



Review article

Advancing cellular immunotherapy with macrophages

Alok K. Mishra ^{*}, Sunil K. Malonia ^{*}

Department of Molecular, Cell, and Cancer Biology, UMass Chan Medical School, Worcester, MA 01605, USA

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ABSTRACT

Cell-based immunotherapies have become an exciting avenue for cancer treatment, particularly CAR T cells, which have shown great success in treating hematological malignancies. However, the limited success of T cell-based approaches in treating solid tumors has sparked interest in alternative cell types that could be used for solid tumor immunotherapy. Recent research has pointed to macrophages as a potential solution, given their ability to infiltrate solid tumors, exhibit a strong anti-tumor response, and persist long-term in the tumor microenvironment. Although early attempts with *ex-vivo* activated macrophage-based therapies failed to translate into clinical success, the field has revolutionized with the recent development of chimeric antigen receptor-expressing macrophages (CAR-M). While CAR-M therapy has reached the clinical trial stage, several challenges still need to be overcome before the therapy can become a reality. Here we review the evolution of macrophage-based cell therapy and evaluate recent studies and developments, emphasizing the potential of macrophages as cellular therapeutics. Furthermore, we also discuss the challenges and opportunities associated with using macrophages as a basis for therapeutic interventions.

1. Introduction

Cancer is a heterogeneous disease resulting from the accumulation of genetic, genomic, and epigenetic alterations in normal cells, leading to uncontrolled cell growth and tumor formation [1,2]. The immune system can recognize and eliminate aberrant or transformed cells. However, cancer cells have evolved various mechanisms to evade or suppress the immune response [3]. Immunotherapy, a type of cancer treatment that leverages the immune system to recognize and attack cancer cells [4], has emerged as a breakthrough in cancer treatment, with several new drugs and therapies receiving approval from the US Food and Drug Administration (FDA). Several immunotherapy-based approaches have been developed in recent years, including checkpoint inhibitors, cancer vaccines, adoptive cell transfer, and cytokine therapies, each of which works in different ways to stimulate the immune response [5]. Adoptive cell therapy (ACT) is a type of immunotherapy that involves usage of patient's own immune cells, that are engineered or stimulated to recognize and attack cancer cells. ACTs have shown considerable promise in treating a variety of malignancies and have emerged as a potential immunotherapy approach for cancer [6]. Among ACTs, genetically engineered T cells expressing chimeric antigen receptors, CAR-T cells, have gained tremendous popularity, especially for their efficacy in treating hematological malignancies [7]. However with

limited efficacy in solid tumors, CAR T cells encounter numerous hurdles when it comes to solid tumors [8]. Some of these include, presence of heterogeneous tumor antigen expression, physical barriers and immunosuppressive tumor microenvironments (TME) that adversely impact the stimulation, activation, accessibility, infiltration, and durability within the tumor microenvironment [9]. Although six CAR-T cell therapies have been licensed for therapeutic use [10], none have been approved for the treatment of solid cancers. Recently, there have been advancements in expanding the CAR platform beyond conventional T cells to other leukocytes, such as CAR-expressing NK and gamma-delta ($\gamma\delta$) T cells. These subsets of lymphocytes possess innate immune functions that could broaden their capability to eliminate tumors beyond the capacity of conventional CAR T cells [11]. Additionally, they can target cancer cells independently of the major histocompatibility complex (MHC), reducing the chance of alloreactivity. Moreover, due to their interaction with antigen-presenting cells, CAR $\gamma\delta$ T and NK cells can effectively link the innate and adaptive immune systems, leading to enhanced immune responses against cancer. However, the large-scale propagation of these immune cells, either *ex vivo* has limited the widespread adoption of CAR NK and CAR $\gamma\delta$ T cell-based therapies [11]. In recent years, macrophages have gained significant attention as a tool and target for cancer immunotherapy [12]. The continuous trafficking/recruitment of monocytes within TMEs and the constant replenishment

^{*} Corresponding authors.E-mail addresses: alok.mishra@umassmed.edu (A.K. Mishra), sunil.malonia@umassmed.edu (S.K. Malonia).

of tumor associated macrophages (TAM) in solid tumors make macrophages not only a valuable tool for cell-based anticancer agents but also a vehicle for delivering anticancer arsenal into the otherwise impenetrable tumor sites. Macrophages are innate immune cells with robust phagocytic and cytotoxic properties that can initiate and amplify an adaptive immune response by recruiting T cells, presenting antigens, providing co-stimulation, and secreting cytokines. Unlike CAR-T cells macrophages exhibit the ability to penetrate and persist within TME, modulate activity of other immune cells, promote long-term anti-tumor immunity, and protection against antigen-negative relapse through an adaptive immune response, which makes them a favorable alternative for cell-based therapy [12]. In addition to this, macrophages have a longer lifespan and low toxicity than other immune cells, which can contribute to their sustained anti-tumor effects and are relatively safe to use in cell-based therapies [13,14]. Moreover, macrophages can be genetically engineered to specifically target and infiltrate tumors, providing a localized therapeutic effect, reducing off-target effects, and improving efficacy [15]. It remained, however a hurdle to develop engineered macrophage-based therapy until recently developed viral vector-based platforms for transducing human macrophages [16,17]. The development of CAR macrophages is a relatively new area of research in the field of immunotherapy. However, despite the potential of macrophages as cell therapy, there are still many challenges that need to be overcome before it becomes a widely available and effective treatment option for cancer. In this review, we summarize the current state of macrophage-based cell therapies, including CAR-M development, applications, and prospects as a potential immunotherapeutic approach for solid tumors.

