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Review article

Adoptive cell therapy for solid tumors beyond CAR-T: Current challenges and emerging therapeutic advances

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ABSTRACT

Adoptive cellular immunotherapy using immune cells expressing chimeric antigen receptors (CARs) is a highly specific anti-tumor immunotherapy that has shown promise in the treatment of hematological malignancies. However, there has been a slow progress toward the treatment of solid tumors owing to the complex tumor microenvironment that affects the localization and killing ability of the CAR cells. Solid tumors with a strong immunosuppressive microenvironment and complex vascular system are unaffected by CAR cell infiltration and attack. To improve their efficacy toward solid tumors, CAR cells have been modified and upgraded by "decorating" and "pruning". This review focuses on the structure and function of CARs, the immune cells that can be engineered by CARs and the transformation strategies to overcome solid tumors, with a view to broadening ideas for the better application of CAR cell therapy for the treatment of solid tumors.

1. Introduction

Cancer-related conversations are associated with nervousness and fear because people consider cancer to be synonymous with pain and death. Traditional treatments, including surgery, radiotherapy, and chemotherapy, are the main approaches for early-and mid-stage cancer [1–3]. However, patients undergoing these treatments usually experience high malpractice rate, side effects, and reduced life expectancy [4,5]. To achieve better therapeutic results, multiple conventional treatments are used simultaneously; however, the results remain unsatisfactory [6–8]. Therefore, there is a critical need for effective cancer treatment.

Tumor cells are usually considered as normal cells in the body that are stimulated due to various factors (such as congenital factors, chemical stimulants, physical stimulants and viral stimulants), hence activating uncontrolled growth [9–11]. Therefore, avoiding accidental injury to surrounding normal cells during treatment is a major challenge in cancer therapy. Immune cells play an important anticancer role. For example, T cells usually identify the "enemy" by recognizing the major histocompatibility complex I (MHC I) of other cells [12–14]. However, tumor cells can escape the surveillance of immune cells by blocking similar recognition mechanisms, leading to unrestricted proliferation and metastasis [15,16]. Therefore, many researchers hope to achieve tumor suppression or even clearance by revitalizing immune cells, known as immunotherapy [17,18].

Although immunotherapy has proven its potential value in recent years, it also has many shortcomings, including inefficient immune response, high off-target toxicity, restricted persistence, insurmountable tumor heterogeneity, and immunosuppressive microenvironment [19–21]. Antibody–antigen interactions are commonly found in humans and are a highly specific reaction [22,23]. This reaction is often used in cancer therapy combined with a series of monoclonal antibodies [24,25]. Most antibodies inhibit the proliferation of cancer cells by binding to their surface receptors; however, it is difficult to achieve the killing of cancer cells [26,27].

Considering the perceptions mentioned earlier, to implement killing

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of tumor cells by antibodies with the lowest possible off-target toxicity, researchers have proposed grafting antibodies, which bind to receptors on the surface of tumor cells, onto the cell surface to synthesize a 'smart cell' that can efficiently recognize and kill cancer cells termed chimeric antigen receptor (CAR) engineered cells [28]. The introduction of modified cells into the patient's body for cancer treatment is often referred to as a form of adoptive cell therapy [29]. These engineered cells have become a new focus for eliminating tumors. The basic structure of a CAR generally consists of a tumor-associated antigen (TAA)-binding region (usually derived from the single-chain variable fragment [scFv] segment of a monoclonal antibody antigen-binding region), hinge area, transmembrane region, and immunoreceptor tyrosine-based activation motif [30,31]. Ideal TAAs should be prevalent on tumor cells and are expected to be expressed minimally in normal tissues to avoid off-target toxicity [32]. Selection of appropriate TAAs, as target antigens for localization, plays a decisive role in the specificity, efficacy, and safety of the final CAR [33,34].

Owing to its characteristic high specificity and no restriction by MHC, CAR cell therapy has gained increasing attention. Initially, CARs were mostly used to transform T cells and have been applied to NK cells and macrophages [35,36]. Recently, CAR cell therapies, especially those of CAR-T cells, have achieved excellent results in treating hematologic tumors and have been approved by the US Food and Drug Administration and the European Medicines Agency [37,38].

However, concerns, including antigen heterogeneity, limited safety, immunosuppressive tumor microenvironment (TME), complex tumor vascular system, suboptimal CAR cell delivery, and complicated production, have severely hindered the development of CAR cells against solid tumors [39,40]. Therefore, in this review, we introduce the basic structure of CARs and its different cell types. Therefore, in order to provide a wider range of research ideas, we mainly summarize the modification strategies for solid tumors, analyze the strengths and weaknesses of the various approaches and hope that these experiences will enlighten a wider range of researchers.

2. Basic structure and development of CAR

CAR (Fig. 1) has three functional domains, namely extracellular, transmembrane, and intracellular structural domains [41,42]. Functionally, the extracellular structural domain comprises an antigen recognition domain that binds antigen and a segment of the hinge that acts as a linker, whereas the intracellular domain contains a co-stimulatory and a signal-transduction structural domain. For prolonging the in vivo retention time of CAR cells and enhancing the therapeutic effect for tumor treatment, CARs have now been developed to the fifth generation. The difference between generations mainly focuses on the upgrading of intracellular structures and the introduction of cytokines. It should be noted that although the principle of CARs construction is unified, most of the current CARs are mainly designed for constructing CAR-T cells and may not be the best choice for other cells. In addition, the optimal method to introduce CARs via transfection varies by cell type.

2.1. Antigen recognition structural domain

The antigen recognition structural domain is the basis of CAR-specific binding to tumor antigens, and the main structure used is the scFv [43,44]. It is usually composed of the variable light (VL) and heavy (VH) regions of monoclonal antibodies linked by peptides that possess specificity for the antigen [45]. Theoretically, the structural design of VL-linker-VH is more in line with the natural structure of antibodies. Interestingly, both VL-linker-VH and VH-linker-VL showed similar results for the final recognition result [46]. For the linker, the sequence -(Gly-Gly-Gly-Gly-Ser)$_3$- with -(Gly-Gly-Gly-Gly-Ser) - is the most preferred [47–49], whereby such amino acid sequences can link VH to VL while remaining flexible. This allows the functional regions of VH and VL to still pair together to constitute monovalent antigen-binding sites [50].

The scFv fragment is monovalent, and the bivalent scFv can be made by linking two scFv fragments.The bivalent scFv generally has two structures, one forms a single peptide chain containing two heavy chain variable regions and two light chain variable regions, called tandem antibodies (tandem di-scFvs); the other is to shorten the length of linker from 15 to 3–12, so that the VH and VL functional regions from two different molecules pair with each other to form a dimer structure, called diabodies (diabodies composed of variable regions from two different antigens are called bispecific antibodies). Similarly, tandem antibodies (tandem di-scFvs) with three heavy chain variable regions and three light chain variable regions can be produced.Triabodies can also be formed by further shortening the Linker length so that the VH and VL functional regions from three different molecules are paired with each other. The scFv multimer has an increased antigen-binding valence and

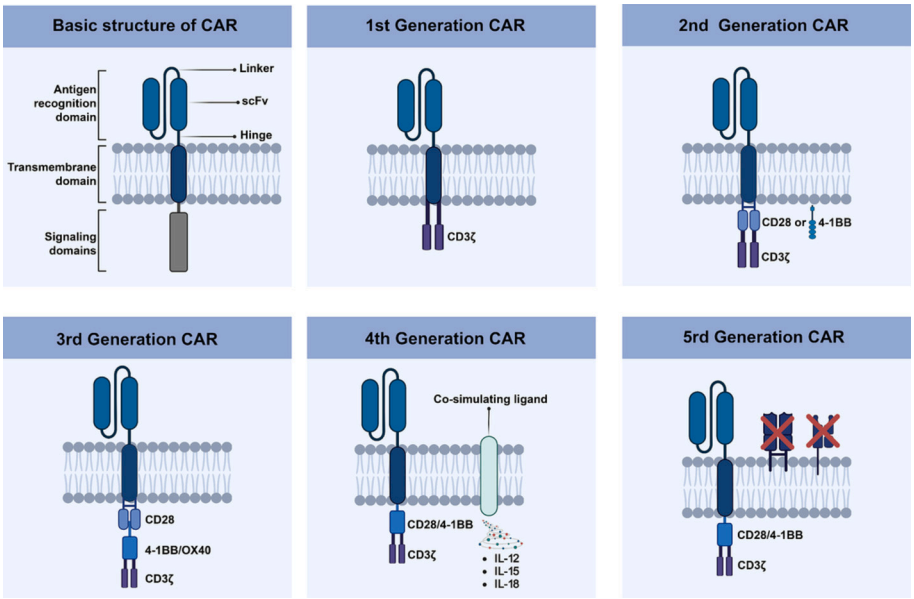


Fig. 1. The basic structure of CARs by generations.

higher affinity than the normal scFv fragment [51]. Unlike bispecific antibodies, bispecific diabodies have more advantages and a high specificity [52,53]. First, the deletion of fragment crystallizable (Fc) fragment minimizes specific binding of Fc receptors (FCR) to immune cells. Second, the shorter amino acid sequence minimizes host rejection of the heterologous antibody. Third, the smaller molecular weight is advantageous for tumor tissue penetration. Finally, diabodies do not require cell hybridization, thus avoiding the use of chemical cross-linking agents and conferring a higher safety profile with lower immunogenicity. Given these advantages, human-derived bispecific antibodies may be worthy of more development and exploitation.

Overall, the presence of scFv confers or enhances the ability of CARs to recognize and bind antigens. It not only relieves T cells of their dependence on MHC, thereby preventing immune escape by tumor cells, but also grants immune cells the ability to recognize non-peptide antigens.

In the structure of CAR, scFv determines the specific recognition of CAR-T cells and is a crucial structural domain. One of the key factors for the realization of CAR function is the affinity of scFv for its cognate antigen [54]. In general, scFv-based CAR-T cells have an affinity for their targets that is several orders of magnitude greater than that of unmodified TCR-T cells. Fine-tuning of CAR affinity also reduces binding to low levels of antigens on normal tissues and reduces on-target off-tumor toxicity, while maintaining sufficient effector function to eliminate antigen overexpressing malignant cells. Another advantage of scFv-based CAR cells is that they do not rely on antigen presentation by MHC to recognize target antigens, which can overcome tumor escape from down-regulated MHC molecules and enable CAR cells to recognize non-peptide antigens such as glycolipids or tumor-specific glycosylation. In addition, ScFv-based CAR-T cells could theoretically be redirected to any antigen. However, scFv-based CARs also have many obvious disadvantages [55,56]. First, since some antigens are not strictly expressed in tumor cells, “miss-killing” may occur during treatment, in which a portion of normal cells that express the same antigen are killed [57,58]. Second, scFvs are less stable and susceptible to external factors, including temperature and pH. Therefore, changes in the production process and in vivo environment may cause damage to scFvs [59]. Additionally, the VH and VL regions of different scFvs are prone to crosstalk with each other, resulting in the loss of the original function of binding to the target antigen and enhancement of the immunogenicity of CARs [60,61]. Particularly, in CAR-T cells, aggregation of CAR induces tonic signaling, accelerates T cell depletion, and even reduces T cell persistence in vivo [62,63]. Meanwhile, the immunogenicity of murine-derived or humanized scFv has been confirmed [64]. Notably, in addition to scFv-based design, other fabrication strategies, including natural receptor/ligand-based or nanoantibodies, have made significant contributions to the development of CARs [65–67].

2.2. Hinges

The hinge domain has been mainly responsible for connecting the antigen recognition domain to the transmembrane structural domain [68]. The origin, length, flexibility and composition of the hinge domain influence the antitumor activity of CAR and the appearance of side effects [69]. The optimal length of the hinge domain depends on the location and accessibility of the antigen and target epitopes. CAR cells will be more easily activated if the target epitope is located close to the target cell membrane. Adjusting the length of the hinge zone can enable CAR cells and target cells to be at an optimal intercellular distance, favoring the formation of immune synapses that might otherwise attenuate CAR signals. It was found that for CD19, carcinoembryonic antigen (CEA), and interleukin 13 receptor (IL13Rα2), short hinge domains activate CAR-T more readily than long hinge domains. For ROR1, mucin 1 (MUC1), neural cell adhesion molecule (NCAM), and 5 T4, the long hinge domain is required to overcome spatial barriers to access the target antigen [70]. Therefore, the optimal hinge domain length varies depending on the target epitope.

