources of macrophages for therapeutic purposes and drug carriers.

(Fig. 2): 1) Macrophage cell lines including RAW264.7 [45], J774A.1 [46] and THP-1 [47]; 2) Primary macrophages extracted from animals or human blood, including BMDMs, differentiation of blood monocytes in medium containing macrophage colony-stimulating factor (M-CSF), pre-existing macrophages from body cavity lavages of tissue-resident macrophages such as alveolar macrophages, histiocytes of the spleen and interstitial connective tissue, peritoneal macrophages and Kupffer cells of the liver [48–50].

2.2.1. Macrophage cell lines

In macrophage cell lines, RAW264.7 is a commonly used macrophage model for the loading and delivery of anti-tumor drugs [51–54]. RAW264.7 cells were first introduced as macrophage-like cancer cells established from murine tumors by Raschke et al., in 1978 [55]. They can bind to metastatic cancer cells via α4 and β1 integrins of RAW 264.7 macrophages and interact with vascular cell adhesion molecule-1 (VCAM-1) of cancer cells [56]. Based on this ability, RAW 264.7 macrophages are widely used as tumor-targeting vehicles to carry cargos deep into tumor niches. Fu and collaborators used RAW264.7 cells as vehicles to deliver doxorubicin (DOX) to tumor tissues [51]. After systematic administration, the DOX-loaded macrophage system showed significant tumor-tropic ability on 4T1 mouse breast cancer cells, with good anti-cancer effect in tumor inhibition, life extension, metastasis inhibition, and reduced toxicity. In addition to peripheral tumor targeting, macrophages offer an alternative therapeutic strategy for the treatment of brain tumors because of their ability to transport across the BBB and infiltrate into the tumor site. The BBB remains a challenge, hindering the application of numerous therapeutics in the treatment of brain diseases. Pang and collaborators exploited macrophages as a "Trojan horse" to carry drug-loading nanoparticles (NPs) across the BBB and offload them to brain tumor sites [57]. DOX was encapsulated in poly(lactic-co-glycolic acid (PLGA) nanoparticles to avoid damage to macrophage vehicles. After RAW264.7 macrophages were incubated with DOX-loaded PLGA nanoparticles, nanoparticle-loaded RAW264.7 cells reduced liver accumulation, increased drug distribution in the brain, and improved the therapeutic effect of glioma.

Although much progress has been made in the study of RAW264.7 macrophage-mediated drug delivery, the potential danger of injecting RAW264.7 cells has emerged. Accompanied by immunogenicity and pro-tumorigenic properties, administration of RAW264.7 cells may lead to unexpected tumor growth in mice. The main reason for this phenomenon may be due to the improper cell-dose. Tumor growth was confirmed to be cell dose-dependent, and 1×10^4–5×10^4 cells per mouse were more conducive to the development of immune tolerance [58]. Therefore, the suitability of RAW264.7 cells for macrophage-based drug delivery or cell therapy remains in question.

2.2.2. The primary macrophages

Unlike the RAW264.7 cell line, primary macrophages have the characteristics of low immunogenicity and excellent biocompatibility. Studies on the safety of autologous macrophages in humans have shown that the most common reported side effects are mild fever and chills, with no serious side effects in 11 human clinical trials, even at the highest dose, of about 10^9 cells, which is the maximum number of macrophages that can be extracted and cultured from a patient in one leukapheresis, or at maximum frequencies of up to one dose every 24 h for 3 days. In these autologous studies, no immune rejection events were observed [24,42]. Accordingly, the abovementioned primary macrophages include BMDMs, peritoneal macrophages (PMs), spleen macrophages (SPMs), and alveolar macrophages (AMs), which are widely engaged in drug delivery vehicles or therapeutics. Evans and his collaborators used BMDMs as vehicles to deliver nanoparticles loaded with tirapazamine (TPZ) for selective solid tumor treatment [59]. Compared with nanoparticles alone, macrophages can significantly improve TPZ transport and accumulation in hypoxic areas of solid tumors, because macrophages can enhance tumor site accumulation and better extravasate and infiltrate deep tumor tissues. In vivo efficacy studies confirmed a 3.7-fold reduction in tumor weight after loading macrophages compared to TPZ alone or the nanoparticle form. In another work, Huang et al. developed a multifunctional non-genetically engineered macrophage (Oxa(IV)@ZnPc@M) as a near infrared (NIR) light-activated drug carrier to enhance chemo-photodynamic synergistic therapy by carrying nanomedicine containing oxaliplatin prodrugs and photosensitizers [60]. After incubation with the nano-drug Oxa(IV)@ZnPc, the bone marrow-derived macrophages were polarized into an anti-tumor M1 phenotype, which made the macrophages themselves having the potential to exert anti-tumor effects. By taking advantage of nature trafficking activity, macrophages effectively escorted Oxa(IV)@ZnPc to primary and bone metastatic tumors. It was found that Oxa(IV)@ZnPc@M had obvious distribution at the tumor site 2 h after injection, while Oxa(IV)@ZnPc had obvious distribution at 8 h after injection. After 24 h, Oxa(IV)@ZnPc@M exhibited higher tumor distribution than Oxa(IV)@ZnPc, with 1.9-fold and 1.3-fold higher fluorescence intensity in primary tumor and bone metastatic tumor, respectively. Furthermore, the therapeutic drug in Oxa(IV)@ZnPc@M was released via NIR triggering, and the primary tumor was effectively killed by chemical-photodynamic combination therapy while inducing immunogenic cell death.

In addition to commonly used bone marrow-derived macrophages, tissue-resident primary macrophages are also used as carriers or therapeutics. Choi and his collaborators used peritoneal macrophages as Trojan horses, carrying either the imaging contrast agent iron oxide or the therapeutic liposome doxorubicin to migrate to the tumor site [30]. Macrophages loaded with doxorubicin liposomes have further demonstrated effective inhibition of subcutaneous and metastatic xenograft A549 tumor. In a recent study on the use of genetically engineered macrophages in the treatment of advanced gastric cancer, human PMs were used to transfected the HER2-FcεR1γ-CAR (HF-CAR) gene to enhance anti-tumour efficacy, as PMs play a key role in regulating the peritoneal immune environment [61]. Similarly, because synovial tissue macrophages (STMs) predominate immune cells in synovial tissue and play a role in maintaining and reinstating synovial homeostasis, STM subpopulations ($MerTK^{pos}TREM2^{high}$ and $MerTK^{pos}LYVE1^{pos}$) have been shown to have the anti-inflammatory function and repair response of synovial fibroblasts with the potential to produce anti-inflammatory lipid mediators for the treatment of rheumatoid arthritis [62]. Wang and colleagues employed AMs as a carrier to deliver perfluorine-15-crown-5-ether nanoparticles (PFCE NPs) to the alveolar space and pulmonary interstitium to further infiltrate into lung tumors [63]. Subsequently, Nguyen et al. isolated and differentiated macrophages from the spleens of BALB/c mice as cell vehicles that could avoid an immune response when injected into a host animal [45]. Meanwhile, considering the premature off-target release of the drug, a double-targeted microrobot based on primary macrophages was designed to co-load citric acid (CA) coated superparamagnetic nanoparticles (CA-MNP) and DOX-containing thermosensitive liposomes (TSLPs) into the macrophages. The dual targeting of microrobots was guided by both the macrophage's innate tumor homing ability and CA-MNP triggered by an external magnetic field. The DOX-containing TSLPs were responsive to near-infrared radiation, ensuring that the microrobot delivers the drug precisely on demand at the tumor site.

With the rapid development of primary macrophage-based cell therapy, its shortcomings have gradually come into view. The low production of monocytes or macrophages is a disadvantage for use in clinical trials. Moreover, unlike immortal and infinitely proliferating cell lines, monocytes have a short half-life of only 20 h in the blood, exiting the cell cycle after 7–10 days of proliferation and differentiation. And macrophages differentiated from monocytes or isolated from tissue lavage are usually short-lived, unable to proliferated when differentiated in vitro, and difficult to genetically modify [64,65]. Therefore, more clinical research is needed for the maturity of this strategy.