2. Evolution of macrophages-based cell therapies

In the early 1970s, Isaiah Fidler first proposed the concept of macrophage-based cell therapy as a potential method to prevent tumor metastasis. He observed that the administration of *ex vivo* activated syngeneic macrophages in B16 melanoma xenograft significantly reduced pulmonary metastases in mice [18]. Over the years, macrophages have been explored for their therapeutic potential in three primary ways, (1) using *ex vivo* activated macrophages, also known as macrophage-activated killer (MAK) cells, (2) use of macrophages as a delivery system for targeted delivery of cytokines, plasmid DNA, and other therapeutics directly to the tumor site (3) and chimeric antigen receptor-expressing macrophages (CAR-M), which have been recently developed. Table 1 shows a timeline of milestones achieved in the evolution of macrophage-based cell therapies for cancer treatment.

3. Macrophage-activated killer (MAK) cells

Scientists explored the use of *ex vivo* activated macrophages as a potential treatment for cancer in the early 1980s. By using activated macrophages, it was hoped that educated macrophages would migrate naturally to tumors and modify tumor microenvironment to stimulate endogenous immunity. German researchers, Andreesen's group pioneered the use *ex vivo* cultured macrophage as an anticancer therapy. They treated 15 patients with advanced cancers that had not responded to standard treatments, using monocyte-derived macrophage cell therapy. The macrophages were stimulated with IFN γ to induce the M1 phenotype before being administered to patients. While the primary tumor did not regress, some patients showed stable disease for up to 6 months post-therapy. Reported side effects were also limited to low-grade fever and discomfort at the injection site [16]. Although subsequent studies demonstrated antitumor activity against cell lines *in vitro* and in preclinical models using a similar procedure, a head-to-head comparison trial with Bacillus Calmette-Guerin vaccine for bladder cancer did not show improved tumor control with macrophage cell therapy models [17]. The limited effectiveness of these non-engineered macrophages might stem from their failure to identify tumor-associated

Table 1
Timeline of macrophage based cell therapy evolution.

Time Period	Milestones	Ref.
1970–80s	Researchers begin exploring the potential use of macrophages for cancer immunotherapy	[18,19]
1987	The first use of <i>ex vivo</i> cultured macrophages as an anticancer therapy was attempted. 15 patients with advanced cancers who had previously received standard treatment without success were treated with <i>ex vivo</i> cultured monocyte-derived macrophages.	[20]
1988	IFN γ activated macrophages also called as MAKs were tested against cell lines <i>in vitro</i> and in preclinical models.	[21]
2006	Chimeric CD64 receptor expressing monocytes were generated using adenovirus delivery of transgene in primary human monocytes to target CEA-expressing tumor cells <i>in vitro</i> and <i>in vivo</i>	[22]
2009	Macrophages were used as delivery vehicle to deliver cytokine to achieve antitumor effect	[23]
2011–13	A lentiviral vector based on HIV-1 that contains Vpx accessory protein was created to enhance transduction efficiency of myeloid cells, which are typically challenging to transduce.	[24,25]
2016	Vpx-LV lentiviral expression system was validated for creating transduced monocytes and macrophages derived from monocytes.	[15]
2016–2017	Society for Immunotherapy of Cancer (SITC,2016) and proceedings of American Association for Cancer Research (AACR, 2017) annual meetings 2017 featured the earlier reports on the use of Chimeric antigen receptor macrophages (CARMA) for adoptive cellular immunotherapy.	[13,26]
2018	Morrissey et al. published a study that demonstrated the ability of anti-CD19 and anti-CD22 CARs to stimulate phagocytic pathways in murine macrophage cell lines and bone marrow-derived macrophages. The study confirmed the capability of CARs to facilitate CAR-mediated phagocytosis of tumor cells and antigen-bearing beads. Chimeric macrophages were generated to target extracellular matrix (ECM) to treat breast cancer model in animal model	[27]
2019	Chimeric macrophages were generated to target extracellular matrix (ECM) to treat breast cancer model in animal model	[28]
2020	A translational approach utilizing chimeric macrophages was developed, demonstrating high-efficiency adenovirus transduction and significant anti-tumor response in preclinical models.	[29]
2021	Preclinical evaluation of antitumor response of CAR-M in combination with immune checkpoint blockade	[30]

antigens and phagocytose cancer cells, or their possible transformation from the IFN γ -primed M1 phenotype to an M2 TAM phenotype due to immunosuppressive tumor microenvironment. Despite these limitations, a phase I trial administering cytotoxic macrophages through hepatic artery infusion to seven patients with metastatic liver disease was found to be safe and well-tolerated, with only mild flu-like symptoms as side effects [31]. Subsequent studies found that radiolabelled macrophages can persist in metastatic sites for over a week after injection, demonstrating the feasibility of administering autologous macrophages intravenously without adverse effects injection [32]. Although these initial efforts did not result in successful clinical applications, they paved the way for the adoption of myeloid cell therapies for future cancer therapies [33].

4. Macrophages for targeted drug delivery

Macrophages are crucial players in solid tumors due to their ability to constantly replenish the pool of TAMs and traffic within the TME. This unique characteristic makes them an excellent candidate for delivering anticancer agents to otherwise impenetrable tumor sites [12,34]. TAMs have been successfully employed for controlled release of antitumor therapeutic nucleotides or cytotoxic drugs using nanocarriers, resulting in specific tumor destruction without inducing systemic inflammation. For instance, a macrophage-mediated biomimetic delivery system

