Programming CAR T cells to enhance anti-tumor efficacy through remodeling of the immune system

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Abstract Chimeric antigen receptor (CAR) T cells have been indicated effective in treating B cell acute lymphoblastic leukemia and non-Hodgkin lymphoma and have shown encouraging results in preclinical and clinical studies. However, CAR T cells have achieved minimal success against solid malignancies because of the additional obstacles of their insufficient migration into tumors and poor amplification and persistence, in addition to antigen-negative relapse and an immunosuppressive microenvironment. Various preclinical studies are exploring strategies to overcome the above challenges. Mobilization of endogenous immune cells is also necessary for CAR T cells to obtain their optimal therapeutic effect given the importance of the innate immune responses in the elimination of malignant tumors. In this review, we focus on the recent advances in the engineering of CAR T cell therapies to restore the immune response in solid malignancies, especially with CAR T cells acting as cellular carriers to deliver immunomodulators to tumors to mobilize the endogenous immune response. We also explored the sensitizing effects of conventional treatment approaches, such as chemotherapy and radiotherapy, on CAR T cell therapy. Finally, we discuss the combination of CAR T cells with biomaterials or oncolytic viruses to enhance the anti-tumor outcomes of CAR T cell therapies in solid tumors.

Keywords CAR T cells; immunoregulatory molecules; endogenous immune response; solid malignancies

Introduction

Along with surgery, chemotherapy, and radiotherapy, immunotherapy is considered an important pillar of cancer treatment. Immunotherapies, such as those using chimeric antigen receptor (CAR) T cells and checkpoint inhibitors, utilize the host's own immune system to exert anti-tumor effects. Immunotherapy was the subject of the 2018 Nobel Prize in Physiology or Medicine for its favorable efficacy in cancer treatment.

CAR T cell therapy is a precise immunotherapy in which genetically engineered T cells identify tumor cells expressing specific antigens. CARs are recombinant receptors composed of three parts: an extracellular antigen recognition domain, a T cell receptor (TCR) transmembrane and hinge domain, and an intracellular signal transduction

domain. CAR-expressing T cells can elicit specific immune responses by specifically recognizing a tumor-associated antigen (TAA) and activating T cells via the signal transduction and hinge domain. In recent years, CAR T cells have shown encouraging results in both preclinical and clinical studies [1–6]. The US Food and Drug Administration (FDA) and European Medicines Agency approved two CAR T cell treatments for cancer immunotherapy, namely, tisagenlecleucel, and axicabtagene ciloleucel in 2017 and 2018, respectively [7–9]. Although CAR T cells have improved the outcomes of patients with hematologic malignancies, numerous challenges remain in the treatment of solid malignancies, including the difficulty in identifying ideal target tumor antigens, restricted trafficking of CAR T cells, decreased persistence and expansion of CAR T cells, antigen-negative relapse, and the susceptibility of CAR T cells to the immunosuppressive tumor microenvironment (TME). Preclinical studies are exploring strategies to overcome the above challenges and to increase the efficacy of CAR T cells in solid malignancies. To overcome the challenge posed by the

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lack of ideal antigens in solid tumors, researchers have altered the CAR designs to include dual and triple CARs [10–13]. T cells are transduced with two or three CARs capable of simultaneously recognizing two or three antigens. Ruella *et al.* reported that dual CD19 and CD123 CAR T cells demonstrated superior *in vivo* activity against B cell acute lymphoblastic leukemia (B-ALL) compared with single CAR T cells [12]. Recently, Bielamowicz *et al.* showed that trivalent CAR T cells (cotargeting human epidermal growth factor receptor 2 (HER2), interleukin (IL)-13 receptor α 2 (IL13R α 2), and ephrin type-A receptor 2 (EphA2)) captured nearly 100% of tumor cells tested in their cohort [13].

The introduction of suicide genes or transient mRNA to CAR T cells can control the lifespan of these cells to reduce treatment-related toxicity [14–18]. Furthermore, local administration of CAR T cells has been explored to enhance T cell trafficking into tumor tissues [19–22]. Varying methods have been explored to deliver chemokine receptors into CAR T cells because effective trafficking is also interrupted by the mismatch between the chemokine receptors expressed by CAR T cells and the chemokines expressed by tumors [23].