2.3. Selection of macrophage source for therapeutic purposes

In order to achieve ideal drug delivery and therapeutic effects, an appropriate source of macrophages should be considered for drug loading and delivery in vivo, by considering comprehensively the pathological characteristics of the disease and the natural properties and functions of macrophages from different sources. It is well known that macrophages from different sources have different heterogeneous phenotypes. The heterogeneity of macrophages plays an important role in their characteristics and biological functions. Therefore, identifying the phenotype and functional differences of macrophages from different sources can provide a basis for selecting macrophage types for different therapeutic purposes. Bone marrow-derived macrophages have been commonly used due to their homogeneity, long lifespan, relatively high cell isolation yield and ability to be transfected [66]. Moreover, BMDMs exhibit superior phagocytosis and the strongest proliferation capacity compared to macrophages isolated from the spleen or peritoneal cavity [67]. However, the application of BMDMs in experimental studies remains difficult due to its phenotypic and functional instability in vivo [68]. Compared to BMDMs, there has been relatively little research using macrophages from other organs, such as SPMs and PMs. All three types of macrophages have similar spherical and deeply stained nuclei, but SPMs and PMs contain much more cytoplasm than BMDMs. In addition, PMs exhibit a larger size and lysosome content than SPMs. Unlike BMDMs, SPMs showed mild manifestations and PMs had no proliferative capacity, suggesting that macrophages from the spleen and peritoneum may be more functionally and phenotypically stable. In terms of surface molecular expression, the three macrophages showed different levels of expression of molecules involved in antigen presentation. BMDMs highly expressed clusters of differentiation 115 (CD115) and glucocorticoid receptor 1 (GR-1), PMs highly expressed major histocompatibility complex class II (MHC II) and CD86, and SPMs highly expressed macrophage 1 antigen (Mac-1) and MOMA-2. Comparing the cytokine expression profiles of the three macrophages, BMDMs produced higher levels of suppressive cytokines interleukin-10 (IL-10) and tumor necrosis fact-alpha (TNF-α) under resting conditions, suggesting that in vitro generated BMDMs may be more likely to have a M2 phenotype, whereas SPMs maintain high levels of proinflammatory cytokines interleukin–6 (IL-6), interleukin-12 (IL-12) and TNF-α [67].

Therefore, RAW 264.7 and SPMs, PMs or BMDMs polarized to M1 macrophages in vitro are theoretically most suitable for tumor-targeting or therapeutic applications, and BMDMs or PMs polarized to M2 phenotype are appropriate for diseases that give off inflammatory signals, such as acute or chronic inflammation, rheumatoid arthritis, atherosclerosis, Alzheimer's disease, Parkinson's disease. In general, macrophages derived from disease sites are more suitable for drug delivery and treatment of the disease. However, Harris and his colleagues reported the therapeutic efficacy of M2 macrophages derived from the spleen rather than bone marrow in alleviating inflammation and repairing kidney damage [69–71]. This may be due to the loss of function and phenotype of bone marrow-produced M2 macrophages in vivo, which is associated with their greater ability to proliferate. We have demonstrated the objective efficacy of bone marrow-derived M2 macrophages in Alzheimer's diseases and rheumatoid arthritis (unpublished data). Therefore, how to maintain the therapeutic phenotype of macrophages for an extended period in vivo is an issue that remains to be addressed, which will be discussed in the next section.

3. Drug loading of macrophages

Macrophages are the largest type of leukocytes, with an average diameter of 25 μm. This large size provides sufficient loading space for a variety of drugs with different physical and chemical properties, and provides great convenience for modifying drugs. For example, cytoplasmic compartments provide sufficient internal space for hydrophilic drug loading, and the lipid bilayer of the cell membrane is particularly suitable for hydrophilic drug loading [1] (Fig. 3). Nevertheless, low drug loading capacity of cells still remains a drawback of macrophage-based cell therapy.

3.1. Drug loading strategies of macrophage-based drug delivery systems

3.1.1. Loading drugs into the cytoplasmic space of the macrophages

Macrophages are able to internalize drugs or drug-loaded particles directly through phagocytosis (Fig. 3 (i)). Loading drugs or particles into the cell cytoplasm provides multifaceted advantages. The half-life of the drug contained within macrophages is longer because the encapsulated drug is not rapidly eliminated by renal excretion or liver metabolism, is not immediately recognized by the immune system, and is not cleared by the endogenous reticuloendothelial system (RES). In addition to extending the circulatory half-life, macrophages can slow the release of drugs [26,27]. Furthermore, encapsulation of drugs in the internal volume of cells is the simplest and most economical route and is also the preferred route for industrial production.

The amount of drug that macrophages can carry depends mainly on the phagocytosis effect. Most drugs that are loaded directly into macrophages are generally highly cytotoxic to macrophages [25,30]. Hence, loading drugs into macrophages with the aid of nano or microcarriers rather than loading free drugs is an effective approach. In addition, nano- or micro-carriers can slow or delay the release of drugs from the cells before the macrophages reach their target, thereby maximizing the therapeutic effect of the drugs on the disease. The various physicochemical properties of the particles, in particular their size, shape, surface charge, hydrophobicity and mechanical flexibility, determine the effectiveness of cellular uptake [72–74].

Size: In terms of the properties of particles, particle size is considered to be the most critical factor. There is increasing evidence that large particles are phagocytized more efficiently by macrophages [25,74]. Nowacek and collaborators found that particles around 1 μm accumulate to a greater extent (2.56 times) than smaller drug carriers (500 nm) [75]. Tabata and co-authors also demonstrated that polystyrene spheres with a diameter of 3 μm are preferentially phagocytosed by macrophages [76]. Larger particles may condense into spherical structures within the cell membrane, possibly membrane-bound vesicles, which can be phagocytized within a short period of time [77]. In addition, contact angle measurements showed that larger particles are more hydrophobic, which would provide evidence for higher macrophage uptake [78]. Particles with smaller size may exhibit higher levels of exocytosis after being engulfed by macrophages [79]. However, some studies exhibited the contrary results. Zhao et al. showed that the size of carboxylat-modified polystyrene latex beads is negatively correlated with nanoparticle absorption, with smaller nanoparticles loading more efficiently in macrophages [80]. Macrophage origin and material properties may account for this disparity.

Surface charge: Surface charge also plays a key role in macrophage uptake. In general, charged particles are more rapidly engulfed by macrophages compared to neutral particles, possibly due to electrostatic interactions between particles and phagocytes. And positively charged nanoparticles accumulate to a greater extent in macrophages than negatively charged particles [81,82]. However, in addition to dosage, surface charge, especially positive charge, may be the most critical particle property in determining cytotoxicity [83].

Shape: The shape of the particles also affects how they are internalized by macrophages. An elaborate study by Champion et al. showed that all shapes can initiate phagocytosis in at least one direction, and that the local particle shape at the initial point of contact with the cell determines the initiation of phagocytosis [84]. For example, the sharper side of an elliptical particle that acts as an attachment point will initiate phagocytosis within minutes, while the blunter side of the same particle will not initiate phagocytosis even after hours. They also revealed that reducing the high curvature region of the particles helps to reduce phagocytosis [85]. Recently, this idea has gained widespread

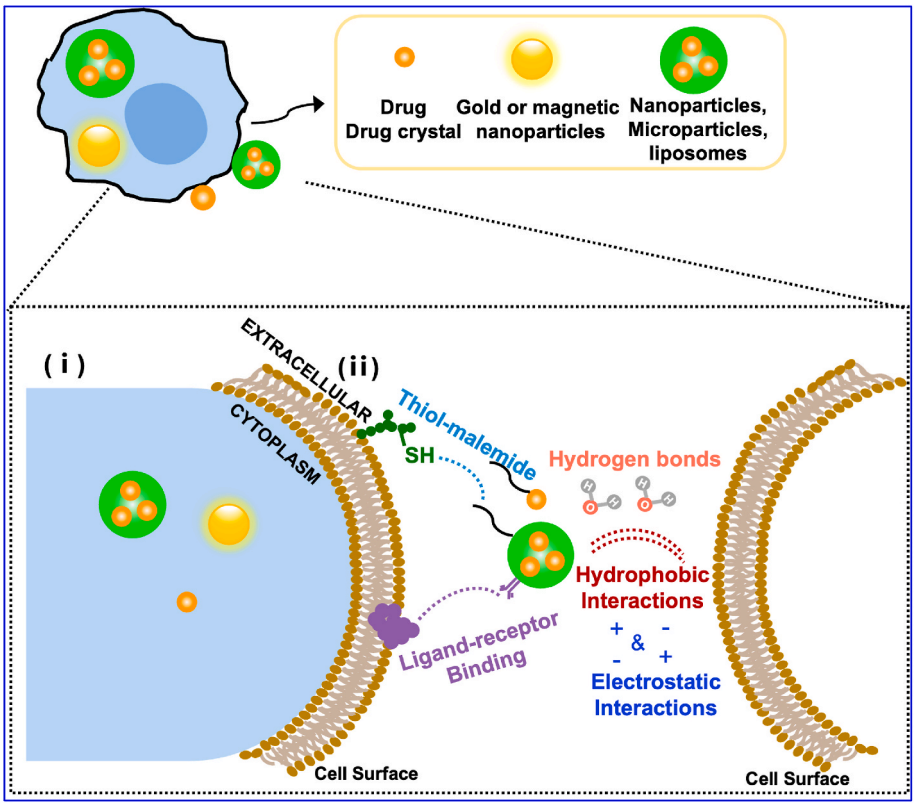

Fig. 3. Schematic illustration of different drug formulations-loading strategies for macrophage-based drug delivery systems, including (i) loading drugs in cytoplasm via phagocytosis, and (ii) attachment on the cell membrane.

recognition, as consistent in vivo studies have shown that long-chain nanomaterials such as filaments [86] and nanorods [87] are more efficient at evading mononuclear phagocyte system (MPS) than traditional spherical particles, and has a longer cycle time. In other words, spherical nanomedicines with the same curvature and contact points may be best suited for macrophage internalization [25].