carrying doxorubicin demonstrated an encouraging anti-cancer effect in a mouse model of breast cancer, indicated by the suppression of tumor growth, prolonged lifespan, and inhibition of metastasis [35]. Another recent study demonstrated a novel drug delivery strategy for glioma therapy using M1 macrophages as carriers. M1 macrophage-loaded nanoparticles (M1-NPs) were efficient in carrying particles into tumor tissues. Doxorubicin-loaded M1-NPs (DOX@M1-NPs) demonstrated significantly enhanced anti-glioma effects with prolonged survival and increased cell apoptosis [36]. TAMs-recognizing nano-complex containing a combination of CpG oligonucleotide (a synthetic TLR9 agonist), anti-IL-10, and anti-IL-10 receptor demonstrated a significant anti-tumor effect in an allograft hepatoma murine model. This targeted delivery system release oligonucleotide-loaded nanocomplexes in acidic microenvironment of the tumor for specific tumor killing without inducing systemic inflammation [37]. Besides this, in tumor-associated myeloid cells, STAT3, a transcription factor that plays well-defined roles in tumorigenesis as well as tolerogenic functions, is responsible for the immunosuppressive activity. The selective targeting of STAT3 in TAMs and other myeloid suppressor cells can be achieved by delivery the STAT3 inhibitor CpG conjugates through the TLR9 in TLR9+ cells [38]. Accordingly, in a glioma model, nanoparticles loaded with paclitaxel as RVG conjugated PTX NPs (RVG-PTX-NPs) were selectively incorporated into brain tumor-associated TAMs, resulting in controlled release of the drug and tumor-specific toxicity [39]. Another innovative approach involves using “backpacks,” which are soft particles that contain the cytokine IFN α on their internal side. These backpacks can be attached to the surface of macrophages, leading to their activation and polarization into the M1 phenotype. A study demonstrated that when backpack-loaded macrophages were injected directly into the tumor site, they were able to maintain their M1 phenotype even in an immunosuppressive TME. This resulted in a significant reduction in tumor growth and metastatic burden in mice treated with these modified macrophages [40,41]. Another strategy involved genetically engineering myeloid cells to express IL-12, a cytokine that promotes type 1 immune responses. When these IL-12-expressing myeloid cells were adoptively transferred into mice, they were able to elicit a type 1 immune response in the lungs, leading to reduced metastasis and primary tumor growth [42]. While these approaches show promise, there are still limitations to be addressed. For instance, the use of backpacks requires further optimization to ensure efficient uptake by macrophages and sustained release of the cytokine. Additionally, the mechanisms underlying the observed anti-tumor effects need to be better understood to inform the development of more effective macrophage-based therapies.

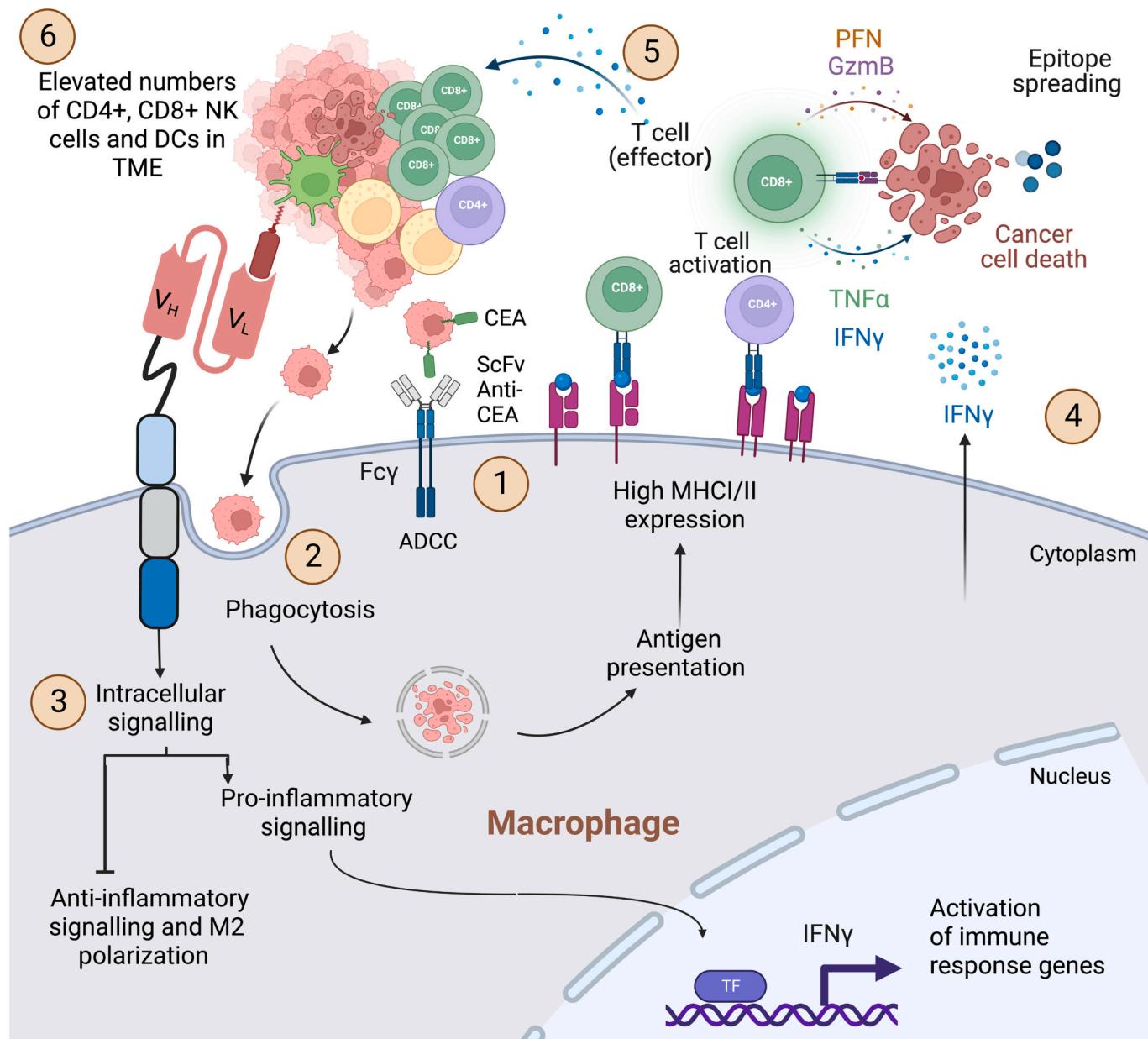
5. Chimeric Antigen Receptor Macrophages (CAR-M)