Additionally, the hostile and immunosuppressive TME impedes the activity of CAR T cells [24–26]. The TME is a complex system that can disturb the anti-tumor function through various immunosuppressive signals [27–29]. Therefore, coordinative innate immune responses inside the TME, including blocking the suppressing immune signals and activating the stimulatory signaling, may elicit effective immune effects (Fig. 1). Blocking suppressive signals, such as programmed cell death-1 (PD-1)/programmed death ligand-1 (PD-L1) signals and immunosuppressive cytokines, potentially heightens CAR T cell-mediated tumor immunity. Cytokines are pleiotropic to the endogenous immune system, and their addition enhances the anti-tumor effect of CAR T cells; to date, several cytokines, including IL-12 [30–32], IL-15 [33], IL-18 [34], and tumor necrosis factor (TNF) receptor superfamily member 14 [35], have been adopted to arm CAR T cells. In addition, co-administration strategies with conventional treatment approaches, such as chemotherapy and radiotherapy, and tumor vaccines/oncolytic viruses (OVs) or biomaterials play an important role in enhancing CAR T cell outcomes in solid tumors. In this review, we surveyed strategies for engineering CAR T cells that not only

Fig. 1 Strategy for engineering CAR T cells through triggering comprehensive endogenous anti-tumor immune responses. T cell costimulatory (left) and/or inhibitory (right) pathways can be targeted to enhance CAR T cell therapy in solid tumors. CAR T cells are genetically engineered to express/secrete cytokines and/or chemokines, such as IL-18, IL-15, IL-12, IL-7, chemokine (C-C motif) ligand 19 (CCL19), and tumor necrosis factor superfamily (TNFSF) members. CAR T cells coordinate with inhibitory signals, including immune checkpoints (PD-1/PD-L1) and transforming growth factor β (TGF- β), to boost immune responses.

augment direct anti-tumor effectors but also trigger comprehensive endogenous anti-tumor immune responses (Fig. 2).

Targeting cytokines to boost immune responses

Combination with IL-12

IL-12 is a heterodimeric cytokine that is physiologically produced in antigen-presenting cells (APCs), neutrophils, and macrophages [36]. As a potent immune stimulatory cytokine, IL-12 is usually used as a single agent or therapeutic adjuvant to initiate innate immune response [37]. In an early phase trial, IL-12 produced toxicity in several tissues [30,37]. IL-12 was delivered directly to the tumor lesion to overcome this shortcoming; this method was proven safe and elicited clinical responses. This result spurred efforts to deliver IL-12 to engineered CAR T cells. Engineered CAR T cells with an inducible and constitutive IL-12 cassette support the anti-tumor cytotoxicity of CAR T cells and the reprogrammed immune response.

Wagner *et al.* engineered Epstein–Barr virus-targeted cytotoxic T cells to constitutively express IL-12 [38], elevating the Th1 cytokine levels and reducing the production of Th2 cytokines. Additionally, IL-12-transduced T cells demonstrated a proliferative advantage in the inhibitory environment. This finding was further confirmed by another study that modified CAR19 T cells to constitutively secrete IL-12 (CAR19/IL-12 T cells) and demonstrated increased cytotoxic capacity and resistance to the suppression of Treg cells [39]. Constitutive secretion

of IL-12 by CAR19 T cells has also been verified by the Brentjen’s group, who showed that CAR19/IL-12 T cells retained a central memory-effector phenotype and significantly increased the anti-tumor efficacy [32].

However, the IL-12 dose is hard to determine by a constitutive expression strategy, and supratherapeutic levels restrict T cell expansion and limit their therapeutic efficacy. These issues favor modified CAR T cells with inducible IL-12 (iIL-12), which is activated and released depending on whether CAR T cells recognize the target cells [40,41]. To date, several preclinical and clinical studies have demonstrated the enhanced anti-tumor activity of iIL-12-secreting CAR T cells.

Chmielewski *et al.* first confirmed that inducible release of IL-12 in carcinoembryonic antigen-specific CAR T cells enhanced the inflammatory response and destruction of tumor cells with antigen loss [41]. Koneru *et al.* further demonstrated that IL-12-secreting mucin 16 (MUC-16) CAR T cells enhanced the anti-tumor efficacy, prolonged the persistence of T cells, and modulated the TME in an ovarian cancer model [42,43]; these results were translated into a phase I clinical trial. Recently, a study on hepatocellular carcinoma showed that inducible expression of IL-12 in glypican-3 (GPC3) CAR T cells enhanced their infiltration and persistence, and that these cells demonstrated potent activity against GPC 3-expressing tumors without evident toxicity [44]. Table 1 summarizes the ongoing clinical trials investigating the combination of CAR T cells with IL-12 in cancer immunotherapy.