The source of the hinge domain also needs to be carefully considered. Currently, most CAR hinge domains are derived from the hinge of IgG or the CD8α/CD28 extracellular region [71]. IgG-based hinges typically use the -CH_2-CH_3 region of the IgG molecule, mainly IgG1 and IgG4 [72].The main advantage of this hinge is its excellent structural flexibility. However, clinical studies have found that CAR-T cells with IgG-derived hinges lack persistence [73]. This might be explained by the fact that several amino acid sequences within the CH_2 structural domain could bind to Fcγ receptors (FcγRs) on innate immune cells, including monocytes/macrophages, dendritic cells (DCs), neutrophils, and natural killer (NK) cells [74]. This binding interaction may lead to redundant innate immune responses including antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis resulting in CAR-T cell depletion. Additionally, the interaction of Fc with FcγR may also cause ligand-independent tonic signaling, which subsequently results in activation-induced T-cell death (AICD). Existing studies have typically eliminated adverse effects by modifying IgGs-derived hinges, such as replacing the IgG1-CH_2 framework with the corresponding IgG2 amino acid or deleting the CH_2 domain outright [75,76]. The researchers generated several anti-CD19 CARs by using different IgG4-derived spacers [77]: one with no mutated CH_2 structural domains, one with a complete deletion of the CH_2 structural domains, and the others with single- or double-point mutations in the CH_2 region. They found that CARs without mutated CH_2 were not consistently recognized and persisted in a xenograft model, whereas engraftment and persistence were partially restored after blocking the interaction through the use of intravenously administered immunoglobulin (IVIG). Furthermore, double point mutations or complete deletion of the CH_2 region increased persistence and anti-tumor efficacy compared to CARs containing non-mutated or single mutated CH_2 regions. Unusually, CAR-T cells had no similar therapeutic efficacy or persistence in vivo after similar modifications to their IgG1-derived hinges. Aiming to minimize potential immune interactions induced by the IgG-derived hinges and to achieve the clinically required safety profile, relevant proteins (e.g., CD8 and CD28) that are naturally expressed by T cells can be integrated as hinges into the CAR structure [78]. It was found that CARs containing the CD28 hinge domain produced significantly more inflammatory cytokines and experienced more AICD than CARs containing the hinge domain of CD8α. In addition, more markers of T-cell depletion, such as PD-1 and lymphocyte-activated gene-3 (LAG-3), were found in CAR-T cells containing the CD28 hinge domain. Other researchers reduced the flexibility of the hinge domain by removing two consecutive Gly residues in the CD8-derived hinge domain in CAR cells [49]. They found that this change resulted in better tumor control and lower inflammatory cytokine release in vivo. In addition, CAR-T cells with a less flexible hinge domain contributed to a downward trend in tumor load and prolonged survival. The exact mechanism of the hinge domain's effect on CAR cells requires extensive research to further determine [79].

2.3. Transmembrane domain

As a link between extracellular and intracellular structures, the transmembrane domain (TMD) is usually derived from selected transmembrane receptor proteins. [80] An appropriate TMD has a great influence on the timely and stable exchange of information between the inside and outside of the cell. Currently, the commonly used sources of TMDs in clinical practice are CD4, CD8α, CD28, and CD3ζ. [81–85]. The functional importance of TMD in the expression level and stability of CARs has been fully confirmed. The researchers analyzed the effects of hinge domains and TMDs on the expression level and cytotoxicity of CARs. The results showed that the hinge region / transmembrane domain co-modified CARs had a stronger effect than the hinge region modified CARs. This indicates that the transmembrane region has a high effect on the expression and stability of CARs. The dimerization of CARs mediated by the transmembrane domain and the interaction with

endogenous proteins contribute to the formation of dimers or trimers, which ultimately enhance the activation of T cells [86–88]. Multiple sources of TMD (including FcεRIγ, CD3ζ, CD28, CD16, NKp44, NKp46, NKG2D, DNAM-1, 2 and B4) have been shown to promote signal transduction and T cell activation by mediating the dimerization of CARs [89–92]. Compared with CD8α TMD, CD28-derived TMD is more conducive to the formation of dimers and reduces the antigen density required for T cell activation. TMDs similarly affect the cytokine production of CAR cells. The researchers conducted a series of studies on the specific effects of CD8α-derived TMDs of different lengths on CAR-T cells. The results showed that CARs with TMDs containing 86 amino acids not only produced potent anti-tumor responses but also had a desirable safety profile. And the appropriate modification of CARs TMD can regulate the secretion of cytokines and also effectively improve the toxicity associated with CAR-T cells. With the in-depth study of TMDs, a variety of novel TMDs have been developed [93–95]. Researchers developed TMDs with the killer cell immunoglobulin-like receptor (KIR) and introduced them into T cells, which ultimately showed potent anti-tumor activity. Most of the existing TMDs were developed based on CAR-T therapies [96]. When applied to construct other CAR cells, sufficient evidence and relevant experiments are required to select the most appropriate TMD.

2.4. Intracellular structural domains

2.4.1. Costimulatory domain

In general, full activation of T cells requires at least two different stimulatory signals. The first activation signal is provided by the binding of specific antigenic peptides that bind MHC molecules on antigen-presenting cells (APCs) to TCRs. The other activation signal is generally provided when a stimulatory receptor on the T cell binds to a cognate ligand on the APC. Incomplete antigenic stimulation leads to unresponsive T cells. The first generation of CARs consisted only of the antigen recognition domain connected to FcγR or CD3ζ alone [97,98]. This simple structure, although exhibiting cytotoxicity to target cells both in vivo and ex vivo, showed only limited anti-tumor efficacy and poor persistence in clinical trials [99,100].

For improving T-cell proliferation and persistence, researchers have constructed second-generation CARs by introducing a co-stimulatory element (CD28 or 4-1BB) as a “second signal” for T-cell activation [101–103]. CD28 is a type I transmembrane glycoprotein that improves proliferation, IL-2 release, survival and metabolic activities of naïve T cells by regulating the expression and activity of nuclear transcription factor (NF-κB), nuclear factor of activated T cells (NFAT), and activator protein 1 (AP-1) [102]. It is also involved in cytoskeletal rearrangements, actin polymerization and membrane raft repolymerization into the immunological synapse, thereby maintaining and facilitating TCR-induced signaling.CD28, when used as a co-stimulatory molecule, enhances T-cell killing but persistence remains unimproved [104]. 4-1BB (TNFRSF9, CD137, ILA) is expressed predominantly on activated T cells and stimulates T cells by activating the NF-κ B, c-Jun and p38 downstream pathways to stimulate T cells [105–107]. In contrast to CD28, 4-1BB enhances T cell activity by stimulating the proliferation, cytokine release and cytolytic activity of effector T cells rather than naive T cells and inhibiting AICD [108]. However, 4-1BB shows limited killing ability as a co-stimulatory molecule [109–111].

For enhancing anti-tumor effects and prolonging in vivo persistence, the investigators constructed third-generation CARs by combining two co-stimulatory molecules [112,113]. The co-stimulatory molecules used with high frequency in CARs were derived from either the Ig superfamily (CD28 and ICOS) or the TNF receptor superfamily (TNFRSF) (4-1BB, OX40, and CD27) [114]. It has been shown that CAR-T cells containing CD28-OX40 display higher levels of expansion and cytotoxicity in vitro compared to CAR-T cells lacking OX40. Unfortunately, the clinical performance of third-generation CAR-T was not superior to that of second-generation CAR-T. This also suggests that simply increasing the number of co-stimulatory molecules does not necessarily enhance the activation effect of CAR on immune cells.

For combining immune checkpoint inhibitor therapies and overcoming the immunosuppressive microenvironment of tumors, the fourth generation of CARs builds on the second generation by adding the ability to express specific cytokines [115–118]. Therefore, they are also known as universal cytokine-mediated killing T cells (TRUCK T), which can activate T cells while secreting corresponding cytokines (e.g., IL-12, IL-15, and IL-18) [119–121] to increase the extent of NK cell and macrophage infiltration at the tumor site, which further enhances the anti-tumor effect [122–124]. In addition, considering the issue of CAR-T cell controllability, some researchers have added controllable suicide genes (e.g., certain drug-sensitive genes) to the structure of fourth-generation CAR-Ts to modulate the survival timeframe of CAR-T cells in vivo. Other researchers have proposed designing an active switch for CAR-T cells. For example, by incorporating the light-switching protein LOV2 (light-oxygen-voltage domain 2) into the structure of fourth-generation CARs, researchers have made it possible for CAR-T cells to function only when excited by a specific blue light and to return to a “dormant” state in the dark [125]. Based on dual-target therapy and “ AND “ logic gates, other researchers have modified CAR-Ts to be lethal only when they recognize two antigens, A and B, at the same time. Such logic gates can improve CAR-T's precision and reduce off-target toxicity.

CAR cell therapies are usually engineered in vitro from the patient's own cells and then infused back into the body, which has limited the scale-up and translation of CAR cell therapies, especially CAR-T cells. As a result, researchers have developed a fifth-generation CAR-T, the Universal CAR-T (UCAR-T). UCAR-T empowers CAR-T cells to recognize multiple antigens by utilizing two ‘third party’ systems (BBIR CAR or SUPRA CAR) that partition the extracellular antigen targeting domains and T-cell signaling units [126,127].In addition, Graft-versus-Host Disease (GVHD) is eliminated by in vitro disruption of TCR genes and HLA class I genes in T cells obtained from allogeneic healthy recipients through gene editing techniques (ZFN, TALEN and CRISPR/Cas9) [128]. The main advantage is that there is no need to obtain T cells from the patient for customization, which greatly saves treatment time and cost. However, this type of general-purpose CAR-T currently has high technical barriers and higher safety requirements. The safety of fifth-generation CAR-T is still at an early stage of exploration.

2.4.2. Activation domain

The signal activation structural domain is mainly responsible for the transduction of T cell activation signals. Currently, CD3ζ is the most used source of activation domains in CAR-T cells. The activation structural domain of CD3ζ consists of three immunoreceptor tyrosine-activated motifs (ITAMs), which are highly dependent on the activity of the lymphocyte-specific protein tyrosine kinase (Lck) [129,130]. Upon phosphorylation of Lck each ITAM in CD3ζ recruits zeta-chain-associated protein kinase 70 (ZAP70) to be phosphorylated by Lck. Activated ZAP70 further phosphorylates the membrane adapter LAT, which forms a signaling network with various proteins (e.g. Grb2/Sos and PLCγ). In T cells, the number and type of ITAMs may influence the signaling process. However, researchers have found that the anti-tumor efficacy of a single functional ITAM is sufficient for therapeutic purposes. Furthermore, CARs containing a single ITAM outperformed CARs containing more ITAMs in vivo, limiting T-cell differentiation, increasing the proportion of central memory CAR-T cells, and improving persistence [131]. Apart from CD3ζ, CD3δ/ε/γ could theoretically serve as structural domains for CARs. The researchers replaced the original CD3ζ by three peptide chains, CD3δ/ε/γ, to examine their T-cell activation effects [132]. The results showed that CARs adopting the other three peptide chains could reduce the occurrence of CRS, improve the in vivo persistence of CAR-T cells, and enhance the safety and efficacy of CAR-T therapy. Most of the existing activation domains were developed based on the mechanism of T-cell activation and may require adaptation when applied to other CARs cells.

3. "Family" of CAR cells

Currently, CAR-T therapies are well developed for cancer treatment. Therefore, the utilization of CAR engineering to modify other immune cell types has aroused great interest among researchers. Several mainstream CAR cells, such as CAR-NK, CAR-NKT, and CAR-macrophage (CAR-M), can exert more potent anti-tumor effects through multiple mechanisms (Fig. 2). Moreover, other CAR cell therapies are gradually attracting the attention of researchers (Table 1).