CAR (Chimeric Antigen Receptor) macrophages are engineered macrophages that have been modified to express chimeric antigen receptors on their surface [6,21,25,39]. These receptors consist of an antibody fragment that binds to a specific target antigen, combined with signaling domains that activate macrophage upon binding to the target surface [13,29,33,43]. CAR recognition of tumor antigen can induce several macrophages mediated anticancer mechanisms including, antibody-dependent cellular cytotoxicity (ADCC) [22], phagocytosis [29], upregulation of MHCII expression in macrophages [44], proinflammatory signaling and maintenance of M1 phenotype [45], systemic immune response, epitope spreading and infiltration of adaptive immune cells [33,44] (Fig. 1). In 2006, Biglari and colleagues made an initial attempt to create chimeric antigen receptor-expressing macrophages to target tumor-specific antigens. The researchers harnessed property of macrophages to eliminate cancer cells via ADCC, where the Fc- γ -receptor (CD64) interacts with the Fc portion of the antibody bound to target cells, leading to a cytotoxic response. They developed a chimeric CD64 molecule that incorporated a single-chain Fv molecule, targeted against human carcinoembryonic antigen (CEA), fused with the transmembrane and cytosolic domains of CD64 (Fig. 2). This chimeric

receptor, induced antigen-specific cytokine secretion, which effectively reduced the growth of CEA-positive tumors in nude mice xenografted with MKN45 K (human gastric carcinoma) tumors [22] (Table 1). Later, Morrissey et al., engineered a family of chimeric antigen receptors for enhanced phagocytosis, called CAR-Ps. These receptors contained different cytosolic domains, from Megf10 (multiple epidermal growth factor like -domain 10) and FcR γ (Fc receptors gamma), as well as antigen-recognizable extracellular domains (Fig. 2). Macrophages expressing CAR-Ps recognized and attacked antigen-coated synthetic particles and whole human cancer cells, demonstrating the potential of CAR-M for therapeutic use [27]. However, the therapeutic scale CAR-M production was hindered by efficient transduction of primary human macrophages. To overcome this challenge Klichinsky et al., utilized a non-integrating adenoviral vector Ad5f35, to efficiently transduce CARs into human macrophages. They postulated that since human myeloid cells lack the primary docking site coxackie-adeno virus receptor for traditional Ad5 vectors, alternatively CD46, which mediates docking of group B adenoviruses such as Ad35 [46,47] could be utilized for efficient transduction of macrophages. Their findings demonstrated that Ad5f35 achieved robust transduction of primary human macrophages and monocytes with high CAR expression and viability, and this expression was sustained for up to one month *in vitro* and 62 days *in vivo*. Additionally, Ad5f35 activated the macrophage inflammasome, providing a beneficial proinflammatory priming signal that synergized with CAR activity, resulting in CAR-macrophages locked into an M1 phenotype. Their results suggested that exploiting the inflammatory response induced by adenovirus may be a promising approach for CAR-macrophage therapy [29,33]. Furthermore, they observed a significant increase in phagocytosis by macrophages transduced with CAR targeting CD19, HER2 or mesothelin, as well as anti-cancer responses against CD19+ hematological and HER2+ or mesothelin+ solid malignancies in mouse models [29]. In a recent study by Pierini et al., demonstrate that Ad5f35-mediated gene delivery to murine macrophages can not only enhance the ability of CAR-M macrophages to phagocytose HER2+ cancer cells but also promote a pro-inflammatory phenotype (M1 phenotype). The intratumorally administered CAR-M elicited a systemic immune response and was also able to regress a collateral wild type of tumor in a syngeneic dual tumor model (Fig. 3), suggesting the ability of CAR-Ms to induce a systemic immune response against cancer [44]. Various approaches to CAR-M generation, the vector used, and the target antigen have been listed in Table 2.

6. CAR design for macrophages

CAR-M shares core components with CAR-T, including an extracellular domain that provides specific recognition through a single-chain variable fragment (scFv), such as CEA [22], CD19 and HER2 [29,33]. Additionally, CAR-M includes a hinge domain, a transmembrane domain (CD64 or CD8), and an intracellular domain that facilitates dedicated downstream signaling through (e.g., CD3 ζ and Fc γ R (Fig. 2). To enhance their ability to recognize tumor cells and improve their phagocytic capacity, first-generation CAR-M cells have been engineered with edited CARs targeting specific antigens. The CAR-M design in the first generation was reliant on unique functions of macrophages such as ADCC [22] and phagocytosis [33,48]. A particularly promising approach to CAR-Ms involves targeting cancers that express HER2, while also activating the CD147 signaling domain to stimulate matrix metalloproteinases [28]. These enzymes can effectively degrade extracellular matrix of the tumor. Although initial *in vitro* results were not particularly impressive in terms of anti-tumor activity, subsequent experiments involving the infusion of CAR-147 cells into mice bearing aggressive 4T1 syngeneic breast tumors showed significant inhibition in tumor growth [28]. The CAR development for macrophages witnessed further progress with the inclusion of intracellular signaling domains in the second generation. In the third-generation CAR-M which is in development there is an anticipation of significantly enhancing the