Surface coatings: Surface coatings are also thought to have an important effect on phagocytosis of macrophages. Particles functionalized by mannans, non-specific complement opsonization, and specific antibody conditioning can be effectively internalized into macrophages through receptor-mediated endocytosis. In addition, an electrostatically neutral and hydrophilic poly(ethylene glycol) (PEG) is currently the most common shell-forming polymer used for injection of nanocarriers, and it is reported that it should not be considered in the context of cell-mediated delivery as far as possible because it can restrict cellular uptake as demonstrated by microspheres carrying high-density surface PEG chains [88].

3.1.2. Loading drugs onto the surface of macrophages

In addition to loading drugs inside macrophages, loading drugs onto the surface of macrophages is an alternative approach. The cell membrane is composed of rich chemical groups such as proteins, lipids, and carbohydrates, primarily charged or active groups that allow drugs or drug-loaded particles to be loaded onto the cell surface by covalent or non-covalent conjugation methods (Fig. 3(ii)). Covalent methods typically utilize reactive chemical groups on the cell surface, such as amino groups on membrane proteins, thiol groups on cystine residues, and glycosyl groups on glycoproteins or glycolipids [89,90]. Among them, N-hydroxysuccinimide ester is the most commonly used reactive group to form stable amide bonds between cell membranes and drugs or particles. In addition, orthogonal chemical groups can be artificially introduced onto cell membranes by treating cells with metabolic compounds such as azido-glycan and azido-phosphatidylcholine.

This approach allows more than 100 nanoparticles with diameters in the range of 100–300 nm to be covalently linked to cell types commonly used in cell therapy without causing toxicity or affecting innate cellular functions [91]. Covalent bonds provide the strongest link between macrophages and drugs, enhancing the stability of cell-drug interactions and avoiding the detachment and uptake of particles in non-target tissues such as the liver and spleen [92]. However, covalent methods face some disadvantages. Firstly, nanoparticles conjugated with glycosyl on glycoproteins occurs randomly, which can negatively affect the biological activity of the proteins [93]. Secondly, maleimides and particularly activated esters are unstable in aqueous medium and can be conjugated randomly to proteins in the cell culture medium [94]. Thirdly, during the maleimide-based conjugation, an additional blocking step is required for unreacted maleimides to prevent them from subsequently binding to unwanted proteins [95]. Fourthly, when conjugating large sterically hindered objects such as cells and nanoparticles, the formation of amide and thioether bonds tends to be sluggish at low concentrations and during slow diffusion of the reaction partners [93]. Finally, the perturbation of cellular physiology inherent to such strategies may interfere with important cellular functions governed by cell surface molecules and damage the cell membrane [96]. In addition, covalent methods are generally associated with reduced drug loadings and elevated immunogenicity [25].

Non-covalent methods rely on electrostatic interactions, hydrogen bonding, van der Waals forces, and hydrophobic interactions to load drugs or drug-loaded particles onto macrophage cell membranes. They require only minimal modification of cells and nanoparticles, providing that nanoparticles are either positively charged or sufficiently hydrophobic [92]. The main advantage of this approach is that it is convenient and does not compromise important cellular functions [97]. At the same time, the non-covalent methods are not strong enough to retain nanoparticles on the cell membrane and have relatively low stability due to the fluidity of the lipid bilayer. The attachment between particles and

cell is difficult to control and can result in unpredictable behavior and detachment from the cell membrane due to blood flow-induced shear stress or cell-cell interactions in vivo [97].

Another non-covalent approach involves ligand-receptor interactions and biotin-avidin cross-linking [76]. Ligand-receptor interactions are mediated by naturally present receptors on the surface of cells and do not require cell modification. They are reliable, reproducible, and simple if target cells are purified. In other words, since many same receptors are present on numerous cells, if the target cell is not purified, it can lead to non-specific attachment to undesired cells. Meanwhile, this method is not powerful enough if an unsuitable ligand is selected for site-specific conjugation with receptors [93]. In addition, another disadvantage is that ligand-receptor interaction may activate or trigger unwanted biological responses [92]. The noncovalent avidin-biotin cross-linking is another method for loading drug carriers on the cell surface. Unlike other noncovalent methods, this link is highly specific and one of the strongest known in biological systems, with association constant (Ka) of up tp 10^{15} M^{-1} [1]. Finally, the method has the advantages of low cost, simple, rapid, high stability, high temperature resistance, proteolytic enzyme resistance, and extreme pH value resistance. Although this approach does not affect cell function, the main drawback is the use of avidin, which is immunogenic in vivo [97].

Recently, Wang and colleagues reported a novel, facile, and cell-friendly noncovalent host-guest interaction for cell hitchhiking [98,99]. Leveraging the high binding affinity of host-guest pairs based on β-cyclodextrin (β-CD) and adamantane (ADA) (typically 10^4~10^5 M^{-1}), macrophage-hitchhiking conjugates, macrophage-liposome and macrophage-macrophage conjugates, were obtained via β-CD-ADA interactions between β-CD-modified macrophages and ADA-modified hitchhiking liposome, and between β-CD-modified macrophages and ADA-modified hitchhiking macrophages, respectively. The cell conjugates could remain stable for at least 1 h, and driven by the inflammatory tropism of macrophage, they achieved efficient delivery in the acute inflammatory foot and lung inflammations (where it is usually fast to reach) in mice, and aorta plaque tissues in atherosclerotic mice. Considering the relatively weak of binding affinity between β-CD and ADA, Wang's group further constructed "supramolecular macrophage-liposome (M-L)" mediated by stronger cucurbit [7] uril (CB [7])-ADA host-guest interactions to replace β-CD-ADA. The supramolecular M-L remained stable for at least 4 h, which was sufficient for in vivo hitchhiking delivery of liposome carrying toxic drugs to the tumor [100].

Immobilization of any drug or particle on the surface of macrophages for a long period of time, avoiding internalization, is a key and important feature of this strategy. Macrophages are a type of phagocyte that usually engulfs any materials attached to their surfaces. As aforementioned, particle size, surface chemistry, shape, orientation and mechanical flexibility significantly affect their susceptibility to be phagocytosed. Thus, elongated, high-aspect ratio particles and very flat disk-shaped particles show reduced phagocytosis compared to spherical particles [85]. Meanwhile, the study also suggests that the high flexibility and low modulus of the particles also contribute to low internalization frequency [76].

3.2. Challenges of macrophage drug loading strategies

Nonetheless, both drug loading methods face challenges in loading large quantities of drugs to macrophages. It is well known that the plasma membrane is essential for cell function and plasticity, and the loading of drugs or drug-carrying particles onto the membrane may adversely affect cell signal transduction, adhesion, and transendothelial migration. In addition, all surface anchoring methods are generally associated with reduced drug load and increased immunogenicity. According to Irvine et al., only 5 % of the plasma membrane can be occupied by nanoparticles without significantly affecting cell function [90,101]. This implies a drug loading rate of less than 1.0 μg per million cells [22]. Considering that 1–10 million macrophages are injected in a cell infusion procedure, the amount of drugs that can be delivered using this method is very limited.

Compared with the surface hitchhiking strategy mentioned above, phagocytosis of drugs or particles into inner cells is more conducive to enhancing drug loading capacity and avoiding occupied particles from affecting cell membrane function [14,102]. But loading drugs into the cytoplasm of macrophages has also been considered challenging or unfeasible. Most chemotherapy drugs are highly toxic to macrophages. Incubation of macrophages with high concentrations of the drug results in immediate cell death, while incubation at sublethal doses causes underloading of the drug. A large body of literature has been published on packaging drugs into liposomes or polymer nanoparticles rather than using free drugs to avoid potentially toxic drug cargoes that kill macrophages before delivery [30,103], but the maximum number of particles that macrophages can take up is limited without affecting cell function. In addition, after internalization, particles are sequestered in phagocytic lysosomes of macrophages, and drug carriers can be rapidly degraded by abundant hydrolytic enzymes and reactive oxygen species (ROS), then releasing high concentrations of drugs that may be toxic to cells [104]. Further investigations are needed to explore solutions to improve the loading capacity of macrophages while maintaining cell functions.