3.1. CAR-T

As lymphoid stem cells derived from bone marrow, T cells differentiate and mature in the thymus and are distributed to immune organs and tissues throughout the body via the lymphatic and blood circulation [133]. T cells specifically recognize "non-me" or tumor neoantigen

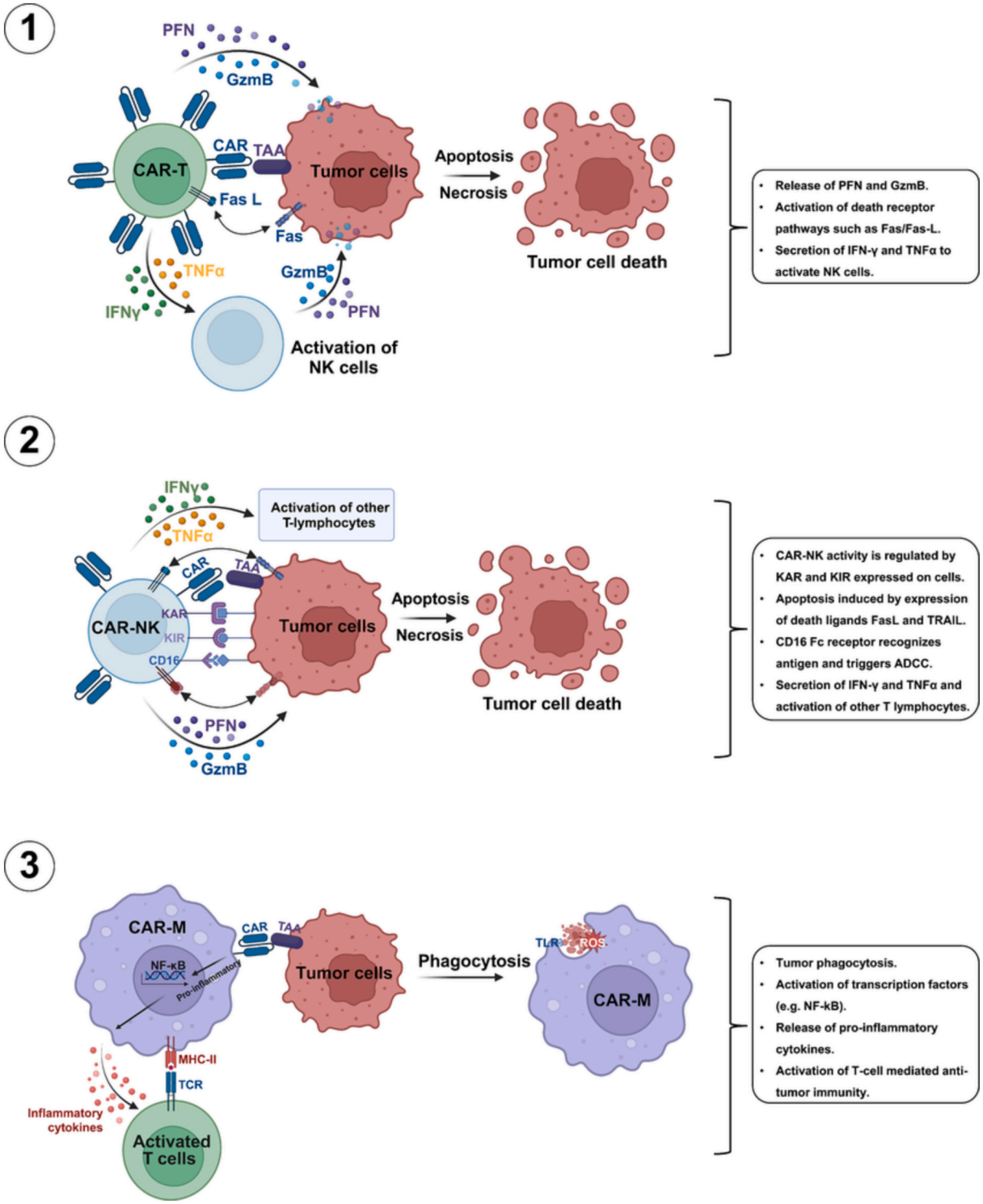


Fig. 2. Killing mechanism of mainstream CAR cells. Abbreviations: PFN, perforin; GzmB, granzyme; IFN-γ, Interferon-gamma; TNFα, Tumor necrosis factor-alpha; TRAIL-R, TNF-related apoptosis-inducing ligand; KIR, Killer inhibitory receptors; KAR, Killer activation receptor; ADCC, Antibody-dependent cellular cytotoxicity.

Table 1
CAR cell family members.

Type	Features	Clinical trials		
		Identifier	Diseases	Recruitment Status
CAR-T 	Advantages: • Longer cycle time • Broad spectrum anti-cancer • Longer anti-cancer memory	NCT05353530	• Glioblastoma	Not yet recruiting
		NCT03851146	• Advanced Cancer	Completed
		NCT05373147	• Solid Tumor	Recruiting
		NCT04185038	• Central Nervous System Tumor • Diffuse Intrinsic Pontine Glioma • Diffuse Midline Glioma	Recruiting
	Challenges: • Immune escape • Adverse effects such as CRS, neurotoxicity • Lengthy preparation process (autologous cells) • Transplanted disease and GvHD	NCT04162340	• T Cell Lymphoma in Relapse • Refractory T-Cell Lymphoma	Recruiting
		NCT05370430	• Mantle Cell Lymphoma	Not yet recruiting
		NCT04653649	• Hodgkin Lymphoma • Adult T Cell Lymphoma	Recruiting
		NCT03638167	• Central Nervous System Tumor • Glioma • Ependymoma • Medulloblastoma • Germ Cell Tumor • Atypical Teratoid/Rhabdoid Tumor • Primitive Neuroectodermal Tumor • Choroid Plexus Carcinoma • Pineoblastoma	Recruiting
		NCT05312411	• Osteosarcoma	Recruiting
		NCT03874897	• Advanced Solid Tumor	Recruiting
		NCT02932956	• Liver Cancer	Active, not recruiting
		NCT03423992	• Glioma • Malignant Glioma of Brain • Recurrence Tumor	Recruiting
		NCT04795882	• Multiple Myeloma	Not yet recruiting
		NCT02905188	• Hepatocellular Carcinoma	Active, not recruiting
		NCT03302403	• B Cell Lymphoma • B Cell Leukemia • Myeloma • Hepatocellular Carcinoma • Pancreatic Carcinoma • Adenocarcinoma of Esophagogastric Junction	Active, not recruiting
		NCT03884751	• Hepatocellular Carcinoma	Completed
		NCT03890198	• Gastric Cancer • Pancreatic Ductal Adenocarcinoma	Terminated
		NCT05013372	• T-cell Non-Hodgkin's Lymphoma	Not yet recruiting
		NCT04283006	• Lymphoid Hematological Malignancies	Recruiting
		NCT03500991	• Central Nervous System Tumor, Pediatric • Glioma • Ependymoma • Medulloblastoma • Germ Cell Tumor • Atypical Teratoid/Rhabdoid Tumor • Primitive Neuroectodermal Tumor • Choroid Plexus Carcinoma • Pineoblastoma	Recruiting
		NCT03198052	• Lung Cancer • Other Cancer	Recruiting
		NCT04581473	• Gastric Adenocarcinoma • Pancreatic Cancer • Gastroesophageal Junction Adenocarcinoma	Recruiting
		NCT04541368	• Relapse Multiple Myeloma	Not yet recruiting
		NCT04603872	• Multiple Myeloma • Non-Hodgkin's Lymphoma	Recruiting
		NCT05131763	• Hepatocellular Carcinoma • Glioblastoma, Medulloblastoma • Colon Cancer	Recruiting
		NCT04572308	• T-cell Acute Lymphoblastic Lymphoma	Completed
		NCT04513431	• Stage III Colorectal Cancer • Colorectal Cancer Liver Metastasis	Not yet recruiting
		NCT04706936	• Multiple Myeloma	Recruiting
		NCT05396300	• Colorectal Cancer • Esophageal Cancer • Stomach Cancer • Pancreatic Cancer • Metastatic Tumor • Recurrent Cancer	Recruiting
		NCT04727008	• Multiple Myeloma	Not yet recruiting

(*continued on next page*)

Table 1 (*continued*)

Type	Features	Clinical trials		
		Identifier	Diseases	Recruitment Status
CAR-NK	Advantages:	NCT04887012	• B-cell Non-Hodgkin Lymphoma	Recruiting
		NCT05410717	• Stage IV Ovarian Cancer	Recruiting
	• Natural tumor killing ability		• Refractory Testis Cancer	
	• No threat of GVHD and CRS		• Endometrial Cancer Recurrent	
	• Low risk of off-targeting	NCT05213195	• Refractory Metastatic Colorectal Cancer	Recruiting
	• Wide range of sources	NCT05194709	• Advanced Solid Tumors	Recruiting
	• Simple preparation process	NCT04639739	• Non-Hodgkin Lymphoma	Not yet recruiting
		NCT05008536	• Refractory Multiple Myeloma	Recruiting
	Challenges:	NCT05410041	• Non Hodgkin Lymphoma	Recruiting
		NCT04847466	• Gastroesophageal Junction Cancers	Recruiting
	• Short cycle time		• Advanced HNSCC	
	• Complex NK cell subpopulation	NCT05020678	• Non-Hodgkin Lymphoma	Recruiting
	• Unknown adverse effects	NCT04796675	• Non-Hodgkin's Lymphoma	Recruiting
		NCT02839954	• Hepatocellular Carcinoma	Unknown
			• Non-small Cell Lung Cancer	
			• Pancreatic Carcinoma	
			• Triple-Negative Invasive Breast Carcinoma	
			• Malignant Glioma of Brain	
			• Colorectal Carcinoma	
			• Gastric Carcinoma	
		NCT05137275	• Locally Advanced or Metastatic Solid Tumors	Recruiting
		NCT03941457	• Pancreatic Cancer	Unknown
		NCT03056339	• B Lymphoid Malignancies	Active, not recruiting
		NCT04991870	• Recurrent Glioblastoma	Not yet recruiting
CAR-M	Advantages:	NCT05007379	• Breast Cancer	Not yet recruiting
		NCT04660929	• HER2 Overexpressing Solid Tumors	Recruiting
	• Natural ability to penetrate TME			
	• Ability to reverse TAM			
	• Ability to phagocytose cancer cells			
	• Ability to present antigens			
	• Less off-target toxicity			
	Challenges:			
	• Limited cycle time			
	• Restricted cell numbers and sources			
	• Poor cell differentiation and proliferation			
Other	Advantages:	NCT05114837	• Lymphoma	Not yet recruiting
		NCT03294954	• Neuroblastoma	Recruiting
CAR-cells	• More possibilities	NCT03774654	• Relapsed or Refractory B-Cell Malignancies	Recruiting
		NCT02656147	• Lymphoma	Unknown
	Challenges:	NCT04702841	• Malignant Tumors	Recruiting
		NCT04842812	• Advanced Solid Tumors	Recruiting
	• off-target effects	NCT04556669	• Solid Tumors	Recruiting
	• Adverse reactions	NCT02830724	• Pancreatic Cancer	Recruiting
	• Cell origin		• Renal Cell Cancer	
	• Safety		• Breast Cancer	
			• Melanoma	
			• Ovarian Cancer	

peptides presented by MHC-I molecules on the surface of target cells, mainly through their surface receptor TCR, and rapidly trigger the immune function of T cells to kill target cells [134–137]. Particularly, T cells are the “heroic fighters” in the body against infections and tumors. In other words, T cells are the “heroic fighters” of the body against infections and tumors. As mentioned earlier, CAR-T is an expression vector that has been genetically engineered to express T-cell killing activation signals on T-cell membranes to form chimeric antigen receptors, thus allowing T cells to recognize tumor cells independent of MHC-I [138–140]. T cells are thus transformed from ordinary soldiers to super warriors.

CAR-T therapies have recently emerged among the most promising tumor immunotherapies. This technology has been successfully applied in the treatment of several hematologic malignancies, especially those of B-cell hematologic tumors [141,142]. CAR-T cell therapies for B-cell maturation antigens in patients with multiple myeloma have shown favorable clinical responses and excellent safety outcomes [143,144]. However, in solid tumors, the effectiveness of CAR-T therapy remains limited owing to the immunosuppressive environment and poor targeting. T cell trafficking and migration are seriously affected by the abnormal vascular system in solid tumors, poor levels of chemokines in the tumor environment, and bad chemokine receptor expression on CAR-T cells [145–147].

Furthermore, the extracellular matrix (ECM) barrier around solid tumors and the thick collagen fiber network surrounding some tumors prevent CAR-T cells from invading the tumor. Even if CAR-T cells can penetrate into the tumor, the immunosuppressive TME affects their potency, subsequently leading to CAR-T dysfunction, premature depletion, or even failure to kill tumor cells [148–150]. Highly active Treg cells, myeloid-derived suppressor cells (MDSC), tumor-associated macrophages, neutrophils, and several immunosuppressive factors (especially transforming growth factor [TGF]-β, IL-10, IL- 4, prostaglandin E2, indoleamine 2,3-dioxygenase, and adenosine) affect CAR-T cell activity and function directly or indirectly through distinct and complex mechanisms [151–155]. Thus, restoration of CAR-T cell activity has been repeatedly demonstrated by inhibiting the production of immunosuppressive factors in TME [156–159].