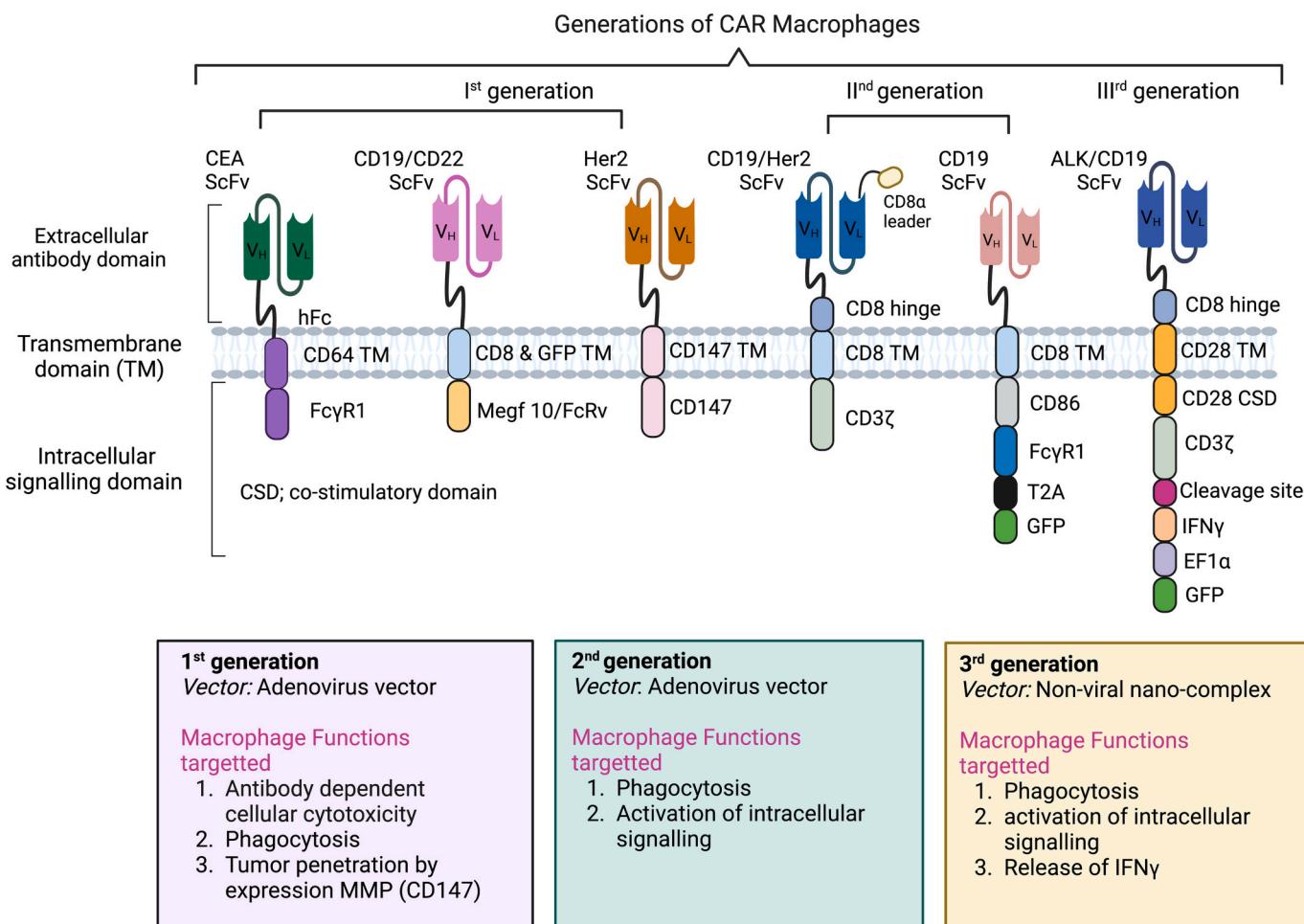


Fig. 2. CAR design for macrophages. The generations of CAR macrophages are depicted, illustrating the evolution of CAR design for macrophages over time. Each generation represents an improvement in the design of CARs, leading to enhanced efficacy and specificity in targeting cancer cells. The bottom panel shows information on the vectors used and the ‘functions of macrophages targeted’ in each generation of CAR design.

efficacy of anticancer treatments. To overcome challenges associated with previous generations, a novel approach has been adopted in the design of the third generation. This involves reprogramming CAR-M *in vivo* using a nonviral vector, which offers advantages such as cost-effectiveness and a simplified manufacturing process [50]. Kang et al., conducted a study where they introduced genes encoding CAR and interferon- γ (IFN- γ) into macrophages *in vivo* using macrophage-targeting polymer nanocarriers. The inclusion of the IFN- γ gene aims to enhance the antitumor potency of CAR-M by transitioning it from an M2 phenotype to an M1 phenotype, further augmenting its effectiveness against tumors [49] (Fig. 2).

7. Therapeutic CAR-M production

To successfully translate cell therapies to the clinic a scalable and reproducible manufacturing process is essential. The CAR-M therapy is based on a 1-week process that starts with the patient’s blood [29]. Firstly, monocytes are mobilized through the administration of subcutaneous G-CSF (granulocyte colony stimulating factor) before leukaapheresis and the selection of CD14+ monocytes. These monocytes are then differentiated into macrophages *ex vivo* and transduced with Adf535, which encodes the CAR transgene. Finally, the resulting CAR-M cells are cryopreserved and infused into the patients (Fig. 4). Recent advances in gene engineering, such as the discovery of Vpx-LV [15,25] and Adf535 [29,44] as effective vectors for primary human macrophage engineering, have made it possible to use synthetic biology approaches

to redirect macrophage effector function against tumors. Moreover, efforts are being made to improve the technology by transducing chimeric adenoviral vector Ad5f35 directly into CD14+ monocytes, reducing the processing time from a week to less than a day. CAR-monocytes express CAR with high efficiency (>90 %), retain high viability (>95 %), and possess potent anti-tumor activity. CAR-monocytes differentiate into M1-like CAR macrophages with strong pro-inflammatory effector functions and are protected against M2 switching by immunosuppressive factors. CAR-mono cells can also differentiate into monocyte-derived CAR-DCs with an activated phenotype. *In vivo*, CAR-mono cells have remarkable long-term CAR expression and persistence and induce anti-tumor activity in various HER2+ solid tumor xenograft models [50].