3.3. Improvement of drug loading of macrophage-based drug delivery systems

The theoretical drug load of macrophages can be modeled according to the equation:

$$m_{drug} = n\rho\left(\frac{4}{3}\pi r^3\right)DLE\%$$

The amount of drug inside the macrophage (m_{drug}) will vary according to the number of drug-loaded particles (n), drug-loaded particle density (ρ), particle size (r), and drug-carrying efficiency (DLE%, the percentage of drug in the particle). Based on these factors, many strategies can be developed to improve the loading efficiency, such as optimizing the physicochemical properties of particles that influence the aforementioned phagocytosis processes, maximizing the number of internalized particles without affecting cellular function, increasing the drug loading capacity of single particles, or controlling drug release from particles. Zhang et al. reported a silicon-based drug nanocapsule technology that overcomes the difficulty of loading a sufficient amount of drug into macrophages without interfering with cell function [22]. The designed nanocapsules are composed of a drug-silica complex filling and a solid silica sheath. Because the silica coating is difficult to be degraded and oxidized by enzymes and ROS in macrophages, it protects the drug from exposure and buys time for macrophages to transport it to the tumor and release the drug in place *in situ*, and avoids the adverse effects of high drug content on the macrophage carrier. Using this approach, DOX as a representative drug can be loaded into macrophages, up to 16.6 pg/cell, and is released at a minimum within the first 6–12 h of cell entry, allowing macrophages to act as an effective carrier to enrich drugs in tumors without killing them prematurely.

In order to improve therapeutic agent loading capacity for diagnosis and therapy, An et al. proposed a strategy to adjust the surface charge of gold nanoparticles, which are the most attractive probes for photoacoustic (PA) imaging and photothermal therapy (PTT), to improve their uptake by macrophages [105]. Small gold nanorods (AuNRs) with different surface charges (cationic, neutral and anionic) were designed, and anionic-AuNRs exhibited the highest phagocytosis numbers in RAW.264 macrophages. The anionic-AuNRs loading capacity of RAW264.7 was 98 μg/10^6 cells. Furthermore, using the tumor homing and penetration behavior inherent in macrophages, in vivo PA imaging confirmed that anion-AuNR carrying macrophages

(Anionic-AuNRs@RAW) were biased toward hypoxic areas of tumors, and anionic-AuNRs@RAW showed enhanced PTT efficacy in tumor-bearing mice. Nowacek and Gendelman et al. developed nanoscale drug crystals (NanoART) loaded into macrophages to protect drugs from degradation and improve intracellular drug loading capacity [75]. HIV antiretroviral therapy/Crystalline drugs were high-pressure or wet-milling homogenized with various poloxamer or phospholipid surfactants to form drug crystals. After phagocytosis by macrophages, the drug-carrying capacity of macrophages was as high as 45 μg/10^6 cells. NanoART dissolves slowly, and ensures macrophages still contain the vast majority of the drug when they migrate to target tissues.

Aside from approaches of increasing drug-load in macrophages, strategies for improving drug loading in other cell types could also provide insights into how to improve drug loading in macrophages. Martinez et al. debuted a multifunctional, highly drug-loaded, multicomponent delivery platform that combines MSCs with multistage nanocarriers (MSV) mediated payload delivery [106]. MSVs are fabricated utilizing biocompatible and biodegradable nanoporous silicon, which have shown a remarkable ability to load payloads, and the combination with drug-loaded MSVs resulted in a five-fold increase in the payload release of MSCs without negatively impacting cellular functions. Another strategy is to use drug molecules directly to create nanoparticles to minimize the incorporation of inert carrier materials, greatly increasing the drug load content. Shen and his collaborators designed the prodrug to form high-loaded nanocapsules, which conjugated the hydrophobic drug camptothecin (CPT) to a short oligomer chain of ethylene glycol (OEG), to form amphiphilic phospholipid mimics prodrugs, and further assembled them into stable liposome like nanocapsules with a CPT loading content up to 40 % or 58 wt% [107]. Likewise, Chai et al. prepared docetaxel (DTX) drug nanocrystals (NC) by a filming-rehydration method with F127 (a non-ionic copolymer surfactant) as the sole excipient. As coated with red blood cell (RBC) membrane, the RBC membrane-coated drug NCs exhibited high drug loading [108]. This has inspired further exploration of macrophages as drug depots to deliver more drugs to target sites and improve treatment efficiency.

4. Sustained modulation of macrophage phenotype

Unlike red blood cells, neutrophils and cancer cells as drug carriers, which can only enhance the blood circulation of drug-carrying particles or their active targeting ability, macrophages have been widely used as therapeutic agents for cancer or inflammatory diseases due to their high immune response properties and remarkable plasticity which can induce therapeutic phenotypes. However, to a large extent, it is high plasticity and flexibility that also can hinder the therapeutic effectiveness of macrophages, as macrophages always respond quickly to their microenvironment after in vitro polarization to the therapeutic phenotype and quickly return to a harmful pathological state. For example, the phenotype of regenerative macrophages disappeared within 3 days after administration of regenerative macrophages into a mouse model of preclinical spinal cord injury [109]. Similarly, Cao et al. found that when macrophages were educated to an anti-inflammatory M2 phenotype in vitro prior to administration into a mouse model of adriamycin-induced nephropathy, M2 macrophages lost their anti-inflammatory phenotype within 2 days and upregulated inflammatory markers within 7 days [110]. Several clinical trials of tumor therapy using anti-tumor macrophages polarized with pro-inflammatory cytokines in vitro have failed because macrophages eventually revert to the tumor-promoting M2 phenotype once embedded in the tumor microenvironment [42]. Therefore, strategies must be developed to promote and limit macrophages to therapeutic phenotypes in order to leverage the powerful therapeutic effects of macrophage-based therapies in the clinic without amplifying local disease.

4.1. Functional polarization of macrophages

Macrophages undergo multiple forms of functional activation in response to different environmental stimuli and signals. Activated macrophages are generally classified into two broad categories, often referred to as classically activated M1 macrophages and alternately activated M2 macrophages [13,111]. Depending on the activating stimulus received, M2 macrophages can be further divided into four distinct subgroups: M2a, M2b, M2c, and M2d [112,113]. This phenomenon of two different M1/M2 phenotypes is known as “macrophage polarization” (Fig. 4).

M1 macrophages are typically polarized by pathogenic products such as lipopolysaccharide (LPS) and type 1 (Th1) cytokines, including interferon-gamma (IFN-γ) and TNF-α. They upregulate interferon regulatory factor (IRF5), increase levels of pro-inflammatory cytokines such as TNF-α, interleukin-1 alpha (IL-1α), IL-6, IL-23, IL-1β, and cyclooxygenase-2 (COX-2) levels, and decrease IL-10 levels, thereby promoting the recruitment of other leukocytes and the generation of ROS and nitric oxide (NO). In addition, M1 macrophages can activate T cells and up-regulate the expression of CD86 and CD80. Functionally, M1 macrophages are involved in the clearance of pathogens and cancer cells, have strong anti-bacterial and anti-tumor activities, are associated with ROS-induced tissue damage, and affect tissue regeneration and wound healing [50,114]. Conversely, M2 polarization is induced by type 2 (Th2) cytokines IL-4 or IL-13 through activation of signal transducer and activator of transcription 6 (STAT6), which promotes tissue repair and remodeling by producing transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). M2 macrophages also perform anti-inflammatory functions by releasing IL-10 [115–117]. In addition, M2 macrophages possess pro-angiogenic and pro-fibrotic properties and are associated with tumor growth [118]. The phenotype of polarized M1-M2 macrophages can, to some extent, be reversed in vitro and in vivo. In several inflammatory conditions or tissue injuries, macrophage phenotypes undergo a series of dynamic changes. M1 macrophages are involved in the initiation and maintenance of inflammation, and M2 phenotypes are associated with the resolution of chronic inflammation [119]. However, growing evidence challenges this simple model, with the observed coexistence of macrophages with different activation states and mixed phenotypes in selected preclinical and clinical conditions, indicating that the M1/M2 macrophage ratio does not accurately reflect the pathological state of the tissue [114,120,121].

4.2. Strategies for sustaining modulation of macrophage phenotype

Recently, researchers have focused more attention on maintaining macrophage phenotypes for therapy and a growing amount of literature has been published with respect to modulating and controlling the phenotype of macrophages [122–124]. Current approaches to sustaining macrophage phenotypes mainly utilize three strategies: 1) phagocytosis of microparticles (>1 μm diameter) instead of nanoparticles by macrophages; 2) “backpack” macrophage surface modification; 3) gene-modified macrophages.