Thus, a lasting accumulation of IL-12 in the CAR T cell overcomes the limitation of CAR T cell therapy by remodeling the innate immune response.

Fig. 2 CAR T cells engineered to co-express/release immune-stimulatory molecules or block immune-inhibitory molecules indirectly mobilize the endogenous immune response by enhancing the activation and recruitment of immune cells, such as M1 macrophages, natural killer (NK) cells, T cells, and dendritic cells (DCs), and by overcoming the inhibitory microenvironment.

Table 1 Summary of ongoing clinical trials investigating the combination of CAR T cells with cytokines and chemokines in cancer immunotherapy

Module	Antigen	Phase	Clinical trial ID	Disease condition	Status	Sponsor
IL-12	MUC16ecto	I	NCT02498912	Serous ovarian, PPC, FTC	Recruiting	Memorial Sloan Kettering Cancer Center
IL-12	EGFR	I	NCT03542799	Metastatic colorectal cancer	Not yet recruiting	Shenzhen Second People's Hospital
IL-7 and CCL19 or IL-12	Nectin4	I	NCT03932565	NSCLC, breast cancer, ovarian cancer, bladder cancer, pancreatic cancer	Recruiting	The Sixth Affiliated Hospital of Wenzhou Medical University
IL-15	GD2	I	NCT03721068	Relapsed/refractory neuroblastoma	Recruiting	UNC Lineberger Comprehensive Cancer Center
IL-15	GD2	I	NCT03294954	Children with neuroblastoma	Recruiting	Baylor College of Medicine
IL-15	CD19	I/II	NCT03579927	MCL, recurrent/refractory DLBCL, recurrent/refractory FL, refractory B cell NHL	Not yet recruiting	M.D. Anderson Cancer Center
IL-15	CD19	I	NCT03774654	Refractory B cell NHL, refractory B cell SLL, relapsed adult ALL, relapsed CLL, relapsed NHL	Not yet recruiting	Baylor College of Medicine
IL-7/IL-15	CD19	I/II	NCT02652910	Recurrent adult DLCL, recurrent FL, recurrent MCL, stage III/IV adult DLCL, stage III/IV FL, stage III/IV MCL	Recruiting	Xinqiao Hospital of Chongqing
IL-15	CD19	I/II	NCT03056339	B-lymphoid malignancies, ALL, CLL, NHL	Recruiting	M.D. Anderson Cancer Center
IL-7 and CCL19	CD19	II	NCT03929107	B cell lymphoma	Recruiting	Wenbin Qian
CCR4	CD30	I	NCT03602157	Lymphoma, NHL, immune system diseases, immunoproliferative disorders, lymphatic diseases, lympho proliferative disorders, neoplasms, cutaneous lymphoma, cutaneous ALCL, MF, SS, lymphomatoid papulosis	Recruiting	UNC Lineberger Comprehensive Cancer Center

Abbreviations: NCT, clinicaltrials.gov identification number; NHL, non-Hodgkin lymphoma; FL, follicular lymphoma; PPC, primary peritoneal carcinoma; FTC, fallopian tube carcinoma; B cell SLL, B cell small lymphocytic lymphoma; ALCL, anaplastic large cell lymphoma; MF, mycosis fungoides; SS, Sezary syndrome; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; DLCL, diffuse large cell lymphoma; CCR, chemokine receptor; CCL, chemokine ligand.

Combination with IL-15

The prosurvival cytokine IL-15 is produced by monocytes, macrophages, and DCs. IL-15 reverses T cell anergy, inhibits activation-induced cell death, and enhances the anti-tumor activity of T cells [45–48]. Notably, studies demonstrated that elevated levels of IL-15 in the TME of patients with rectal cancer are associated with increased T cell infiltration and increased survival. As an immunotherapeutic agent, IL-15 is required for the production of congenital T cells, which are involved in immune surveillance and impede tumor growth [45,49–51]. Hsu *et al.* investigated the biological activity of constitutive IL-15 expression in T cells and discovered that IL-15-expressing T cells showed enhanced persistence, continued proliferation, and resistance to apoptosis [52].