8. Challenges and possible solutions

8.1. Ex-vivo expansion of macrophages

Unlike T lymphocytes, it is challenging to expand macrophages *ex vivo*. In addition, some of the patients may not be able to undergo monocyte-apheresis, and prior cancer treatment regimens may also negatively affect the number of functional monocytes in peripheral circulation. However, recent studies have revealed alternative sources of macrophages that can be engineered into functional CAR-M cells for therapeutic purposes. For instance, the use of CAR-iMac, which are derived from induced pluripotent stem cells (iPSCs). These CAR-iMac cells showed proinflammatory phenotypes, enhanced phagocytosis,

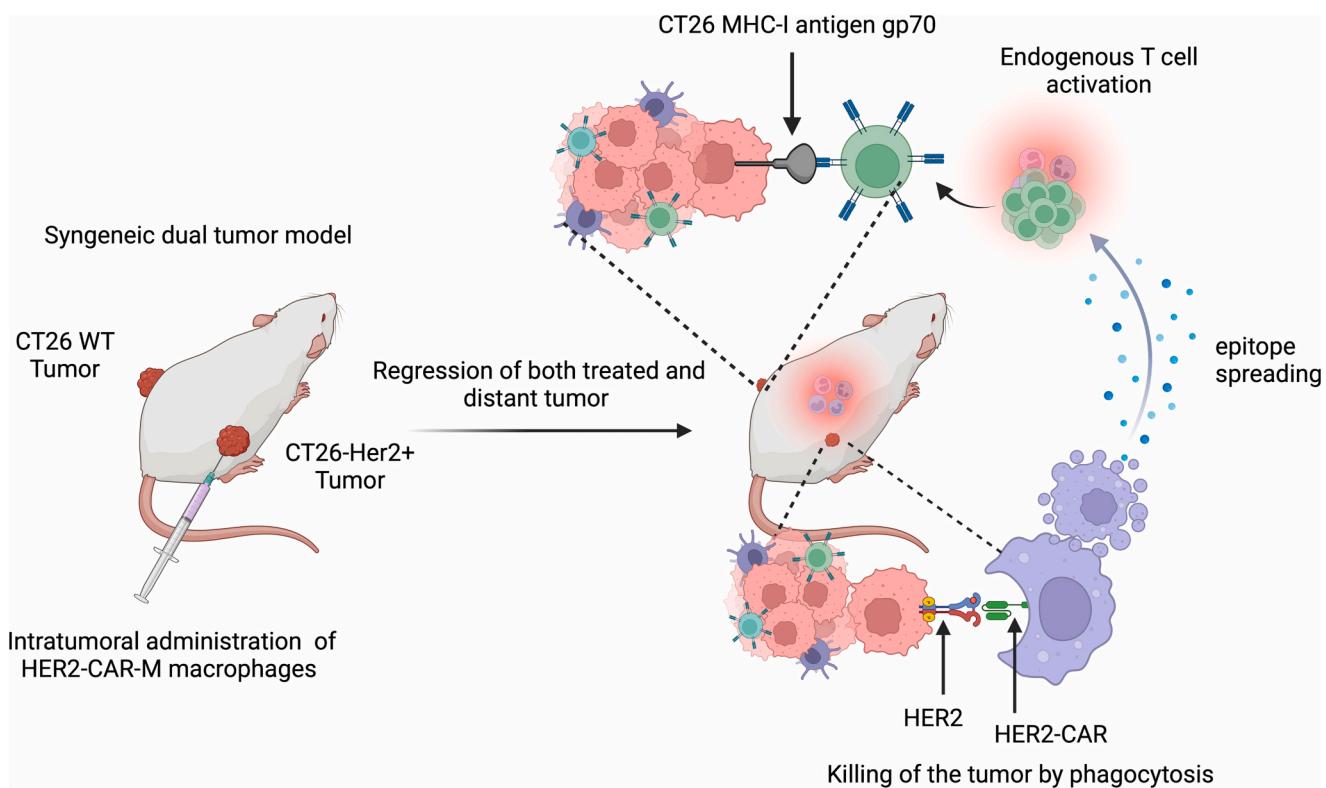


Fig. 3. CAR-M induces systemic immune response and endogenous T cell activation.

The interaction between chimeric antigen receptor macrophages (CAR-M) and the tumor microenvironment, as well as their effect on the endogenous adaptive immune system has been demonstrated in an immunocompetent syngeneic mouse model by Peirini et al. The researchers used murine bone marrow-derived macrophages that were engineered to express an anti-human epidermal growth factor receptor 2 (HER2) CAR using the chimeric adenoviral vector Ad5f35. *In vivo* experiments using mice engrafted with CT26-HER2+ tumors showed that treatment with CAR-M resulted in significant tumor control and improved survival compared to control groups. This was accompanied by an increase in intratumoral immune infiltration and enhanced epitope spreading. Moreover, researchers demonstrated the abscopal effect of CAR-M treatment on contralateral tumors. As shown above, when mice were simultaneously engrafted with CT26-HER2+ and CT26-Wildtype (WT) tumors on opposite flanks and treated with HER2+ tumors only with CAR M intratumorally, a significant reduction in both the contralateral tumors was observed suggesting that CAR-M treatment not only controls the targeted tumors but also stimulates a systemic anti-tumor immune response that impact distant tumors as well [44].

Table 2
Outline of various macrophage-based cell therapies; preclinical studies and clinical trials.