Additionally, therapeutic regimens combining checkpoint inhibitors to modify CAR-T cells to eliminate the influence of the immunosuppressive microenvironment have strengthened the anti-tumor efficacy of

CAR-T cells and enhanced their survival in several preclinical models [160,161]. To enhance the TME penetration and anti-tumor properties of CAR-T cells, researchers have modified the surface of CAR-T cells with hyaluronidase and checkpoint-blocking antibody α-programmed death-ligand (PDL)1 by combining bioorthogonal reactions and click chemistry, and their therapeutic efficacy and safety have been evaluated in two solid tumor models (Fig. 3) [162]. Bioorthogonal reactions can occur in living cells or tissues without disturbing the biochemical reaction itself and are therefore often employed in combination with click chemistry. Notably, considering the development of photothermal therapy, enhancement of the therapeutic effect of CAR-T on solid tumors by modulating the TME with nanophotosensitizers and constructed nanophotosensitizer-engineered CAR-T biohybrids (CT-INPs) has been proposed [163]. The anti-tumor properties of CT-INPs in Raji tumor-bearing mice have also been investigated. The combination of CT-INPs and laser treatment showed more potent and durable anti-tumor properties than CAR-T without photosensitizers. Recently, there has been a growing interest in a novel form of CAR-T cells called 'micro-medicine' CAR-T. This concept is based on a specific type of CAR-T cells known as synthetase-armed killer (SEAKER) cells. These SEAKER cells possess the ability to target tumor cells like immune cells, while also expressing a synthetase enzyme. This enzyme activates a prodrug that is administered systemically, resulting in a powerful anticancer effect at the tumor site [164,165]. Another concern of researchers is that CAR-T often induces CRS and neurotoxicity. It has been demonstrated that monocyte hyperactivation during CRS is a result of CAR T cell-monocyte interactions. Therefore, reducing monocyte hyperactivation by controlling CAR T cell-monocyte interactions could provide a potential solution for the treatment of CRS and neurotoxicity. The investigators thus found that surface in situ polyethylene glycolized CAR T cells could eliminate CAR T lymphocyte overactivation of monocytes during CRS and prevent subsequent inflammatory effects by blocking cell-to-cell interactions between CAR T cell nuclei, monocytes, and tumor cells [166].

As synthetic biology and genome editing technologies emerge, it has become imperative to optimize engineered CAR design to unlock the complete anti-tumor potential of CAR-T cells while overcoming obstacles in clinical practice, including side effects [167–169]. Over the past decade, investigators have extensively explored solid tumor targets for CAR-T, including B7-H3, CAIX, carcinoembryonic antigen (CEA) CAM5, CD133, CD171, EGFR, EGFRvIII, Fra, PTPN 2, GD2, GPC3, human epidermal growth factor receptor 2 (HER2), CD27, IL13Ra2, MUC1, PSMA, ROR1, and VEGF-R2, which have provided multifaceted ideas for attacking solid tumors [170–176]. The maturation of cell engineering and gene editing technology has also helped in improving CAR-T cell efficacy and safety, significantly [177]. Although treatment of solid tumors using CAR-T is still a struggle, the development of new targets and

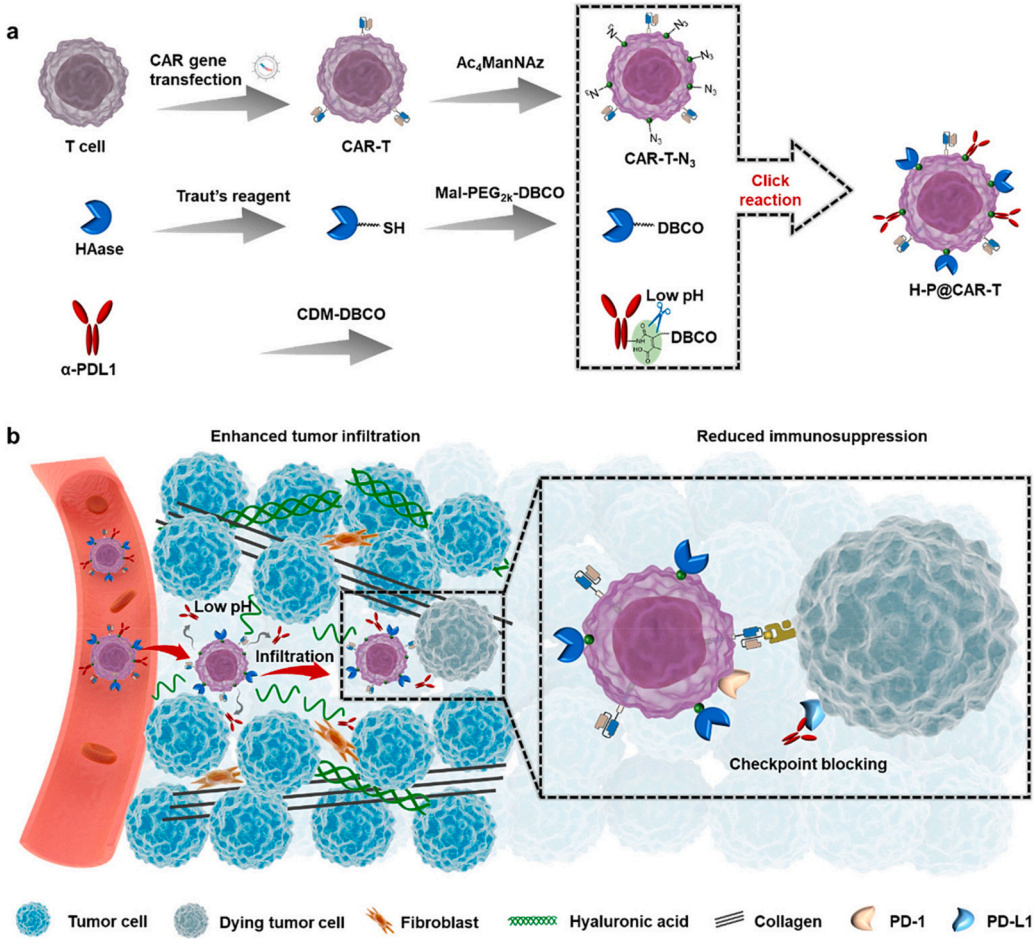


Fig. 3. Schematic representation of hyaluronidase (HAase)- and α-programmed death-ligand (PDL) 1-engineered CAR-T cells (H-P@CAR-T) used to enhance immunotherapeutic efficacy in solid tumors. (a) Checkpoint-blocking antibody α-PDL1 and HAase, which degrades tumor extracellular matrix (ECM), are both engineered on the surface of CAR-T cells through metabolic glycan biosynthesis and click chemistry reaction. α-PDL1 is conjugated to tumor extracellular acid-reactive maleic acid amide bonds and will be released in the low pH tumor microenvironment (TME). (b) H-P@CAR-T cells disrupt the tumor ECM by degrading hyaluronic acid, which in turn enhances tumor infiltration. Meanwhile, the checkpoint-blocking antibody α-PDL1 was released from H-P@CAR-T cells in a dominantly acidic TME, reversing the immunosuppression of the PD1-PDL1 pathway and thus enhancing anti-tumor activity.

innovations in CAR engineering technologies are expected to improve the outcomes in patients with advanced solid tumors, further supported by a huge number and variety of clinical trials which may significantly verify the efficacy and applicability of CAR-T.

3.2. CAR-NK

In the past two years, in addition to CAR-T therapies, natural killer (NK) cell-based therapies have also been gaining increasing attention. NK cells, as essential immune cells in the body, are not only involved in anti-tumor, antiviral infection, and immune regulation, but also in hypersensitivity reactions and autoimmune diseases [178–180]. They are often considered as "cousins" of T cells having a similar mode of killing target cells with a contrasting feature, where NK cell activity is not restricted by the MHC [181,182]. Therefore, NK cells may be safer, more effective, and faster, and hence a potential candidate for cellular anticancer therapy. CAR-NK utilizes genetic engineering to add a receptor to NK cells, thereby aiding in tumor cell recognition and its simultaneous activation to kill tumor cells.

The CAR significantly increases the specificity and efficacy of NK cells. Based on unique anti-tumor properties, CAR-NK has several advantages over CAR-T [183,184]. Firstly, contrary to CAR-T cells, CAR-NK cells greatly retain the inherent ability to identify and target tumor cells by their natural receptors. In other words, CAR-NK have both CAR-mediated targeted killing ability and their own anti-tumor properties, which in turn can identify and kill tumor cells with down-regulated or absent CAR targets, reducing the possibility of tumor cells escaping killing and improving therapeutic efficacy [185]. Next, it has been proven that allogeneic CAR-NK cells are free from immune rejection within days to weeks. Therefore, CAR-NK have not demonstrated the same safety issues as CAR-T, especially the troubling cytokine release syndrome, in any of the clinical trials [186]. Moreover, NK cells are not required to be strictly HLA-matched and have no potential to cause GvHD, unlike the CAR-T cell immunotherapy [187–189]. In particular, allogeneic NK cells are available from a wide range of origins, including peripheral blood, umbilical cord blood, induced pluripotent stem cells, NK-92, and other NK cell lines [190,191], whereas CAR-T cells are mostly obtained from patients' autologous or healthy human donor cells.

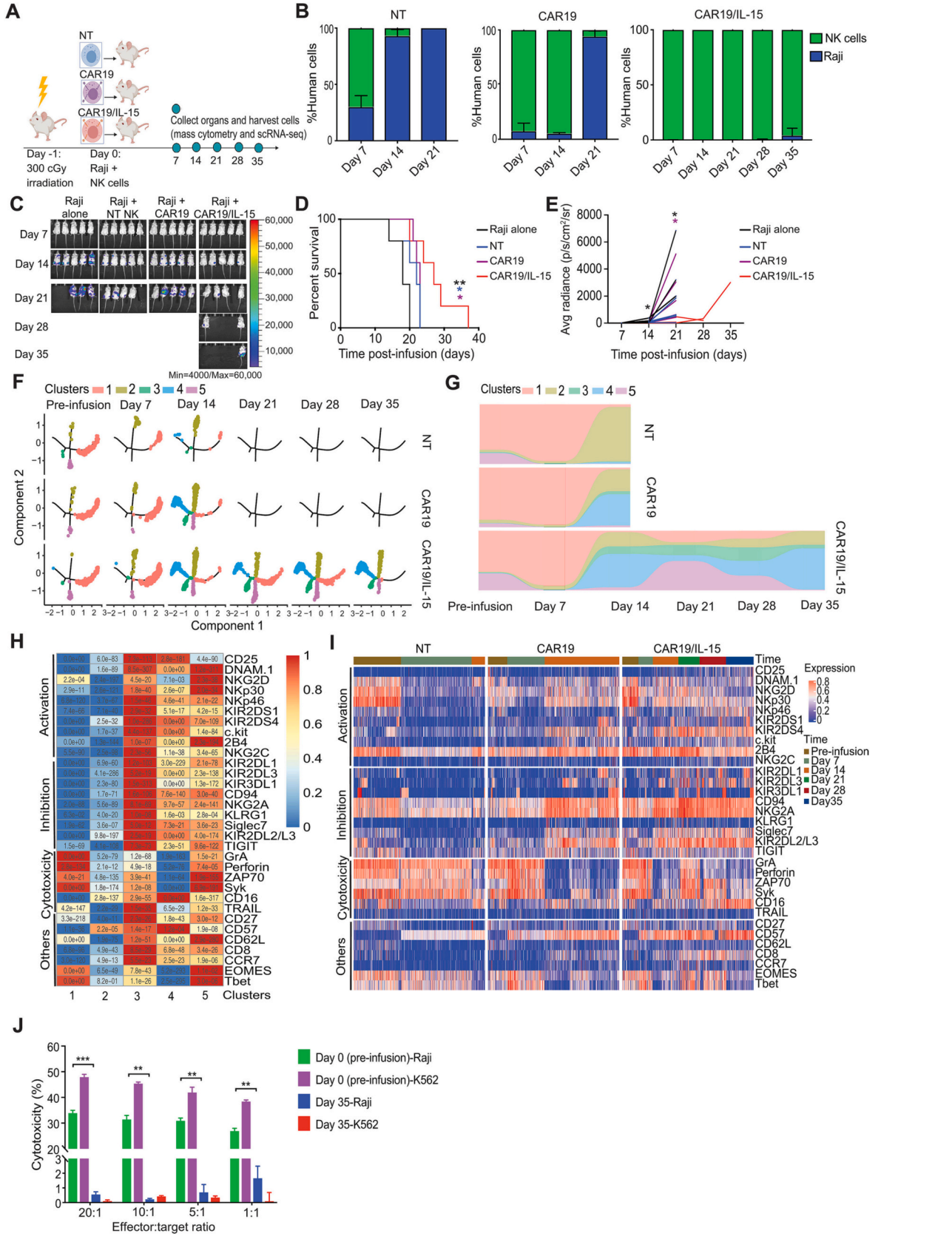
CAR-NK has emerged as a major therapeutic success in recent years, demonstrating unique advantages in the treatment of tumors. Results from phase I/IIa trials have proven the clinical efficacy of CD19 CAR-NK cell therapy in patients with relapsed/refractory non-Hodgkin's lymphoma and chronic lymphocytic leukemia, without serious toxic effects (NCT03056339). Over recent years, investigators have also screened for available targets for CAR-NK in metastatic solid malignancies expressing tumor-associated antigens including HER2, PSMA, mesothelin, ROBO1, HLA-G, or MUC1, including prostate, ovarian, pancreatic, and non-small cell lung cancers [192–195]. The immunosuppressive TME is a natural barrier that protects tumors from infiltration and elimination by immune cells. Targeting immune checkpoint protein (ICP) molecules and constructing CARs that can convert immunosuppressive signals into activating signals is a promising potential strategy [196]. Recognizing that HLA-G is an ideal ICP, researchers constructed an anti-HLA-G CAR-NK against the scFv of HLA-G [197]. Subsequent experiments demonstrated that CAR-NK effectively inhibited xenograft tumor growth and prolonged median survival in an in situ mouse model. Recently, an emerging prognostic tumor marker and potential immunotherapeutic target, CD276 (B7-H3) has been designed to modify CAR-NK cells [198–202]. Based on this, investigators constructed CAR NK-92 cells that could target CD276 (B7-H3) and comprehensively evaluated their ability to overcome the immunosuppressive TME [203]. The results indicated that CD276-CAR NK-92 cells induced specific cellular inactivation in melanoma cell lines with a notable ability to resist various immunosuppressive effects of the TME. Additionally, CAR-NK modified against c-Met, FRα, GD2, DR4, CXCR1, and HER2-associated antigens have also shown good anti-tumor effects [204–207]. Interestingly, using a variety of in vivo tumor models and clinical data, the researchers revealed that activation of the chimeric antigen receptor (CAR) in NK cells facilitates the transfer of CAR cognate antigens from tumors to NK cells. This impairs the ability of CAR-NK cells to bind to target cells and results in fratricide of trogocytic antigen-expressing NK cells ($NKTROG^{+}$) and NK cell hyporesponsiveness. The researchers responded to this phenomenon by designing a dual-CAR system [208] consisting of an activated CAR targeting homologous tumor antigens and an NK self-recognition inhibitory CAR, which delivers a 'don't kill me' signal to NK cells upon engagement with their TROG siblings. In addition, for CAR-NK cell therapy, the mechanisms driving resistance and disease recurrence have not been fully elucidated. Researchers have revealed a previously unidentified mechanism of resistance through in vivo studies, investigating mechanisms that may be associated with resistance to CAR-NK cell therapy, characterized by transcriptional signatures indicating loss of metabolic adaptations associated with NK cell dysfunction [209]. Furthermore, arming CAR-NK cells with IL-15 enhanced metabolic capacity and effector function and improved anti-tumor efficacy (Fig. 4).