Recently, research demonstrated that transgenic expression of IL-15 is an appealing strategy to enhance CAR T cell function. A study by Hurton's group revealed that co-expressing CAR19 with a membrane-bound chimeric

IL-15 augmented T cell survival, increased the persistence of a memory stem cell phenotype in T cells, and resulted in superior anti-tumor effects *in vivo* [45]. Krenciute's group also verified that transduction of IL-15 in IL-13R α 2-CAR T cells in solid tumor resulted in substantial proliferative capacity, and this IL-15-transduced IL13R α 2-CAR T cells showed a strong proliferative capacity, high cytokine secretion capacity, and pronounced survival advantages in glioblastoma [53].

However, similar to IL-12, high exposure to IL-15 causes aberrant proliferation of T cells and adverse events, such as autoimmune disease [45]; in addition, chronic stimulation by IL-15 promotes the proliferation and survival of several types of tumor cells [54–56]. Therefore, co-expressing a suicide gene (e.g., inducible caspase 9 (iCaspase9)) with cytokine transgenes has become a reliable method for eliminating IL-15 transgenic cells as required. Hoyos' group incorporated the IL-15 gene and the iCaspase9 suicide gene into CAR19 T cells (iC9/CAR.19/IL-15); as predicted, these cells enhanced the

expansion and reduced the expression of the inhibitor signal, and iCaspase9 effectively cleared the T cells [33]. This “safety switch” has increased the potential clinical applicability of IL-15 in CAR T cell immunotherapy. Table 1 summarizes the ongoing clinical trials investigating the combination of CAR T cells with IL-15 in cancer immunotherapy. In summary, IL-15 provides a desired signal for the long-lasting immunosurveillance and preserves the therapeutic potential of CAR T cells.

Combination with IL-18

IL-18 is an IL-1 family cytokine that regulates the immune system by stimulating the interferon- γ secretion. Clinical administration of IL-18 is safe, and several adverse events have been observed in patients treated with IL-18 [57]. Systemic administration of IL-18 can cause inflammatory responses [58], making the release of IL-18 a promising function of engineered CAR T cells.

Hu and colleagues reported that IL-18-secreting CAR19 T cells have improved anti-tumor efficacy in hematologic malignancies [59], and this effect has also been verified in solid tumors. Chmielewski's and Abken's groups demonstrated that IL-18 expression in CAR T cells can alter the immunosuppressive microenvironment by activating DCs, recruiting NK cells, inhibiting Treg cells, and increasing tumor-associated M1 macrophages. IL-18 released by CAR T cells eradicated the pancreatic cancer and metastatic lung cancer in solid tumor models [34]. IL-18 secretion promotes CAR T cell proliferation and persistence and the anti-tumor activity *in vivo*. Furthermore, IL-18-strengthened CAR T cells can evoke potent endogenous anti-tumor immune responses [60]. However, all published data are from the preclinical models, and we anticipate the translation of these results into clinical trials.

Combination with other cytokines

In addition to the above cytokines, the IL-7 signal is a potent stimulator of T cell proliferation and expansion [61], and the expression of IL-7R α improves the function and efficacy of CAR T cells in solid tumor environments. As reported, CAR T cells co-expressing an IL-7 cytokine receptor (C7R) had increased T cell proliferation and survival and enhanced anti-tumor activity in metastatic neuroblastoma and orthotopic glioblastoma xenograft models [62]. Table 1 summarizes the ongoing clinical trials investigating the combination of CAR T cells with IL-7 in cancer immunotherapy.

IL-21 is another cytokine that is combined with CAR T cells. In melanomas, IL-21-producing CAR T cells increased T cell persistence, elevated memory CAR T cell survival, and were highly efficacious in promoting tumor eradication [63].

Targeting chemokines

In addition, CAR T cells have been engineered to constitutively or conditionally secrete cytokines, and numerous methods have been used to engineer CAR T cells with chemokines to restore the endogenous immune system and to potentiate their anti-tumor efficacy [33,42,59,64,65].

Chemokines are secreted proteins that mediate immune cell trafficking to specific tissues, and their expression is positively correlated with tumor-infiltrating lymphocytes (TILs) and with postoperative survival [66]. Chemokines must match their receptors, allowing T cells to penetrate into tumors. However, chemokine receptors expressed by T cells often mismatch tumor chemokines, thereby affecting T cell infiltration into the tumor bed [67]. Given the major role of chemokines and their receptors in regulating trafficking and homing of T lymphocytes, varying methods have been explored to engineer chemokine receptors into CAR T cells to improve CAR T cell migration and infiltration and to enhance the infiltration of other immune cells, such as T cells and DCs, into tumors [68–77].