Source of monocytes	CAR construct	Vector	Target antigen	Cancer model	Anticancer mechanism	Reference
<i>Preclinical studies</i>						
Primary human monocytes	chimeric scFv-CD64	First generation Adenoviral	CEA	MKN45 K (human gastric carcinoma engrafted in nude mice)	ADCC	[22]
J774A.1 Macrophages	CAR with variable cytosolic domains-CAR-p ^{Megf10} , CAR-p ^{FcRY}	Lentivirus	CD19	CD19+ Raji B cells	ADCP Trogocytosis	[27]
Primary human monocytes	CAR-HER2- CD3ζ CAR-meso- CD3ζ	Adenovirus	HER-2 mesothelin	i.v. infused lung metastasis and IP carcinomatosis model of SKOV3 in NSG mice	ADCP	[29]
Murine mammary cells 4 T1	CAR-HER2-CD147	Lentivirus	HER-2	HER2-4 T1 tumor in BALB/c mice	ECM degradation by expressing MMPs	[28]
Cord blood (CB)-derived HSPCs	CEA-DAP12/CD3ζ	Lentivirus	CEA	HT1080 cells	ADCP	[51]
Primary human monocytes	CAR-HER2- CD3ζ+Anti-PD1	Adenovirus	HER-2	CT26-HER2 syngeneic mouse model	ADCP+ Checkpoint inhibition	[30]
<i>Clinical trials</i>						
CT-0508 (PBMCs) TEMFERON; Autologous CD34+ HSPC-derived myeloid cells expressing IFNα2	Anti-HER2-CAR Tie-2	Adenovirus-Lentivirus	HER-2+ TME Targeted release of IFNγ	Solid tumors (Phase I) Glioblastoma multiforme (Phase I/IIa)	ADCP IFNγ mediated killing	NCT04660929 NCT03866109 [52]

and anticancer activity *in vivo*, suggesting that iPSC-derived macrophages could be used as a source of therapeutic scale CAR-M production in the future [12,34]. Another study suggests an *ex vivo* generation of

functional CAR-Ms using human hematopoietic stem cells (HSPCs). The researchers were successful in introducing CAR in cord blood (CB)-derived HSPCs which showed typical macrophage morphology,

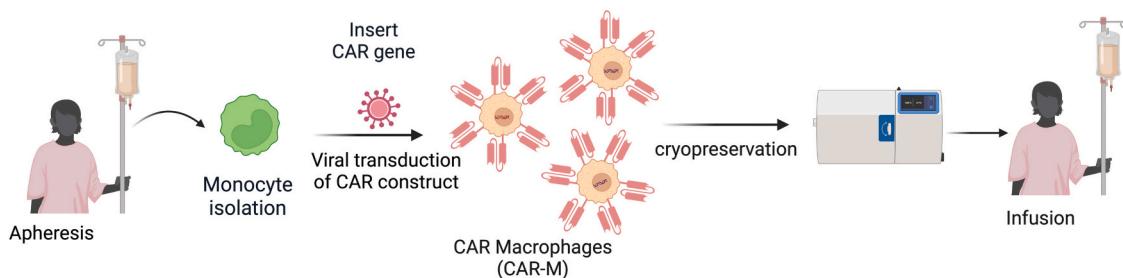


Fig. 4. Macrophage-Based CAR Therapy for Cancer.

A patient's blood is collected through apheresis, and the macrophages are separated from other blood components. The isolated macrophages are then transduced with viral vector carrying CAR construct. Once the macrophages are equipped with antigen specific CAR they are reinfused into the patient's body, where they can target and destroy cancer cells. This approach is designed to harness the body's own immune system to fight cancer.

phenotype, functional properties, as well as considerable *ex-vivo* cell expansion during differentiation to CAR-M. The HSPC-derived CAR-M cells containing CEA-DAP12/CD3 ζ -CAR showed enhanced phagocytosis of CEA $^+$ HT1080 (fibrosarcoma) cells, indicating HSPCs as a viable alternative source of macrophages for producing CAR-M [51]. Moreover, umbilical cord blood is a valuable source of hematopoietic stem cells and has been utilized to produce CAR-T cells [53]. It can also serve as an allogeneic source of macrophages for the generation of CAR-M cells [54].

8.2. Genetic manipulation

Myeloid cells are difficult to transduce owing to their innate immune function. These cells are highly sensitive to foreign nucleic acids or viral invasion, making it challenging to introduce a transgene or manipulate their genetic makeup [55,56]. Expression of SAMHD1, a restriction factor specific to myeloid cells further reduces their susceptibility to lentiviral vector transduction [56]. Myeloid cells have a complex and diverse gene expression profile, which complicates the transduction process. Additionally, myeloid cells are challenging to expand in culture due to their terminally differentiated forms. The innate immune functions and unique characteristics of myeloid cells make their transduction more challenging compared to other cell types, such as T cells or fibroblasts. To address these issues, lentivirus vectors expressing VPX protein have been developed [25]. VPX is a viral accessory protein that has been shown to improve transduction efficiency of macrophages by reducing innate immune response. VPX binds to SAMHD1, a protein that restricts the replication of lentiviruses in non-dividing cells such as macrophages, and promotes degradation of SAMHD1, enabling efficient transduction of macrophages [57]. As mentioned above, monocytes instead of terminally differentiated macrophages have been demonstrated to be efficiently transduced by adenovirus and differentiated into active CAR-macrophages [58]. However, the mechanisms underlying macrophage recruitment, differentiation, and polarization in solid tumors are not fully understood, which limits our ability to design effective strategies to genetically manipulate these cells. Moreover, macrophages exhibit strong intrinsic immune regulatory capacity that can suppress immune responses [59], and hinder their use as anticancer agents. Therefore, genetic modifications must be carefully designed to avoid unwanted immune regulatory effects and maintain their pro-inflammatory and anti-tumor activity. Despite these challenges, recent advances in gene-editing technologies and viral vector-based platforms have provided new opportunities to engineer macrophages for therapeutic purposes, and ongoing research in this field holds promise for the development of new treatments for solid tumors.