Numerous clinical researches on CAR-NK for solid tumors are also underway. However, the breakthrough achievements in CAR-NK therapy are currently limited to preclinical studies, and clinical research that have progressed are not extensive and lack sufficient data to support them. The clinical efficacy of NK cells may be limited by their short lifespan in humans, low cytotoxicity, and the inclusion of cell subsets with different functional properties, thus suggesting a need for further exploration to select appropriate cell subsets for CAR-NK [210–213]. This includes ways to prolong in vivo persistence and overcome functional failure to further enhance their efficacy. It is believed that with the improvement of NK cell knowledge, the continuous development of clinical research and the innovative application of combined therapy, CAR-NK cell therapy will occupy a stage in the treating solid tumors in the future.

3.3. CAR-M

Solid tumors prevent the infiltration of immune cells into the heterogenous tumor by constructing a TME with immunosuppressive properties, thus making them resistant to attack by CAR-T cells. Even if some T lymphocytes are capable of infiltrating solid tumors, their immune response may be limited by immunosuppressive cells or suppressors in the microenvironment, and the complex heterogeneity limits the efficacy of CAR-T therapies targeting a single antigen. Macrophages are the body's first responders to viral infections and engulf the invaders [214,215]. Upgrading modified macrophages may be a way to develop cellular therapies for effective treatment of solid tumors. It is worth mentioning that genetically engineered CAR-M cells not only target and engulf tumor cells but also alter the microenvironment near the tumor by secreting pro-inflammatory cytokines and present the tumor antigens to the T cells to activate their anti-tumor immune response, namely " One Arrow, three Eagles" [216,217].

Macrophages, as key effector cells of innate immunity, are more likely to infiltrate tumors in an immunosuppressive microenvironment and have a powerful phagocytic effect which distinguishes them from other cell therapies (including T cells and NK cells) [218–220]. Moreover, as antigen-presenting cells, they can activate the patient's own adaptive immune system [221,222]. These properties give macrophages the opportunity to surmount the barriers of CAR-T therapy. Related studies have also confirmed the ability of CAR-M to infiltrate tumors, influence the TME, reduce tumor burden through phagocytosis, and improve overall survival in animal models of solid tumors [223,224]. However, autologous monocyte-derived CAR-M have been found to be limited in number and functionally impaired due to the effects of prior therapy [225–228]. To overcome this hurdle, investigators chose primary human hematopoietic stem and progenitor cells as an alternative cell source for the preparation of CAR-M [229]. The experimental results



(caption on next page)

Fig. 4. (A) Schematic timeline of experiments ($n = 13$ to 15 mice per group; 5 mice were followed for survival and 8 to 10 were assigned for single-cell analyses; 2 mice per group were sacrificed at each time point). (B) Bar plots of NK and Raji cell percentages in samples collected at multiple time points from mice treated with NT, CAR19, or CAR19/IL-15 NK cells. (C) Bioluminescence imaging ($n = 5$ mice per group). Kaplan-Meier plots (D) showing mice survival and average radiance (E). Black asterisks: Raji alone versus CAR19/IL-15. Blue asterisks: NT versus CAR19/IL-15. Purple asterisks: CAR19 versus CAR19/IL-15. (F) Trajectory evolution of NK cell products from pre-infusion (day 0) to day 35 post-infusion ($n = 8$ mice per group, 2 mice analyzed at each time point). No data were available at days 21 to 35 in NT and CAR19 groups due to limited in vivo persistence. (G) Relative proportion of NK cell clusters. (H) Heatmap showing the average expression levels of the proteomic markers for the five clusters. *P* values in each square were calculated using unpaired *t*-test by comparing the levels of marker abundance for cells in their cluster versus cells from all other cluster. (I) Heatmap of protein expression at different time points across products. (J) 51Cr-release assay of CAR19/IL-15 day 0 or 35 days after infusion against K562 or Raji. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. The P values were determined by log-rank (Mantel-Cox; D) and unpaired t-test in (E) and (J). Copyright © 2019. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confirmed that the prepared CAR MΦs have enhanced cytokine secretion capacity as well as desirable phagocytic properties, making them suitable for cancer therapy. Chimeric adenoviral vectors were shown to break the inherent resistance of primary human macrophages to genetic manipulation and confer a sustained pro-inflammatory (M1) phenotype. The resulting CAR-Ms reduced tumor load and prolonged overall survival in two solid tumor xenograft mouse models and induced a pro-inflammatory tumor microenvironment and enhanced anti-tumor T

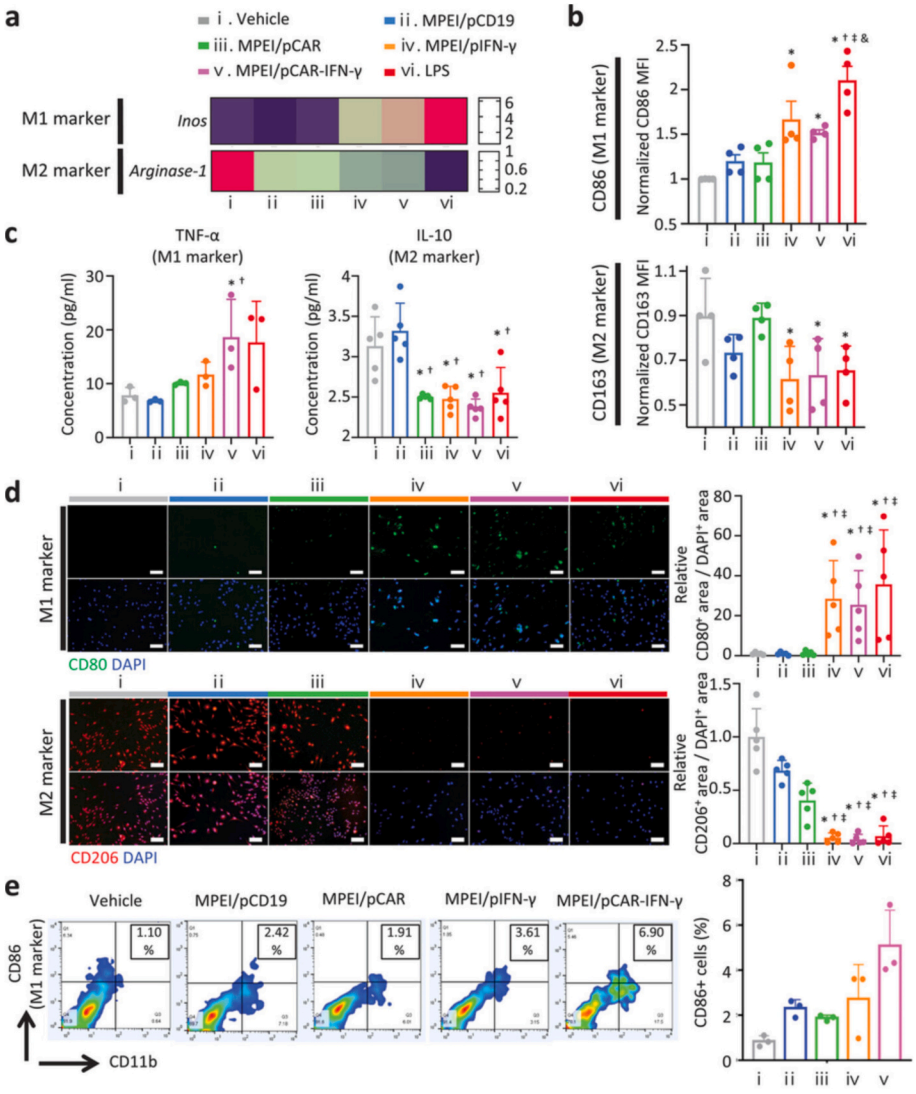


Fig. 5. M2- to M1-phenotypic shift in macrophages upon MPEI/pCAR-IFN-γ transfection in vitro. mRNA and protein expression levels of M1 (iNOS, CD86, TNF-α, and CD80) and M2 (arginase-1, CD163, IL-10, and CD206) markers in M2 macrophages post in vitro treatments with vehicle (= gene transfection buffer) (group i), MPEI complexed with a plasmid encoding anti-CD19 CAR (MPEI/pCD19, group ii), anti-ALK CAR (MPEI/pCAR, group iii), IFN-γ (MPEI/pIFN-γ, group iv), or MPEI/pCAR-IFN-γ (group v), as evaluated using a) qRT-PCR at 24 h ($n = 7$–8), b) flow cytometry at 48 h ($n = 4$), c) ELISA at 72 h ($n = 3$–5), and d) immunofluorescence staining at 72 ($n = 5$). MPEI/pCAR-IFN-γ transfection successfully programmed M2 macrophages to M1 phenotype. Nuclei were stained with DAPI (blue). Scale bars: 100 μm. LPS-treated macrophages served as the positive control (group vi). The expression of the mRNA of interest was normalized to that of GAPDH. e) Flow cytometric analysis of M1-associated marker (CD86) expression in vehicle-, MPEI/pCD19-, MPEI/pCAR-, MPEI/pIFN-γ-, or MPEI/pCAR-IFN-γ-transfected M2 macrophages post co-culture with Neuro-2a cancer cells (n = 3). $*p < 0.05$ versus vehicle, †p < 0.05 versus MPEI/pCD19, ‡p < 0.05 versus MPEI/pCAR, p < 0.05 versus MPEI/pIFN-γ, and p < 0.05 versus MPEI/pCAR-IFN-γ. Statistical significance was calculated using one-way ANOVA with Tukey's significant difference post-hoc test. ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide. Copyright © 2021 Wiley-VCH GmbH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cell activity in a humanized mouse model [230]. Inducing the generation of CAR cells in vivo through gene vectors is now one of the mainstream ideas in research. Researchers have chosen low-cost and safe non-viral vectors to mediate the delivery of genes, which program M2 TAMs in TME in vivo into M1 macrophages with CAR-expressing and anti-tumor phenotypes for the treatment of solid tumors. In vivo injection of a nanocomplex consisting of a macrophage-targeting nanocarrier and CAR-interferon-γ-encoding plasmid DNA induces the generation of CAR-M1 macrophages and endows them with CAR-mediated cancer phagocytosis, anti-tumor immunomodulation and inhibition of solid tumor growth (Fig. 5).