4.2.1. Phagocytosis of microparticles

Unlike the aforementioned rapid exocytosis in which nanoparticles are internalized within macrophages, microparticles are more likely to remain inside the cell for a period of time, allowing the drug to be released in a controlled manner and maintaining the macrophage phenotype. Spiller’s group employed the anti-inflammatory drug dexamethasone (Dex) loaded with PLGA particles to regulate macrophage phenotypes intracellularly [125,126]. After phagocytosis by monocytes, PLGA microparticles can be stored in the cell for at least 5 days, and even on day 7, low levels of Dex were detected in the extracellular culture medium. Due to the retention of internalized microparticles and drugs within the cells, the M2 phenotype persisted for up to 7 days, similar to

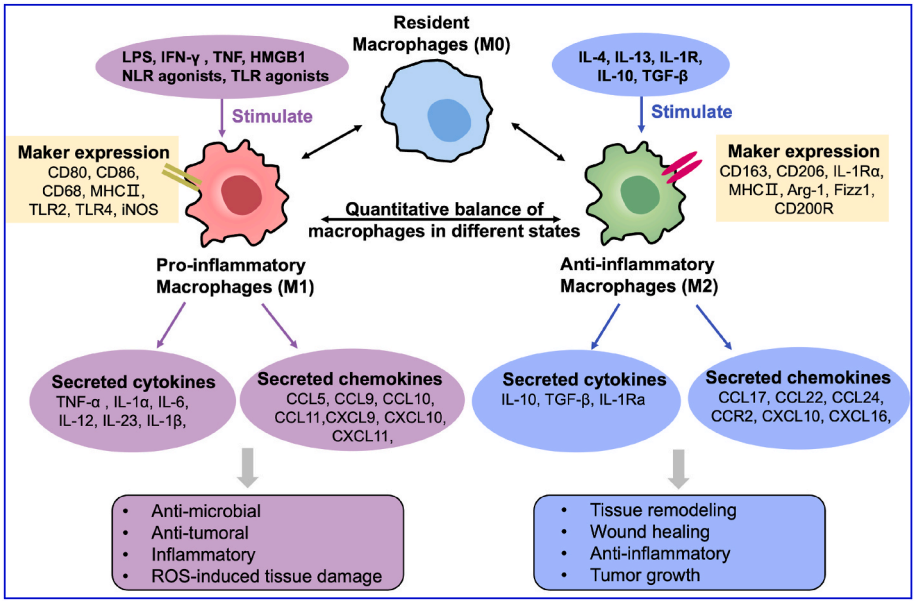

Fig. 4. Different biological and physiological features of M1 and M2 macrophage phenotypes.

cells treated with continuous free dexamethasone. We have demonstrated similar results for the regulation and maintenance of macrophage phenotypes using drug-loaded PLGA microspheres (unpublished data). After being engulfed by BMDMs, PLGA microspheres containing curcumin remained inside macrophages for seven days, releasing the drug and directing macrophages toward M2 polarization for eight days, even in an inflammatory environment.

Macrophages containing microparticles can effectively increase the retention and effect of drugs on cell carriers. However, the main problem is that granular substances and intracellular drugs need to avoid phagosomes to reduce drug degradation. In addition, the issue of whether much stiffer and larger particles affect other functions of macrophages, including migration and targeting ability, needs to be addressed, which will be discussed in the next section.

4.2.2. *Macrophage surface modification — the "backpack"*

Attaching drugs to the surface of cell carriers can overcome the shortcomings of intracellular drug degradation. It enables release of a range of drugs in a controlled and sustained manner, making it easier for macrophages to maintain a favorable phenotype. Mitragotri and colleagues reported an engineered disk-like nanoparticle known as a "backpack" that adheres firmly to the surface of macrophages and regulates cell phenotypes in vivo [127]. Compared with the sphere-shaped particles, the discoidal anisotropic shape allows the backpack to escape phagocytosis and degradation by macrophages for a longer period of time. With coated with IFN-γ, backpacks exhibited controlled and slow drug release kinetics and consistently directed macrophages toward anti-tumor M1 phenotype polarization for at least 5 days, even in the strongly immunosuppressive environment of a mouse model of breast cancer. After intratumoral injection of cell backpacks, the phenotype of endogenous tumor-associated macrophages was reversed.

Nevertheless, the potential interference of attachment with the migration process is a concern. The disk-like cell backpack is administered by intra-tumoral injection; they did not determine tumor targeting ability and transendothelial migration processes by intravenous administration. Whether surface attachment affects the recruitment of macrophages by inflammatory chemokines and cytokines remains to be further studied. In addition, immunoadjuvants loaded in the particles have a limited half-life, and may gradually degrade and lose their activity. To overcome such hurdles, Zhang and colleagues very recently reported a bacteria-based backpack that can adhere to macrophages (MΦ@bac) to guide cellular polarization in vivo by leveraging the native immunogenicity of bacteria [128]. The attached bacteria, referred to as backpacks, were not only abundant in proinflammatory factors but also able to proliferate in tumor tissues, resulting in a sustainable activation toward M1 phenotypes of MΦ@bac. Moreover, MΦ@bac exhibited tumor-homing ability comparable to MΦ with M1 phenotypes, repolarized endogenous TAMs and then remodeled immunosuppressive TEM. However, there are also limitations to the surface modified approach. The attachment of this backpack to macrophages may be nonspecific and temporary. Due to shear stress or cell reshaping, it may cause detachment of particles during transport [15,101]. And the released drugs or backpacks, which are directly exposed to enzymes in the blood and interact with the filtering organs, may lead to degradation and loss of control of the macrophage phenotype. Further studies are required to precisely adjust their interactions.

4.2.3. *Gene-modified macrophages*

In addition to non-genetically engineered strategies, gene-modified approaches have shown promise in controlling macrophage phenotypes and enforcing specific therapeutic behaviors, particularly in terms of anti-tumor effects. Gill and his collaborators applied chimeric antigen receptors (CARs) to macrophages and engineered CAR-macrophages (CAR-Ms) by genetically engineering human macrophages with CARs to confer a sustained pro-inflammatory (M1) phenotype and direct their tumor-phagocytosis activity [129]. The transduced CAR-Ms present and maintain the M1 phenotype for at least 40 days, and also express pro-inflammatory cytokines that reprogram M2 macrophages to M1 and enhance anti-tumor T cell activity. A parallel study led by Zhang et al. reported on induced pluripotent stem cells (iPSCs)-derived CAR macrophages (CAR-iMACs) [130]. The expression of CAR polarized macrophages towards pro-inflammatory or anti-tumor phenotypes and enhanced the phagocytosis of cancer cells and anti-cancer activity in vivo. Recently, Zhang's group upgraded the first-generation CD3ζ-based CAR-M and developed the second-generation M (iMACs)-CAR by integrating intracellular CD3ζ and intracellular toll/IL-1R (TIR) domain of toll-like receptor 4 in tandem to construct an antigen-targeting M-CAR, resulting in both target engulfment capacity and antigen-dependent M1 polarization and M2 resistance, also exhibiting a markedly enhanced antitumor effect compared with the first-generation CAR-M [131]. Bei and co-workers also employed CAR-M based on genetically modified human peritoneal macrophages (PMs) expressing a HER2-FcεR1γ-CAR

(HF-CAR), to enhance anti-tumoral effects on gastric cancer [61]. Meanwhile, Shi et al. found that insulin-like growth factor (IGF-2) modulated the innate immune memory of macrophages, enabling them to sustain oxidative phosphorylation, and trained macrophages to become anti-inflammatory phenotypes even upon pro-inflammation stimulation [132]. Furthermore, following administration of IGF-2-preprogrammed peritoneal macrophages, they showed a significant effect in treating encephalomyelitis (EAE) and inflammatory bowel disease (IBD), inhibiting inflammation in EAE and IBD mice and promoting Treg differentiation by enhancing PD-L1, reducing severities.

Additionally, Deng and co-authors permanently reprogrammed the phenotype of macrophages without reconversion by leveraging the nanosystem-based CRISPR/Cas9 technology [133]. An X-ray-triggered remotely controlled CRIAPR/Cas9 genome editing nano-system (X–CC9) was constructed by X-ray-sensitive diselenide bonds cross-linked polyetherimide-g-poly(propylene sulfide) (PEI-PPS) nanoparticles loaded with plasmid pCas9-CSF1-R and pCas9-SIRPα (CC9) with a pH-responsive PEG shell. The pH-sensitive PEG corona was de-shielded in responsive to the acidic tumor microenvironment and the diselenide bridges were broken under X-ray irradiation. CC9 was then released for effective genome editing and accurate phenotypic polarization of tumor-associated macrophages (TAMs) from M2 to M1 *in situ*, further significantly improving antitumor efficacy with robust immune responses. It is worth noting that the insertion of genetic material into the host genome is potentially harmful, and the transfection rate of gene edited cells was low and immunogenicity was enhanced. Therefore, further development on this front is absolutely needed.