9. CAR-Ms beyond cancer

Though CAR-M is a new and emerging approach in cancer immunotherapy and has shown promising potential in the treatment of solid

tumors [13,33]. However, recent research has expanded the scope of CAR macrophages beyond cancer, demonstrating their potential in treating infectious diseases such as COVID-19. A study by Wenyan Fu et al., proposed a new approach of genetically equipping human macrophages with chimeric antigen receptors (CARs) to alter their phagocytic activity against SARS-CoV-2. The researchers tested different CAR constructs and discovered that CAR_{MERTK} macrophages were able to decrease the virion load without causing an increase in proinflammatory cytokine expression, indicating an "immunologically silent" scavenger effect. These findings open up avenues for further investigation of CARs as a potential treatment for severe cases of COVID-19, particularly in those at high risk of hyperinflammation [60]. Studies have shown that macrophages are essential for the clearance of viral [61], bacterial [62,63], and fungal pathogens [64], and CAR-Ms could potentially enhance their activity, providing a more efficient and targeted approach for the treatment of infectious diseases.

10. Future directions

The advent of CRISPR/Cas9 gene editing technology has enabled researchers to edit the genome of any cell type and precisely modify their genetic makeup [65]. This approach has been used to generate CAR T cells with enhanced anti-tumor activity, improved tumor infiltration, and increased persistence in the tumor microenvironment [66]. Moreover, researchers have used CRISPR/Cas9 to genetically reprogram natural killer (NK) cells for enhanced anti-tumor activity [67,72]. In terms of the advancement of CAR-M technology, CRISPR/Cas9 gene editing can be used to generate macrophages with enhanced phagocytic activity, improved homing to tumors, and inhibition of pro-tumorigenic polarization in the TME. In addition, genome-scale CRISPR screens present powerful tools that can be employed for the identification of the genes that regulate macrophage activity. CRISPR screens have been successfully employed in identification of key genes in the activation and viability of macrophages [68,69]. Moreover, CRISPR screens can identify genes that regulate the expression of key cytokine genes and phagocytic checkpoints such as SIRP α and SIGLEC10. By targeting and manipulating these genes, CAR-M activity against cancer cells can be enhanced.

Likewise, synthetic biology approaches can also be applied to engineer CAR-M with improved anti-tumor activity [70,71]. Synthetic biology approaches have been instrumental in the design and synthesis of various chimeric receptors that have been critical in the development of CAR-T cells. Similar approaches can be used to develop CAR Ms. with enhanced functionality [70–72]. Researchers can design and integrate synthetic genetic circuits [73] into the macrophages to regulate their activity and improve their tumor-killing ability. For example, a synthetic genetic circuit can be designed to trigger macrophages to secrete cytokines when they encounter cancer cells, thereby amplifying anti-tumor response. Alternatively, a synthetic genetic circuit can allow macrophages to release toxic molecules selectively when they get polarized by

the tumor microenvironment, enabling them to exert their effect specifically in tumor. Another advancement could be the optogenetic manipulation of CAR-M for better anti-tumor activity and reduced side effects. Optogenetics is an emerging synthetic biology approach that can be utilized to address the toxicities associated with CAR-M therapy. It has already shown potential in dealing with toxicities in CAR-T cells. One recent development is the design of light-switchable CAR (LiCAR) T cells, which can be activated in real-time through photoactivatable mechanisms to effectively induce tumor cell killing [74]. Maren Hülsemann and colleagues have demonstrated the feasibility of optogenetic manipulation in macrophages [75] which could pave the way for the development of optogenetically engineered macrophages for therapeutic use. The precise control over macrophage activity provided by optogenetics can potentially enhance the safety and efficacy of macrophage-based therapies in future.

Although CAR-M therapy has shown potential, it may not be effective as a standalone treatment. Hence, combining CAR-M with other treatment modalities may achieve additive or synergistic effects. For example, in a syngeneic CT26 tumor model, combining CAR-M with PD-1 blockade improved overall survival with no noticeable signs of cytokine release syndrome (CRS), and multiple safety evaluations confirmed its tolerability [30]. The combination therapy with other immune checkpoint inhibitors can also enhance the activity of CAR-M and overcome tumor-mediated immune suppression. Moreover, the use of CAR-M in combination with other cell-based therapies, such as CAR-T [76] can offer a synergistic effect against cancer cells. Additionally, the use of CAR macrophages with chemotherapy or radiotherapy can enhance their anti-tumor activity by promoting the release of tumor antigens and creating a more immunogenic tumor microenvironment.

11. Conclusion

Macrophages have been recognized as a potential cancer therapeutic agent since 1980s. Genetic modification of myeloid cells, however, presented challenges. In recent years, gene delivery methods, particularly viral vectors, have generated renewed interest in genetically engineered macrophages for therapeutic use. Additionally, macrophages are being explored as a delivery system for anticancer agents. Recent advances in immunotherapy have attracted a lot of attention to CAR-M. Although, CAR-M technology is still in the early stage of development as cancer immunotherapy, its potential in treating infectious diseases beyond cancer is also an exciting prospect. The success of CAR macrophages in reducing the virion load of SARS-CoV-2 without causing inflammation is a significant step in demonstrating the safety and efficacy of CAR macrophages in treating infectious diseases [65]. Despite challenges, the potential of macrophages as a therapeutic agent continues to be explored, and new breakthroughs in this field are expected to have a significant impact on the development of novel therapies for various diseases.

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CRediT authorship contribution statement

AKM and SKM conceptualized the study and wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

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