CAR-M has been recognized as a powerful immunotherapy for tumors, but many issues need to be overcome to achieve the desired results. Compared with T cells and NK cells, macrophages have a limited source, which restricts their future mass production. To address this issue, researchers prepared CAR-iMac with high yield and purity using induced pluripotent stem cells (iPSC), which have high differentiation potential, rapid expansion, and convenient gene editing, as a source [231]. It effectively inhibited tumorigenesis in a solid tumor model. In addition, although CAR-M has demonstrated its effectiveness in the treatment of solid tumors, it still has limited effectiveness in clinical treatment due to the immunosuppressive tumor microenvironment. Therefore, sustained and controllable M1 polarization is one of the key factors for CAR-Macrophage to be able to move toward clinical treatment. The researchers focused on Toll-like receptor 4 (TLR4), which has the function of polarization and macrophage activation, and genetically engineered its intracellular TIR signaling domain into the intracellular location of CARs, constructing Macrophage-specific CARs and developing the second-generation CAR-iMAC [232]. Compared with the first generation CAR-iMAC, the second generation CAR-iMAC maintained a better level of M1 polarization (≈60% CD80 positivity, ≈30% CD163 positivity) after a period of time of in vivo anti-tumor activity. Moreover, other investigators constructed ACOD1-depleted CAR-iMAC by screening for the ACOD1/KEAP1/NRF2 pathway to regulate cellular metabolism and pro-inflammatory activity in macrophages [233]. In mouse models of ovarian or pancreatic cancer, ACOD1-depleted CAR-iMAC exhibited enhanced tumor suppression with elevated survival rates. Finally, the immunosuppressive microenvironment of tumors is a challenge for all immunotherapies targeting tumors. Researchers have developed multiple CAR-M modification strategies to target the immunosuppressive microenvironment. They first found that targeted removal of sialic acid from the surface of tumor cells significantly enhanced macrophage sensitivity to tumor cells. Combined with the strategy of knocking down Siglec-5 and Siglec-10 (immune checkpoints), the researchers further constructed DKO-CAR-iMac, which significantly inhibited tumor growth and prolonged survival in mice in vivo. However, while tumor inhibition could be achieved with either DKO-CAR-iMac or sialidase alone, sialidase was not able to further enhance the efficacy of DKO-CAR-iMac. In addition, there are several issues worth noting. Compared with T cells and NK cells, macrophages fail to achieve the ideal differentiation and proliferation ability, which can seriously limit their therapeutic effect in vivo and future mass production [234]. Once macrophages are overactivated in vivo, their interleukin secretion (IL-1 and IL-6) can easily cause cytokine release syndrome (CRS) reactions. The adverse effects of such reactions have been demonstrated in CAR-T cell therapy.

Tumor vaccines of immune cell origin have recently received much attention. It is mainly aimed at preventing or treating tumors by activating immune cells in vitro and preparing them into tumor vaccines. Compared with ordinary immune cells, CAR-immune cells have stronger anti-tumor effects, including longer-lasting immune memory, higher cytokine levels, and stronger immune activation effects. However, the high cost and relatively complicated preparation process may be the main problems hindering the use of CAR-M for the preparation of tumor vaccines. Several CAR-M therapies are already being tested in the clinics. One example is the completion of the first subject dosing of CAR-M cell therapy CT-0508 in March 2021, one of the few applications of engineered macrophages in clinical research (NCT04660929). As a highly plastic cell, macrophages have the ability to adapt their properties and functions in response to external stimuli and to bridge innate effector functions with adaptive immunity. Further, tremendous advances in genetic engineering have provided greater possibilities to reshape the anti-tumor properties of macrophages.

3.4. Other CAR family members

The multiple immune cells in the body offer a variety of possibilities for chimeric antigen receptor engineering. The complex properties of these cells may also give researchers additional ideas for fighting tumors. Here are a few potential cells that could be used for CAR therapy, which hopefully will give researchers several new ideas.

Treg cells have always served to maintain the body's immune homeostasis and negatively regulate the body's immune response, thus playing a role in maintaining self-tolerance and avoiding excessive damage to the body [235–237]. Unlike the aforementioned CAR cell therapies targeting tumors, CAR-Treg targets autoimmune diseases where the engineered Treg cells can accumulate in susceptible tissues to suppress their autoimmune response [238–240]. The use of CAR-Treg in tumor therapy is still in its infancy. Current studies have found that the proportion of CAR Treg cells (CAR-Treg cells are inadvertently generated in the absence of a step of exclusion from the heterogeneous T-cell population in leukocyte isolation products) is negatively correlated with CAR-T cell expansion, but not tumor load (lactate dehydrogenase LDH levels) [241]. These data demonstrate that CAR-Treg cells, may serve as a new biomarker of clinical efficacy and toxicity and it is possible that this subpopulation may be involved in modulating the body's response to CAR-T cells. Furthermore, some necessary barriers must be broken before CAR-Tregs can be used in the clinic [242,243]. Effectiveness of CAR-T therapy is constrained by the side effects of CRS response and neuronal cytotoxicity, and such adverse effects are not known for CAR-Tregs. Moreover, the screening of CAR-targeting antigens and the development of specific antibodies are among the major limitations. Finally, the rapid depletion of cells may limit the efficacy of CAR-Treg.

Further, natural killer T cells (NKT) are a specialized subpopulation of T cells which express both TCR and NK cell receptors on their surface and can also produce a large number of cytokines, including IL-4 and IFNγ [244–247]. These cells can act as killers against tumor cells directly or indirectly. With these properties and modifications of specific CARs, NKT cells can launch a dual attack on tumor cells specifically through CARs and endogenous TCRs [248]. Chondroitin sulfate proteoglycan 4 (CSPG4), which is overexpressed in 70% of melanomas, is a potent target for its treatment [249–251]. Based on this, CSPG4-specific CAR-NKT was constructed and evaluated for its ability to fight melanoma [252]. Interestingly, CAR-NKT cells exhibited lower cytokine secretion but similar specific cytotoxicity compared to that by CAR-T cells. Like CAR-T, the suboptimal cycling time of CAR-NKT cells hinders their further development. IL-15 has not only been suggested to have a critical role in NKT cell developmental processes and homeostatic balance but also protects them from hypoxia [253–257]. Thus, the in vivo persistence and anti-tumor capacity of CAR-NKT was enhanced by achieving IL-15 co-expression with CAR [258], indicating the safety profile and application of CAR-NKT cells as anti-cancer treatment in clinical settings [259]. Despite the obvious advantages, the number of NKT cells are very low in the blood, affecting the applicability of CAR-NKT therapies [260]. Researchers have made many attempts to address this problem and have proposed several possible solutions. Human hematopoietic stem cells (HSCs) are currently one of the main sources for researchers to generate iNKTs. HSC-iNKTs are very similar to endogenous iNKTs and can effectively inhibit the growth of multiple myeloma and melanoma in vivo [260]. In addition, researchers have also attempted to use iPSCs to obtain large numbers of iNKT cells. iPSCs derived NKTs are significantly superior to iNKTs in terms of cytokine secretion and cytotoxicity [261].

Several CAR-NKT cell therapies are currently in the clinical phase (NCT03294954, NCT03774654), which may provide valuable insights for the development of CAR-NKT.

As one of the players in tumor surveillance and anti-tumor immunity, γδT cells constitute approximately 1–5% of peripheral blood T lymphocytes and are mainly distributed in mucosal and epithelial tissues [262,263]. They recognize antigens without MHC restriction; therefore interference from TCR signaling is not required for consideration [264,265]. Additionally, they perform immunosurveillance by naturally homing to various tissues [266]. Taken together, CAR-γδT has potential value in solid tumor therapy. The transformation of γδT cells into CAR-γδT cells makes it possible to achieve precise recognition of specific antigens and effective clearance of tumor cells, and their use may improve the treatment of solid tumors. In vivo experiments in immunodeficient mice also demonstrated that CAR-γδT cells not only failed to induce xenograft-versus-host disease but also effectively inhibited the growth of B-cell lymphoma, exhibiting both innate and acquired anti-tumor capabilities [267]. Although promising, CAR γδ T cell therapy needs to overcome obstacles in all aspects of biology, preparation and clinical application. First, CAR γδ T cells are more sensitive to activation induced cell death (AICD) than αß T cells, which may affect the choice of CAR gene delivery modality. Gene transfer techniques need to be further optimized in terms of time, frequency and dose when used to produce CAR γδ T cells. Furthermore, the limited number of autologous γδ T cells is not conducive to CAR cell therapy. The novel allogeneic γδ T cell expansion technology may have helped to broaden the source of cell supply. In addition, immune checkpoint targeting strategies for CAR γδ T cells may need to be readjusted due to the unique co-stimulation requirements of γδ T cells. A related clinical study is currently underway (NCT04735471).

Tumor infiltrating lymphocytes (TIL) isolated from tumor tissues have strong tumor homing properties and can be used for enhanced infiltration and targeting [268,269]. The main sources of TILs are freshly excised tumor specimens or allogeneic cells. The process of existing TILs therapies can be described as first isolating lymphocytes from the patient's tumor, then activating and expanding or modifying the isolated cells in vitro, and finally infusing them back into the patient. Apart from melanoma, TILs-based therapy has shown significant potential in malignancies such as non-small cell lung cancer, cervical cancer, bile duct cancer, colorectal cancer, breast cancer, head and neck, sarcoma, gallbladder cancer and others. If TILs are combined with CAR technology in the future, they may also be capable of becoming novel immune cell therapies for managing solid tumors.

4. Strategies for enhancing CAR cells

Currently, immunotherapy is emerging as a highly promising therapeutic modality for tumors. In hematologic tumors, CAR cells have exhibited surprising clinical results. However, they have failed to demonstrate the same effect in solid tumors clinically. Approximately 90% of cancer-related deaths are caused by solid tumors [270]. Because of the high heterogeneity of solid tumors, finding stably expressed tumor-specific antigens on different tumor types or on different cells of the same tumor is extremely hard and challenging [271–273]. Multiple hindrances in the TME (such as abnormal vascular system, accumulated immunosuppressive cells and abundant immunosuppressive factors) have also made CAR cell therapy ineffective in solid tumors [274–276]. As shown in Fig. 6, this section describes the different enhancement strategies of CAR cells when used to treat solid tumors (Table 2).

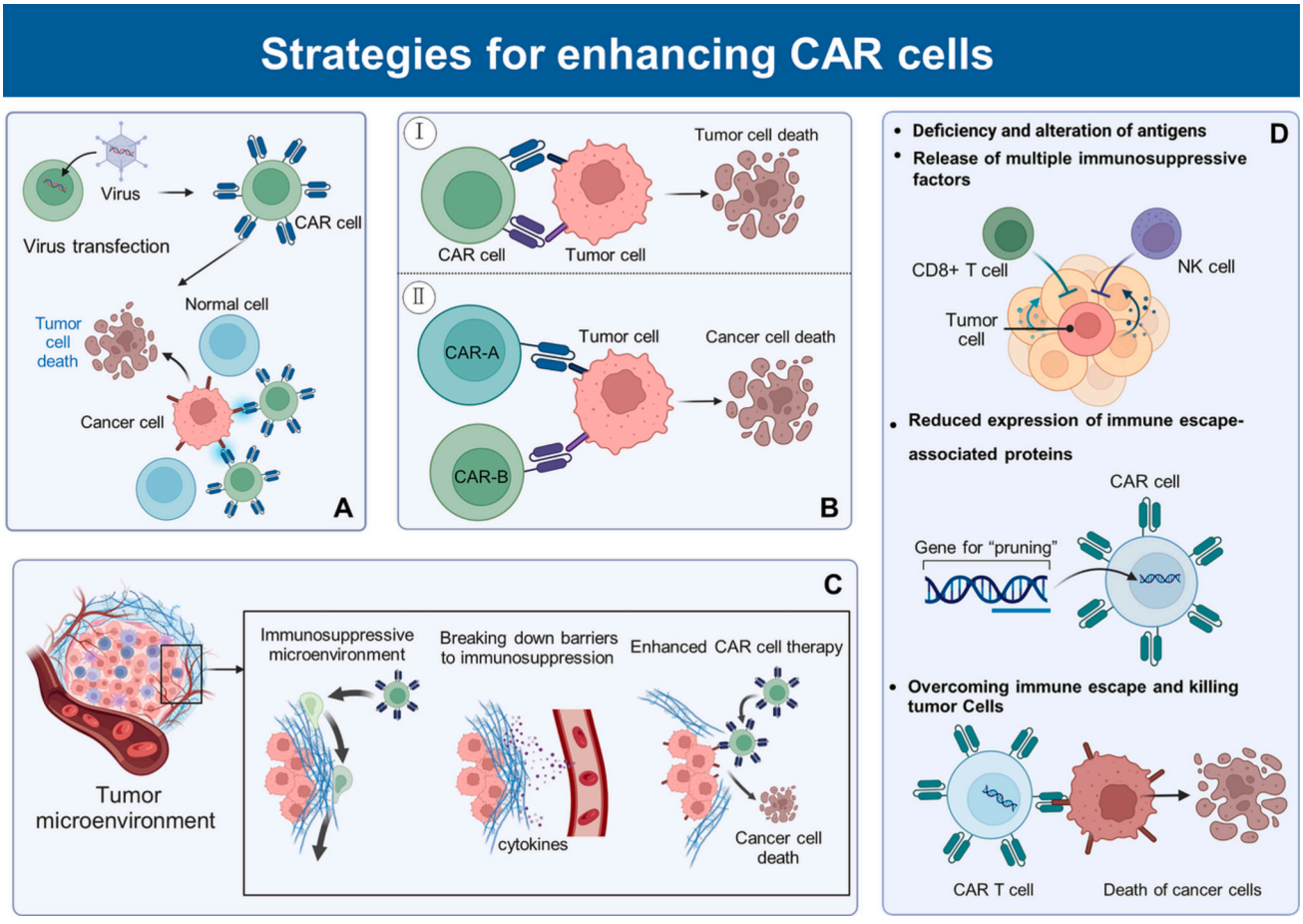


Fig. 6. Multiple strategies for arming CAR cells.