5. The homing of macrophages to diseased tissue

The ability of macrophages to possess and maintain inflammation homing and tumor targeting after drug or particle loading is an important theoretical requirement for macrophage-based cell therapy, essential to improve therapeutic efficacy, reduce the number of transplanted cells and undesirable side effects. A number of studies have shown that the macrophages still possess their homing ability when drugs are packaged inside macrophages [134–136]. For example, in a study on the biological distribution of CAP (CpG-ASO-Pt)-nanosphere-laden macrophages (CAP@M) injected intravenously in a nude mouse model of subcutaneous A549 tumor, imaging results showed that only a moderate amount of CAP nanospheres accumulated at the tumor site due to passive diffusion of CAP nanospheres in tumor blood vessels [137]. In comparison, along with the CAP nanospheres loaded into macrophages, CAP@M showed the greatest migration to the tumor area even 24 h after injection, suggesting that macrophages can deliver the CAP nanospheres to the tumor site. In a macrophage-mediated delivery study carrying the nanozyme catalase, pharmacokinetic results showed that the plasma half-life of catalase increased from 2.5 h to 3.3 h and the time-averaged total body clearance (CL) decreased from 6.7 mL/h to 2.4 mL/h after delivery of catalase in macrophages, and that macrophages loaded with nanozyme increased the delivery of catalase to a range of different tissues [138].

5.1. The multistep process of macrophage transmigration

Macrophage recruitment is specific and highly regulated by several membrane proteins and cytokine/chemokine gradients. Circulating macrophages recognize the endothelium of blood vessels in inflammatory tissue, and interact with the vascular wall through a series of steps including capture, rolling, activation, firm adhesion, crawling and transmigration (Fig. 5) [18,139,140]. During inflammation or infection, endothelial cells of blood vessels upregulate endothelial selectins (E-selectin and p-selectin), vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1), mediating the capture of macrophage from the flowing blood. The transient interaction then causes macrophages to roll up the endothelium. Cytokines and chemokines, which are produced by endothelial cells or derived from inflammatory cells and transmitted on endothelial cells, trigger activation of rolling macrophage integrins, such as leucocyte function-associated molecule-1 (LFA-1, a ligand of ICAM-1), Mac-1 (a ligand of ICAM-1), and very late antigen-4 (VLA4, a ligand of VCAM-1), and bind to adhesion molecules on endothelial cell surface. After firm adhesion,

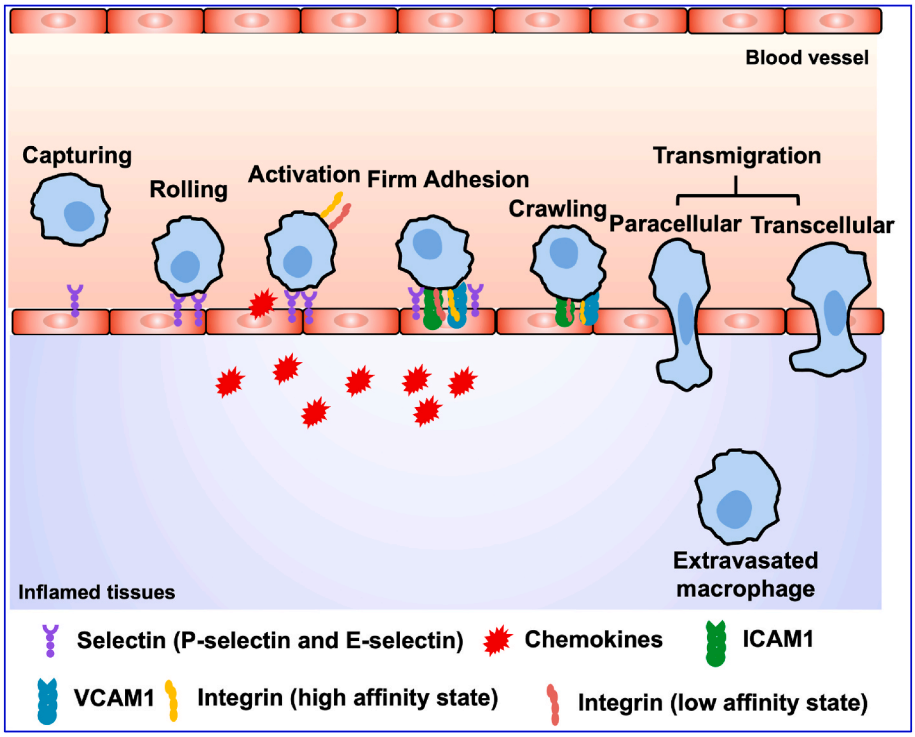

Fig. 5. Schematic illustration of the homing process of macrophage in six major steps: capturing, rolling, activation, firm adhesion, crawling, and transmigration.

macrophages start to crawl on the surface of the vascular lumen until they transmigrate through the endothelial barrier at the appropriate sites.

5.2. The challenge of macrophage recruitment

Whether macrophages can maintain their targeting ability while carrying drugs or drug-loaded particles, in aspects such as migration speed, migration amount, and so on is a matter of concern. In this regard, there is a lack of research identifiable that is in a systematic and quantitative manner. The average diameter of macrophages is 25 μm, which is much larger than the ~10–15 μm diameter of pulmonary capillaries [141,142]. In addition, loading of polymer or inorganic particles increases cell stiffness. Therefore, mechanical entrapment in the lungs occurs often via systemic circulation. Previous studies have documented that after intravenous injection, drug-loaded macrophages are distributed and accumulate in the three major organs of the liver, lung and spleen [60,143,144]. In a clinical trial on the biological distribution of autologous activated macrophages (AAM) in patients with metastatic renal cell carcinoma (RCC), results showed a transient retention of AAM in the lungs, where they predominated in the first hour after intravenous infusion. Later on, macrophages accumulated in the liver and spleen and then decreased from day one and day two, respectively [145]. This limits the access of exogenously introduced macrophage-based therapeutics to target tissues outside the lung and results in the ineffectiveness of the vast majority of therapy being administered. Hence, the homing ability of macrophages to target tissues needs to be further improved.

5.3. Improvement of macrophage recruitment

The most common trains of thought for improving the targeting ability of engineered macrophages include; 1) improving the targeting ability of engineered macrophages themselves; and 2) reducing the entrapment of macrophages in the lung.

5.3.1. Improving targeting ability of macrophages themselves

The current strategy for enhancing macrophage homing ability is to integrate specialized agents that respond to external stimuli, such as high-frequency magnetic fields or near-infrared (NIR). For example, Li et al. elaborately designed artificial reprogrammed macrophages with hyaluronic acid-decorated superparamagnetic iron oxide nanoparticles (HIONs) for augmentable tumor targeting ability and tumor cytostatic effect [134]. Under magnetic guidance, Hions-laden macrophages (HION@Macs) displayed improved tumor-tropic migration and chemotaxis compared to native macrophages. The results indicated that the integration of HIONs into macrophages offered a chance to combine magnetic targeting with the bio-targeting of macrophages. Similarly, Nguyen and colleagues developed dual-targeting macrophages-based microrobots, incorporated with the aforementioned superparamagnetic nanoparticles [45]. After being injected intravenously in tumor-bearing mice, with the help of an external magnetic field, the microrobots navigated towards the tumor site more efficiently than native macrophages at each evaluation time point. At the same time, a reduction of microrobots in the lungs, in the presence of the magnetic field was also observed. Wang et al. reported an in vivo strategy to construct cell-based nanomedicine carriers, where both bacteria membrane coated β-CD-modified gold nanoparticles (GNPs) and ADA-modified GNPs were intravenously injected, selectively phagocytosed by phagocytic immune cells, and subsequently self-assemble into sizable intracellular aggregates via host-guest interactions, inhibiting the leakage of GNPs from immune cells [146]. Moreover, the initial photothermal treatment (PTT) treatment caused tumor damage and increased the inflammatory signal from the tumor tissue, leading to enhanced recruitment of immune cells and significantly improved targeted hitchhiking delivery of GNP aggregates to 1.78-fold that of mice without PTT treatment. These results provide further evidence that the use of external stimuli, combined with the native targeting ability of macrophages, could lead to enhanced migration.

Additionally, on the basis of the macrophage migration process, a potential strategy to improve cell homing efficiency is to use peptides or other molecules responsible for cell rolling, adhesion, or extravasation, such as cell surface adhesion molecule-binding peptides, selectin binding peptides, appropriate antibodies, and modify or overexpress them on macrophages to improve macrophage transmigration.