Table 2
Targeted strategies for solid tumor treatment.

Strategies		Features	Clinical trials	Diseases
Tumor-Specific Targets	HLA-G	• Precise targeting	None	None
	HER2	• Diverse selection	NCT03696030	• Malignant Neoplasm
		• Off-target effects	NCT04903080	• Ependymoma
		• Unknown adverse effects	NCT04995003	• Advanced Sarcoma
			NCT04842812	• Advanced Solid Tumors
			NCT02442297	• Brain Tumor
	CD105		None	None
	GD2		NCT02107963	• Sarcoma • Osteosarcoma • Neuroblastoma • Melanoma
			NCT03721068	• Neuroblastoma • Osteosarcoma
			NCT04196413	• Glioma of Spinal Cord • Glioma of Brainstem
			NCT04539366	• Neuroblastoma • Osteosarcoma
			NCT05298995	• Pediatric Brain Tumor
	CEA		NCT05240950	• Colorectal Cancer • Metastatic Liver Cancer
			NCT03818165	• Metastatic Pancreatic Carcinoma
			NCT04513431	• Stage III Colorectal Cancer • Colorectal Cancer Liver Metastasis
			NCT05415475	• Colorectal Cancer • Esophageal Cancer • Stomach Cancer • Pancreatic Cancer • Metastatic Tumor • Recurrent Cancer
			NCT02850536	• Liver Metastases
			NCT02416466	• Liver Metastases
			NCT04348643	• Solid Tumor • Lung Cancer • Colorectal Cancer • Liver Cancer • Pancreatic Cancer • Gastric Cancer • Breast Cancer
	ROR1		NCT04842812	• Advanced Solid Tumors
			NCT02706392	• Advanced ROR1+ Malignancies
			NCT04348643	• Solid Tumor • Lung Cancer • Colorectal Cancer • Liver Cancer • Pancreatic Cancer • Gastric Cancer • Breast Cancer
	B7-H3		NCT04385173	• Glioblastoma
			NCT05211557	• Ovarian Cancer
			NCT05341492	• Lung Cancer • Triple Negative Breast Cancer
			NCT04077866	• Glioblastoma
			NCT04897321	• Pediatric Solid Tumor
			NCT04483778	• Pediatric Solid Tumor
			NCT05323201	• Hepatocellular Carcinoma
			NCT05241392	• Glioblastoma
			NCT04185038	• Central Nervous System Tumor • Diffuse Intrinsic Pontine Glioma • Diffuse Midline Glioma
			NCT04670068	• Epithelial Ovarian Cancer
	MSLN		NCT04489862	• Non-small-cell Lung Cancer • Mesothelioma
			NCT05373147	• Solid Tumor
			NCT04503980	• Colorectal Cancer • Ovarian Cancer
			NCT03545815	• Solid Tumor, Adult
			NCT03198052	• Lung Cancer
			NCT04981691	• Refractory Malignant Solid Neoplasm
			NCT02414269	• Malignant Pleural Disease • Mesothelioma • Metastases • Lung Cancer • Breast Cancer

(*continued on next page*)

Table 2 (*continued*)

Strategies		Features	Clinical trials	Diseases
			NCT01897415	• Pancreatic Cancer
	Exogenous Antigens		None	None
Bispecific CAR	TanCAR	• Lower risk of off-targeting • Higher safety profile • Unknown adverse effects	NCT04662099 NCT04283006 NCT05412329	• Multiple Myeloma • Lymphoid Malignancies • Multiple Myeloma
	CAR cell co-infusion therapy	• Unstable CAR	NCT03407859	• Therapy Related Leukemia
Reversal of immunosuppressive environment	Immunosuppressive factors or cells	• Improved anti-tumor properties • High potential	NCT04227275 NCT04976218	• Prostate Cancer • Solid Tumor, Adult
	Tumor Vessels	• Little help for CAR function	NCT01218867	• Metastatic Cancer • Metastatic Melanoma • Renal Cancer
	Environmental Factors		None	None
"Pruning"	CRISPR/Cas9	• Reversal of immunosuppression • Unknown side effects	NCT04637763 NCT03545815 NCT04037566 NCT05397184	• B Cell Non-Hodgkin Lymphoma • Solid Tumor, Adult • CD19+ Lymphoma • T Cell Malignancies
	RNA interference		NCT03060356	• Malignant Melanoma • Breast Cancer
Combination therapy	Photoacoustic Therapy	• Improvement of off-target effects	None	None
	Nanotechnology	• Controllable CAR	None	None
	Immunotherapy	• Hard preparation process • Safety and efficacy to be evaluated	NCT04433221 NCT03291444	• Sarcoma • Osteoid Sarcoma • Ewing Sarcoma • Leukemia, Acute Lymphocytic • Leukemia, Acute Myelogenous • Myelodysplastic Syndromes

4.1. Tumor-specific targets

Excellent targeting ability is expected as an essential ability possessed by CAR cells. Selection of appropriate tumor-specific antigens not only enhances the targeting ability of CAR cells but also ensure their safety [277–279]. Consequently, it is a promising strategy to screen for TAAs stringently and convert them into activation signals for CAR cells to overcome the limitations toward solid tumors (Fig. 5A). Here, several TAAs worthy of consideration are discussed.

4.1.1. HLA-G

Human leukocyte antigen G (HLA-G) is a newly expressed TAA in solid tumors. In adults, HLA-G expression is strictly limited to a few tissues, including erythroid precursors and pancreatic islets [280]. HLA-G assists tumor cells to escape the surveillance of the immune system mainly through IL-T2 and IL-T4 and broadly inhibits most cells involved in the immune response [281,282]. Two anti-HLA-G CAR-T cells have been designed by limiting the target to the subtypes of HLA-G with suppressive effects. Both cells were effective in clearing HLA-G–expressing tumor cells in the in vivo experiments, and differentiated memory cells were effective after repeated stimulation of tumor cells [283]. Toward killing tumor cells, CAR-NK cells can achieve their goal in an MHC non-dependent manner compared to CAR-T cells. For this reason, researchers have modified CAR-NK cells that can target HLA-G scFv based on NK cells.In all four solid tumor models in vitro, CAR-NK cells exhibited effective cytolytic effects. For in vivo experiments, the growth of xenograft tumors was inhibited, and the median survival of mice was significantly prolonged. For Ewing's sarcoma, HLA-G had no significant adverse effect on CAR-T [284]. Thus, anti-HLA-G may be a viable and potentially effective target that may be useful for treating numerous tumor types, which may not express known TAA.

4.1.2. HER2

HER2 is overexpressed in >30% of human tumors (including breast, ovarian, endometrial, fallopian tube, gastric and prostate cancers) and is now a potential target for tumor diagnosis and treatment [285–287]. In solid tumors, HER2 overexpression was reported to provide suboptimal prognosis [288,289]. Currently, HER2 has been applied to CAR cells to target breast cancer, gastric cancer, sarcoma, glioblastoma, ovarian cancer, and osteosarcoma [290]. The effectiveness of HER2 has been demonstrated for targeting solid tumors [291–293], and a favorable safety profile of HER2-CAR-T cells has been reported in a clinical study [294].

4.1.3. CD105

CD105, also known as endothelin, is highly expressed on cancer cells and on peri- and endothelial tumor cells. It is essential for tumor angiogenesis and promotion of tumor growth [295,296], and its overexpression was related to poor prognosis for several types of solid tumors [297]. Therefore, CD105 can be used for designing CAR cells against solid tumors. In vivo experiments using anti-CD105 CAR-T cells reported inhibition of tumor growth and enhanced survival of xenograft mice [298].

4.1.4. Tumor-associated carbohydrate antigens

Tumor-associated carbohydrate antigens (TACAs) are a class of highly promising anti-tumor targets. In contrast to normal cells, tumor cells overexpress various TACAs on their surface, which essentially contribute to tumor growth [299–301]. Moreover, high expression levels of TACAs can be found in several types of tumors, suggesting their potential use as broad-spectrum targets against solid tumors [302,303]. Disialoganglioside (GD2) is a TACA-containing glycolipid antigen with consistent low expression in normal tissues but higher expression in various types of cancers, including neuroblastoma and osteosarcoma [304–306]. GD2 did not show a stable expression in other sarcomas, gliomas, neuroendocrine tumors, and epithelial carcinomas [307]. It induces tyrosine phosphorylation, leading to the activation of multiple kinase pathways and promotion of cell proliferation, migration, and invasion [308–310]. GD2 knockdown affected tumor formation in vivo [311]. Taken together, GD2 has emerged as a top antigenic targets in cancer. Several preclinical studies have demonstrated its potential as an anti-tumor target [312–315], and a related phase I/II clinical trial (NCT03373097) is underway.

4.1.5. CEA

CEA, a glycoprotein specifically expressed on tumor cells, is overexpressed in >90% of colon cancers and approximately 50% of breast cancers [316–318], where its high expression levels were associated with a worsening prognosis [319]. Although CEA is expressed in healthy colon tissues, its expression is polarized to the tip of the epithelium and

hardly recognized by circulating antibodies [320].These properties provide a theoretical basis for the use of CEA as an anti-tumor target [321–324]. The clinical applications of CEA are currently being validated (NCT05240950, NCT03818165, and NCT02850536).

4.1.6. ROR1

Because of its overexpression in several types of solid tumors, ROR1 is an attractive target for CAR engineering [325–327]. It was strongly associated with poor prognosis of tumors [328–330], and its potential has been recognized in various tumors (including lung, breast, colon, pancreas, renal, and ovarian cancers) [331–333]. Considering the expression of ROR1 on some normal tissues, researchers have proposed that molecules, including ROR1, expressed on both tumors and normal tissues can be safely targeted using combinatorial antigen recognition to trigger intact T-cell activity. The clinical efficacy of ROR1 as a target for CAR cells applied to all kinds of solid tumors is being evaluated (NCT02706392, NCT04842812, and NCT05274451).

4.1.7. B7-H3 (CD276 or B7RP-2)

B7-H3 protein has restricted expression in normal human tissues, including prostate, breast, placenta, liver, colon, and lymphoid organs [334–336].However, a high percentage of human malignancies exhibiting an aberrant expression of B7-H3 have been revealed [336–338]. High levels of B7-H3 expression in tumor cells were associated with scarce TIL, rapid tumor progression, and poor clinical outcome [339,340]. Based on this, CAR-T cells targeting B7-H3 (named B7-H3. CAR-Ts) have been designed to control the growth of pancreatic ductal adenocarcinoma, ovarian cancer, and neuroblastoma in vitro and in metastatic xenograft mouse models, including patient-derived xenografts [341]. Interestingly, 4-1BB co-stimulation in the structure promoted PD-1 expression in B7-H3.CAR-Ts and had superior anti-tumor activity when targeting PD-L1-expressing tumor cells. B7-H3.CAR-Ts inhibited tumor growth through multiple pathways. Currently, their clinical safety and efficacy is being evaluated in multiple tumor types (NCT05211557, NCT05341492, NCT04077866, NCT04897321, NCT04483778, and NCT05323201).

4.1.8. Mesothelin

As a classical TAA, mesothelin is expressed in many types of solid tumors, including lung cancer and ovarian cancer [342,343]. It exhibits great potential as a therapeutic target for cancer owing to its high expression levels in tumor cells and low expression levels in normal tissues [344,345]. Preliminary insights into the anti-tumor properties of MSLN have been revealed [346–350]. Based on the information from preclinical trials, multiple clinical products were designed and developed and are being evaluated for anti-tumor properties (NCT05373147, NCT02930993, and NCT03030001).

4.1.9. Exogenous antigens

All the above-mentioned tumor-specific antigens have potential endogenous origin. Hence, whether adding exogenous antigens to solid tumor cells to achieve targeting and activation needs to be explored as a novel approach. Theoretically, modifying suitable exogenous antigens in situ in solid tumors could improve efficacy, stability, flexibility, and safety and provide sufficient targets for immune recognition, which would significantly broaden the therapeutic scope and enhance the efficacy of CAR cell therapy. Fusogenic nanosystems as antigen modification vectors were chosen to overcome the lack of target antigen and cellular heterogeneity and establish a non-tumor intrinsic antigen-dependent broad-spectrum anti-cancer CAR-T cell [351]. A novel, fused liposome system containing neutral and positively charged lipid molecules and additional lipid components with an extended conjugated π-electron system were used [352]. The fused liposome system-based CAR-T cells successfully inhibited proliferation and prolonged survival in subcutaneous and peritoneal dissemination tumor models. Bacteria have been shown to selectively colonize the tumor core and preferentially grow in the hypoxic and necrotic tumor microenvironment. Several studies in recent years have shown that different tumor types commonly colonize with different tumor microbiota. Researchers have combined probiotics with CAR-T therapy to create ProCAR, a probiotic-guided CAR-T cell platform [353]. In this platform, CAR-T cells are designed to sense and respond to antigenic targets synthesized by probiotics colonizing solid tumors, enabling in situ discovery, recognition and destruction of tumor cells.

4.2. Bi-specific CAR

During CAR cell therapy, the process may fail, or the cells may lose their antigens. To avoid such errors, synthetic bispecific CAR cells have been designed to achieve precise therapeutic effects [354]. Bispecific CAR cells are generally constructed in two ways (Fig. 5B). One is the CAR monomer, in which two different single-chain antibodies are present “in tandem” on the surface of a single cell, or when two different single-chain antibodies are present monomerically on a single cell (dual-signaling CAR therapy) [355,356]. Another is the sequential or simultaneous infusion of different types of CARs, and its application has been confirmed by numerous studies [357–360]. However, the lack of a constant region of immunoglobulin G can lead to instability of single-chain antibody fragments and even the presence of CAR monomers on individual cells, resulting in oligomerization and aggregation among cells and ligand non-dependent tension signals. The selection of appropriate coexisting epitopes is also another issue that needs to be considered. Clinical trials for solid tumors are underway (NCT04662099, NCT03706547).

4.3. Reversal of immunosuppressive environment

Numerous studies have suggested that an immunosuppressive TME will lead to mis-expansion and rapid depletion of CAR cells in humans [361–364]. As mentioned previously, fourth-generation CAR cells can be modified to produce cytokine-inducing components to eliminate these adverse effects (Fig. 5C). The release of cytokines is achieved by CAR cells after specific binding to antigens on tumor cells. These cytokines activate CAR cells, TIL cells, and other innate immune cells (e.g., macrophages and NK cells) [365]. Currently available cytokines include IL-12, IL-15, IL-18, IL-21, and other ligands for receptors on immune or tumor cells [366–369].Several experiments have demonstrated that fourth-generation CAR cells not only have a desirable circulating lifespan and expansion rate but can also overcome the immunosuppressive TME by activating immune effector cells and promoting their proliferation [370–373]. Meanwhile, the secretion of these cytokines can be helpful to enhance the function of immunosuppressive cells. Additionally, other cytokines (e.g., CD40L or 4-1BBL) can assist CAR cells in weakening the resistance of tumor cells to immune attack. To overcome the challenges related to CAR cell migration toward tumor sites, receptor genes for CCR, CXCR, or other chemokines secreted by solid tumors may contribute to improving CAR cell-homing ability.

Tumors create a barrier rich in immunosuppressive factors and cells through negative regulatory mechanisms, hindering CAR cell attack. TGF-β is a major immunosuppressive factor that significantly inhibits immune surveillance in vivo [374]. The effectiveness of inhibiting TGF-β to reactivate anti-tumor immune responses has been confirmed [375–377], and the therapeutic idea of combining it with CAR-T has been achieved [378]. Elimination of immunosuppressive cells (MDSCs and Tregs) has likewise demonstrated a strong potential [379,380].

The abnormal choroidal system in solid tumors is the important barrier that blocks the infiltration of immune cells and suppresses immune action [381,382]. Therefore, the main features of the vasculature system in complex tumors have been explored. Numerous features were associated with the suppression of immune response. First, tumors contributed to the compromised expression of ICAM-1 on the surface of endothelial cells overexpressing endothelin B receptor by secreting

endothelin-1, which conversely interfered with the infiltration of immune cells [383,384]. Second, because of abnormal growth rate, the tumor vascular system often lacked pericytes to maintain the normal morphology of the vessels, which can lead to vascular incompleteness and affect the delivery and circulation of immune cells within the tumor [385,386]. Lastly, abnormal tumor vascular growth results from overexpression of angiogenic factors, including VEGF and FGF. These excessive cytokines promote angiogenesis while suppressing the expression of many adhesion factors (e.g., ICAM-1, ICAM-2, VCAM-1, and CD34), subsequently affecting CAR cell infiltration [387,388]. Based on the above findings, the anti-tumor effect of CAR cells has been improved by blocking VEGF receptors [389–391]. Although the current clinical results are not satisfactory (NCT01218867), it is possible to enhance the infiltration of CAR cells by improving the abnormal vascular system.

The TME, which is low in pH, hypoxic, and rich in immunosuppressive substances, can significantly affect the normal function of immune cells and even reduce cellular activity [392,393]. Therefore, reversing this abnormal environment is a way to enhance CAR cell function. For instance, hypoxia resists immune cell infiltration and attack mainly through hypoxia-inducible factor (HIF) proteins, including HIF-1α, HIF-2α and HIF-3α [394–396]. Accordingly, enhancement of the anti-tumor response could be achieved by inhibiting HIF expression. Several immunotherapeutic studies are based on such a concept [397,398]. In addition, reducing or exploiting the level of reactive oxygen species in the tumor environment is another strategy to enhance the anti-tumor effect of CAR cells [399–401]. Currently, therapeutic regimens combining CAR cells with inhibitors are still in the development stage but may be implemented in the near future. The complex regulatory mechanisms of the immune system contribute significantly to the homeostasis of the body; however, they are a "double-edged sword."

Reversing the adverse tumor environment has long been considered as a classical tumor treatment strategy. Inhibitory cytokines, poor physiological environment, and complex vascular system can be one of the targets. The effective combination of these targets with CAR cell therapy suggests an endless potential, an area for researchers to fully explore and an effective means to improve solid tumor treatment.

4.4. "Pruning"

In contrast to the approach of adding more elements to CAR cells to optimize their function, removing unnecessary or harmful elements (including PD-1, which helps tumor cells escape) may potentially help enhance anti-tumor effects (Fig. 5D). It is comparable to cutting off dead or diseased branches to encourage the growth and development of plants. The key to the "pruning" strategy lies in stable gene-editing technologies. Among these, the CRISPR/Cas9 system is extremely essential, which can enable secured gene editing in CAR cells. The expression of PD-1 on T cells is upregulated upon encountering antigen. The binding of PD-1 to the ligand PD-L1 leads to immunosuppression, which is one of the main mechanisms of tumor immune escape [402]. In addition, simple knockdown of the PD-1 gene may increase the risk of collapse of peripheral immune tolerance. Therefore, knockdown of PD-1 and TCR genes is the mainstream choice of researchers. Several clinical trials utilizing this technology are currently underway (NCT04637763, NCT03545815, NCT05397184).

Additionally, the immune response has been enhanced by inhibiting diacylglycerol kinase (DGK), whose activation leads to the downregulation of the TCR distal molecule. Knockdown of the two subtypes of *DGK* (*DGKα* and *DGKζ*) led to the activation of ERK signaling, thus synergistically enhancing the proliferation and immune properties of CAR-T cells. [403] Moreover, DGK knockdown conferred protection to CAR-T cells from soluble immunosuppressive factors (including TGFβ and prostaglandin E2) released by tumor cells. Thus, removing the "spy component" of immune cells using CRISPR/Cas9 technology enhanced the anti-tumor effect. However, whether such knockdown will have other adverse effects needs to be considered and explored carefully for clinical application.

RNA interference, a phenomenon in which small double-stranded RNAs specifically degrade or suppress the expression of homologous mRNAs to inhibit or shut down the expression of specific genes, may be another candidate for "pruning." It is often used to explore gene function and tumor therapy [404–406].Downregulation of the expression of PD-1 and CTLA-4 in CAR-T cells by RNA interference had no effect on CAR expression, and anti-tumor assays confirmed that the transfected CAR-T cells exhibited markedly improved anti-tumor ability. Notably, CAR-T cells transfected with siCTLA-4 alone failed to show significant changes. Thus, "pruning" can help immune cells relieve the immunosuppression of tumors and can be considered in combination with other ideas for fighting solid tumors to enhance the anti-tumor ability of CAR cells.

4.5. Combination therapy

Various treatment options have both advantages and disadvantages. When the inherent deficiencies of these options cannot be improved, a combinatorial approach is preferable. But how to select the best combination from the many transformation strategies is something that needs to be proven in practice. For example, to decrease CAR cell off-targeting and reshape the immunosuppressive TME, the combination of CAR cell therapy with photoacoustic therapy or nanoengineering is worth being considered [407–409]. CAR cells are activated when light or ultrasound is applied locally to the tumor; however, when applied elsewhere, they will be harmless. Drug-loaded nanoparticles have also been employed to improve the anti-tumor effect of CAR-T cells, and the efficiency of combination therapy had been confirmed [410]. Additionally, researchers have found that CAR-T cells can reprogram their metabolic pathways and promote interaction between CAR-T cells and the endogenous immune system, thus activating sustained antigen spreading, when stimulated by vaccines [411]. Both the IFN-γ produced by CAR-T cells and the IL-12 produced by dendritic cells are crucial for antigen spreading. The combination of vaccines and CAR-T cells can effectively treat solid tumors with antigen heterogeneity. A single treatment modality is considered to have a limited effect only; however, a multimodal treatment may achieve greater efficacy.

5. Conclusions and prospects

CAR-T cell therapy has emerged as a popular treatment regimen and has brought new hope to defeat tumors. Furthermore, it has provided an insight into the emergence of other CAR cell family members. Optimized CARs design requires a comprehensive understanding of the characteristics of each component individually or in combination to improve the rational construction of CARs cells. The following issues need to be addressed if CAR cells aim to be further translatable in the clinic: cytotoxicity, cellular exhaustion, cellular source, and drug resistance. In addition, CAR cells design strategies should be adapted to solid tumors due to the strongly immunosuppressive microenvironment and complex vascular system. To further enhance the performance of CAR cells, many effective strategies have been proposed, such as screening for more appropriate tumor-specific antigens, reversing the tumor microenvironment and combination therapy. The effectiveness and feasibility of them have been demonstrated in various studies and clinical trials. Furthermore, the likelihood of curing a malignant solid tumor with only one therapy is extremely low; therefore, combination therapies and real-time monitoring approaches should be developed simultaneously. Accurate and stable delivery of CAR cells can be achieved using a wide range of existing biomaterials (films, particles, scaffolds and microneedles) [412]. Biomaterials can help immune cells to reach the tumor site and provide a location for them to reside, proliferate and perform anti-tumor activity [413]. Biomaterials loaded with immunomodulators

can further enhance immune cell activity and modulate the tumor immunosuppressive microenvironment. The mechanical properties, shape, spatial structure and encapsulation technology of the biomaterials are key factors in their ultimate effectiveness. The cost of CAR cell therapy should be reduced as technology advances to be acceptable to most patients.

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CRediT authorship contribution statement

Tingrui Zhang: Writing – original draft, Methodology, Investigation, Formal analysis. **Zongguang Tai:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Fengze Miao:** Methodology, Investigation. **Xinyue Zhang:** Resources, Investigation. **Jiadong Li:** Software, Methodology. **Quangang Zhu:** Writing – review & editing. **Hua Wei:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Zhongjian Chen:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

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

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