5.3.2. Reducing macrophage entrapment in the lung

In order to overcome the pulmonary obstacle and improve targeting, pre-administration of vasodilators such as sodium nitroprusside have been used to increase blood vessel diameter to reduce cell entrapment in the lung [141,147]. Another approach is to use macrophage-derived extracellular vesicles or exosomes instead of macrophages to modify cell size. Exosomes are 40–100 nm particles, smaller than parental cells in diameter, but they inherit most of the characteristics of parental cells such as biocompatibility, targeting ability, and long-term circulation [148]. After intravenous (i.v.) administration, the smaller diameter reduces their entrapment in the pulmonary capillaries. Furthermore, exosomes are also lipid-delimited vesicles that facilitate loading of cargo through active or passive loading mechanisms [149,150]. Gao and colleagues collected M2 exosomes from primary peritoneal macrophages of type M2 for berberine delivery (Exo-Ber), and in vivo imaging studies have shown increased accumulation of Exo-Ber in spinal cord injured tissues and decreased accumulation in the lungs [151]. Similarly, Wu et al. reported that molecularly engineered M2 macrophage-derived exosomes (M2 Exo) have inflammation-tropism and anti-inflammatory capabilities in atherosclerosis imaging and therapy [152], and Mei and co-authors also demonstrated that M1-like macrophage exosomes could effectively enrich in tumor sites [153]. Another promising strategy is to increase cell deformability, allowing macrophages to migrate more efficiently through lung capillaries. In 2022, Wang and colleagues employed enucleated human mesenchymal stromal cells (cargocytes) for targeted delivery of therapeutic agents to diseased tissues [154]. With density gradient centrifugation removing rigid nuclei, “cargocytes” are much smaller, more prone to deformability, and can pass through small contractions more efficiently than nucleated parental MSCs, and show decreased lung trapping and improved biodistribution [155,156].

6. Drug release from macrophages at targeted sites

Drug release from macrophages is also an active area for further exploration. The controlled and full release of drugs from macrophages within the diseased tissue is key to reducing the dose and frequency of administered engineered macrophages, improving the accumulation of drug carriers in the pathological tissue and improving the therapeutic effect, as well as further increase relative safety. Currently, the three most common drug-release mechanisms in macrophages are the passive release of drugs through cell death, slow release of drugs capable of crossing cell membranes, or through exosomal mechanisms [42]. For example, in DOX/liposomal loaded macrophages, macrophage death further releases drugs due to DOX leakage induced by internal degradation of liposomes [30]. In addition to the degradation pathway, macrophages carrying DOX-loaded nanoparticles have also been found to release drugs through exocytosis [57].

6.1. Challenges of drug release from macrophages

One of the challenges of drug release by macrophages is that drugs, especially cytotoxic drugs, or nanomedicine, are usually released uncontrollably or prone to exocytosis, and prematurely leak into the cytoplasm uncontrollably, causing the macrophages to die prematurely before reaching the target site [157]. As with the degradation-mediated drug release mechanism mentioned above, this uncontrolled drug

release situation is far from satisfactory. On the other hand, how to fully release the cargos of macrophages after they reach the target site remains a challenge. Drugs enter macrophages through endocytosis and are sequestered in the phagosomes of macrophages, where they are degraded [76]. Only some drugs successfully escape the phagosomes and are released from the macrophages.

6.2. Strategies for controlling drug release from macrophages

From this perspective, utilizing the cell's response to external conditions, such as magnetic field, ultrasonication, and laser-induced temperature gradient, could provide controlled and adequate drug release. In recent years, various measures have been taken to achieve drug control and full release of engineered macrophages at target sites. Sun and colleagues designed smart nanoparticle loading and NIR controlled self-destructing macrophages for controlling and releasing its payload at tumor sites [157]. Manganese dioxide (MnO_2) shells encapsulated DOX-loaded mesoporous carbon nanospheres (MCNS) to form smart nanoparticles as the inner cores (designed as MDM), which were then ingested by macrophages to obtain nanoparticle loaded macrophages (designated as MMDM). Due to the good pore blocking effect of the MnO_2 shell, undesirable leakage of drugs in MMDM is avoided during the system circulation. After the MMDM migrated to the tumor site, the carrier macrophages were destroyed under NIR, so that the macrophages released sufficient MDM. MnO_2 shells were further degraded in the tumor microenvironment, and DOX was released and absorbed by tumor cells. In a parallel study, Huang et al. made genetically engineered macrophages encoding the EGFP-TNF-α fusion protein and loaded the cells with a NIR-reactive thermal nanogenerator. The release of EGFP-TNF-α triggered by NIR irradiation was spatiotemporally controlled [158].

However, external stimulation requires the use of external devices under continuous medical imaging, as well as expertise in precise manipulation of micromotors in vivo, thus hindering clinical translation, especially in remote clinic applications. Wang and colleagues developed a series of self-propelling cell platforms based on intracellularly engineered macrophages, to overcome the premature efflux of internalized nanomedicine [159,160]. By aid of multiple and strong β-CD and ferrocene (Fc) host-guest interactions, β-CD-capped CuS NPs and Fc-capped CuS NPs at the diameter of around 15 nm were phagocytosed sequentially by macrophages and subsequently self-assembled into 1 μm of large aggregates, thereby inhibiting exocytosis from macrophages [159]. By sensing the inflammatory tumor microenvironment, macrophages carrying CuS aggregates actively targeted tumor tissues, and the oxidation of Fc dissociated the β-CD-Fc host-guest pair, driving disassembly of aggregates and release of small CuS NPs to penetrate deep tissue. In addition, MnO2 NPs modified by host molecule β-CD (MnCD) and MnO2 NPs modified by guest molecule amantadine (AD) (MnAD) have also been shown to self-assemble into micro-sized aggregates intracellularly through host-guest interaction after phagocytosed by macrophages, effectively inhibiting the efflux of nanoparticles [160].

In addition, macrophage surface anchoring is another way to achieve controlled drug release and bypass the membrane barrier. Mitragotri and colleagues are the first to use cell backpacks attached to the surface of macrophages to achieve finely controlled drug release [76]. After loading fitc-bsa-loaded PLGA particles into a backpack, FITC-BSA was released from the backpack in a burst fashion, with about 40 % released in the first 2 h and >90 % released within 24 h, indicating a controlled release [76]. A similar strategy is used to deliver and release catalase to the brain, and the release of catalase from preloaded macrophage backpacks exhibits long-term and sustained behavior [161]. Recently, considering the limitations of conventional surface attachment methods described above, researchers have developed simpler and more benign cell membrane loading methods for drug release. For example, Yang et al. used non-covalent biotin-avidin crosslinking as an alternative method for loading DOX-carrying liposomes (DOX-Lip) onto the surface of macrophages [162]. In this way, compared with free DOX in PBS, DOX-Lip exhibits a more durable release characteristic and does not have a burst initial release. Zhu et al. developed a metal-coordinated loading method in which nanoparticles are adsorbed to the surface of macrophages through a metal-phenol network formed by the coordination of metal ions with phenolic ligands [163].

7. Macrophage-related biomimetic drug delivery systems

In addition to the macrophage-based drug delivery systems discussed above, macrophage-related biomimetic drug delivery systems, including those based on nanomaterials, macrophage cell membranes, and macrophage-derived extracellular vesicles (EVs), have also been developed.

In the past few years, widespread development of drug carriers dominated by nanotechnology-based drug delivery systems, including liposomes, micelles, polymeric or inorganic nanoparticles, solid lipid nanoparticles, has improved the stability and solubility of free therapeutics, facilitated transmembrane transport and prolonged circulation times to improve safety and efficacy [164–166]. However, the number of nanotechnology-based drug delivery systems actually in use in clinical settings is far lower than the extensive research in the field would suggest, partially because of their improved but still unsatisfactory physicochemical and biological properties, such as immunogenicity, short plasma half-life, poor barrier crossing, and tissue penetration [1,167].

With newer developments, macrophage membrane-coated nanoparticles and macrophage-derived extracellular vesicles (EV) have become promising platforms for drug delivery. Macrophage membrane-coated drug delivery systems inherit the membrane proteins of macrophages, such as chemokine receptor proteins and adhesion proteins, which grant them immune escape, longer blood circulation time, and enhanced inflammation tropism effect and adhesion with targeted tissues and cells [168]. Wang et al. reported a biomimetic drug delivery system derived from macrophage membrane coated ROS-responsive nanoparticles [169]. Macrophage membranes not only avoided the clearance of nanoparticles from the reticuloendothelial system (RES) and directed them to the inflammatory tissues, but also sequestered proinflammatory cytokines to inhibit local inflammation. However, at present, the preparation process for macrophage membrane-coated nanoparticles is still at the laboratory research level, being complex and small-scale. Clinical translation is limited by the lack of suitable technologies for mass production of cell membrane. The membrane extraction process has a high cost and low yield and efficiency, along with unstable quantity, density, structure, and activity, and loss or misorientation of membrane proteins, which may affect the function of the membrane and even produce potential negative side effects. In addition, the long-term storage and transportation of cell membrane-coated nanoparticles remains a challenge due to the easy degradation of membrane proteins at 4 °C or environmental conditions [170,171].

As for macrophage-derived EVs, including exosomes, ectosomes, microvesicles, membrane vesicles and apoptotic bodies, similar genetic and protein components to macrophages endow them with low immunogenicity, the ability to transport across certain biological barriers, and an higher ability to target inflammatory tissues or organs than traditional drug delivery systems, even without any modifications [148,172]. For example, compared to nanoparticles, macrophage-derived exosomes coated with murine RAW264.7 had improved blood circulation time and smart organ tropism, and provided powerful chemotherapy to breast cancer cells in tumor tissues [173]. However, due to macrophage capture, EVs remain prone to rapid clearance from the bloodstream after systemic administration. The terminal half-life of EVs was at most 60 min, and less than 5 % of the injected dose of exosomes remained in the blood circulation at 3 h post-injection [174,175]. Due to unresolved issues of purity and identity, comprehensive and standardized

characterization, and quality assurance of EVs remains a huge challenge. Currently, the term EV actually comprises a heterogeneous population with several subtypes. There are no typical EV markers on EVs that are identifiable, making it difficult to distinguish EVs from pollutants [176]. Therefore, macrophages as a carrier for drug delivery represent one of the most promising new directions for development.

8. Conclusion and future perspective

In summary, macrophage-based cell therapies are emerging in rapid development and are widely used in the treatment of diseases with an inflammatory component, including cancer, neurodegenerative diseases, lung injury, rheumatoid arthritis, and arthrosclerosis. The advantages and limitations of macrophage-based cell therapies were summarized in Table 2. On the whole, macrophages are being leveraged for disease therapies following three aspects: (1) Non-genetically engineered macrophages as therapeutics, (2) macrophages as delivery carriers, and (3) genetically modified macrophages having enhanced therapeutic effects. Macrophage-based drug delivery systems are typically constructed using two strategies: phagocytic drugs in the cytoplasm or wrapping drugs around cell membranes. Each approach has its own unique advantages and disadvantages, which should be considered in light of the mechanism of the disease being treated and the characteristics of the drugs and particles used.

In addition to the challenges discussed above, the development of engineered macrophages for therapeutic use still faces several technological problems. To date, the modification or education of macrophages is mostly in vitro, which affects the survival rate, activity and circulation time of exogenous macrophages in vivo [177]. Another problem is the precise polarization of macrophages to the M2 subtype (M2a, M2b, M2c or M2d). Different M2 subtypes have their own functions. Controlling the macrophage phenotype to a specific M2 subtype and to achieve maximum therapeutic effect remains to be further investigated. In addition, quantitative evaluation methods for biodistribution, pharmacokinetics and targeting efficiency of exogenous engineered macrophages after administration are still lacking. Although a series of literatures have reported qualitative analyses of the biodistribution of injected macrophages within various organs, few studies have examined the percentage of doses or the absolute number of macrophages that reach the target tissue, which makes it difficult to assess the targeting efficiency of engineered macrophages. Since the quantification of cell biodistribution provides important information for determining the engineering macrophage regimen, improving the safety of macrophage therapy, and reducing toxicity to other healthy organs, further study of methodology is needed.

Furthermore, although some macrophage-based therapies have entered preclinical applications, there are still many practical issues to consider before these therapies can be transferred from the laboratory to the pharmaceutical industry. Firstly, it is important to note that there may be immune rejection when allogeneic macrophages are injected into patients, although animal studies of allogeneic macrophages have shown promising results in treating disease. Instead, autologous macrophage-based therapy may be preferable, but since the treatment and targeting ability will need to be personally customized, it will be of high cost. Secondly, large-scale manufacturing of macrophages remains a serious obstacle to practical application. Therefore, mass production technology for macrophages needs to be developed. Thirdly, the quality control of human macrophages is difficult. The commercialization of macrophage formulations requires stricter Good Manufacturing Practice (GMP) production conditions. It is important to construct standard protocols for macrophage extraction and macrophage engineering to reduce batch-to batch variability. Finally, it is necessary to develop reliable detection techniques and technical indicators to evaluate the characteristics of macrophages.

As a highly promising emerging therapeutic approach, engineered macrophages overcome many of the limitations of current pharmaceutical, nano-based, or other formulations. New discoveries made in the past few years have opened avenues for effective research into the problems or challenges mentioned in the preceding paragraphs. The solution of these problems or challenges will make it possible for the basic and applied research results of macrophage drug delivery to be translated into the clinic, and will certainly bring benefits to solve presently intractable diseases, such as cancer and autoimmune diseases.

Compliance with ethics guidelines

We have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

Data availability

No data was used for the research described in the article.

Table 2
Advantages and limitations of macrophages as drug carries.

Usage of macrophages	Characteristics	Advantages	Limitations	Reference
Drug carriers	Non-immunogenic	Macrophage cell lines		[30,45,51–54,57,59–63]
		Immortal, convenience	Immunogenicity and pro-tumorigenic properties	
		Primary macrophages		
		Low immunogenicity, excellent biocompatibility	Low production, a short half-life of 20 h in the blood, short-lifespan	
	Phagocytosis	Loading drugs	Limited amount of drug loaded in macrophages	[22,25–27,30,45,75,105,125,126,159,160]
	Surface modification			[76,98–100,127,161–163]
	Inherent inflammation homing	● Migrating to inflammatory sites; ●Crossing blood-brain barrier to target inflamed brain tissue; ●Deep infiltration into the hypoxic areas of tumors that lack blood flow	Ineffectiveness of disease targeting ability	[45,134–138,146]
	Blood circulation	●Prolong blood circulation time of drugs or nanomedicine; ●Protecting drugs or nanomedicine from recognition and clearance by RES	Uncontrolled drug release at targeted sites: ●Prematurely or leaked before reaching the targeted sites; ●Incomplete release in the targeted sites	[76,157–163]
Therapeutics	High plasticity of different phenotypes	Re-educated to therapeutic phenotypes to exert anti-tumor, anti-inflammatory, anti-bacterial activities	Rapid conversion of macrophages from therapeutic phenotypes to detrimental state	[61,125–133]

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Ethics approval and consent to participate

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CRediT authorship contribution statement

Qian Guo: Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zhong-Ming Qian:** Validation, Funding acquisition, Formal analysis, Data curation, Writing – original draft, Writing – review & editing.

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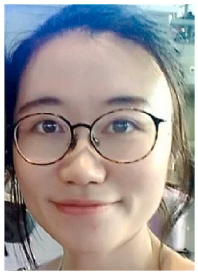
Abbreviations

AAM: autologous activated macrophages
AMs: alveolar macrophages
AuNRs: Small gold nanorods
BBB: blood-brain barrier
BMDMs: bone marrow-derived macrophages
CA: citric acid
CA-MNP: CA coated superparamagnetic nanoparticles
CAP: CpG-ASO-Pt
CAP@M: CAP-nanosphere-laden macrophages
CARs: chimeric antigen receptors
CAR-Ms: CAR-macrophages
CD115: clusters of differentiation 115
COX-2: cyclooxygenase-2
CPT: camptothecin
Dex: dexamethasone
DOX: doxorubicin
DOX-Lip:: DOX-carrying liposomes
DTX: docetaxel drug
ICAM1: intercellular adhesion molecule-1
IFN-γ:: interferon-gamma
IL-1α: interleukin-1 alpha
IL-1β: interleukin-1 beta
iPSCs: induced pluripotent stem cells
IRF5: Interferon regulatory factor
LFA-1: leucocyte function-associated molecule-1
LPS: lipopolysaccharide
M2 Exo: M2 macrophage-derived exosomes
Mac-1: macrophage 1 antigen
MCNS: mesoporous carbon nanospheres
M-CSF: macrophage colony-stimulating factor
MHC II: major histocompatibility complex class II
MnO2: Manganese dioxide
MPS: Mononuclear Phagocyte System
MSCs: mesenchymal stem cells
MSV: multistage nanocarriers
NanoART: nanoscale drug crystals
NC: nanocrystals
NIR: near-infrared
NO: nitric oxide
NPs: nanoparticles
NP-IDV: nanoparticle indinavir
OEG: oligomer chain of ethylene glycol
PA imaging: photoacoustic imaging
PDGF: platelet-derived growth factor
PEG: poly(ethylene glycol)
PLGA: poly(lactic-co-glycolic acid
PMs: peritoneal macrophages
PTT: photothermal therapy
RBC: red blood cell
RCC: renal cell carcinoma
ROS: reactive oxygen species
SPMs: spleen macrophages
STAT6: signal transducer and activator of transcription 6
TGF-β:: transforming growth factor-β
TNF-α:: tumor necrosis fact-alpha
TPZ: triapazamine
TSLPs: thermosensitive liposomes
VLA4: very late antigen-4
VCAM1: vascular cell adhesion molecule-1
VEGF: vascular endothelial growth factor



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