CAR30 T cells co-expressing CCR4 improve the homing of CAR30 T cells to Hodgkin lymphoma (HL) and thereby enhance the anti-tumor effects [78]. In solid tumors, C-X-C motif chemokine receptor 2-expressing CAR $\alpha\beta$ 6 T cells elicited superior anti-tumor activity in the xenograft models of ovarian and pancreatic tumors [79]. CCR2b-expressing disialoganglioside (GD2) CAR T cells showed improved homing to CCL2-secreting neuroblastomas and showed anti-tumor activity *in vivo* [71]. Injection of CCR2b-co-expressing meso-CART cells in an immunodeficient mouse model resulted in increased T cell infiltration and anti-tumor activity [72]. CAR T cells that are simultaneously modified with cytokines and chemokines also exhibit enhanced anti-tumor function. IL-7 enhances the proliferation and survival of T cells [80–82], and CCL19 is a chemoattractant for T cells and other immune cells [83,84]; IL-7 and CCL19-expressing meso-CAR T cells showed improved therapeutic effects against solid tumors, enhanced T cell activation, increased the development of memory T cells, and enhanced the infiltration of T cells and DCs into tumor tissues [67,85]. Table 1 summarizes the ongoing clinical trials investigating the combination of CAR T cells with chemokines in cancer immunotherapy.

Targeting the TNFSF members

Combination with LIGHT

LIGHT is a member of the TNFSF14 and is an immunostimulatory cytokine [86]. Light is mainly

expressed in immature DCs and activated T cells. LIGHT can bind to two different receptors, namely, herpesvirus entry mediator (HVEM), and lymphotoxin β receptor (LT β R), to interact with stromal cells, DCs, NK cells, T cells, and tumor cells [87–89]. LIGHT delivers costimulatory signals to T cells by binding to HVEM [90] and forms secondary and tertiary lymphoid structures by binding to LT β R [89,91].

Notably, overexpression of LIGHT in cancer can increase the number of TILs and their activation status, thereby upregulating the anti-tumor immune responses [92]. Targeting tumor tissue with LIGHT increases the recruitment and retention of tumor effector cells, directly or indirectly inducing strong anti-tumor immunity to inhibit primary tumor growth and eradicate metastasis. LIGHT expressed in tumor cells forms a lymphoid-like structure that directly activates T cells, resulting in tumor regression [93,94]. Fu *et al.* demonstrated that LIGHT expression in primary tumors or liver metastases stimulates lymphocyte proliferation and activates tumor cell-specific immune responses, leading to tumor regression or slowing down of liver metastasis. Recently, this group generated an anti-epidermal growth factor receptor (EGFR)-hLIGHT fusion protein against EGFR-expressing tumor tissues. Strikingly, this fusion protein caused complete regression of EGFR-expressing tumors. Together, these results demonstrate that LIGHT overcomes tumor resistance to PD-1 by sufficiently increasing T cell infiltration and by generating memory cells. These data suggest that a proper combination of LIGHT with immunotherapy may enhance the immunotherapeutic effect [95]. Our team is conducting a study of the combination of LIGHT with CAR T cells to enhance the anti-tumor efficacy of CAR T cells against solid tumors on the basis of the immunostimulatory activity and anti-tumor immune response of LIGHT.

Combination with CD40 ligand (CD40L)

CD40L is another member of the TNF gene superfamily, and as a costimulatory molecule, it is involved in tumorigenesis and tumor immunosurveillance [96]. CD40L binds to its receptor, CD40, which is expressed mainly in various immune and nonimmune cells, including B cells, DCs, and macrophages. The CD40L/CD40 pathway is involved in regulating immune function through various mechanisms, such as via modulating B cell activation and DC antigen presentation, affecting T cell proliferation and cytokine secretion, reversing CD8 T cell depletion, and generating the memory phenotype [97–102]. In addition, the CD40L/CD40 pathway exhibits direct anti-tumor activity in various carcinoma cells, such as breast carcinoma, squamous cell carcinoma, epithelial carcinoma, and glioma cells, by influencing the balance between apoptotic and survival signals [103–105].

CD40L also enhances the anti-tumor activity by

increasing the immunogenicity of malignant cells. This finding was confirmed by William's group, who demonstrated that leukemia cells transduced with an anti-CD40L antibody became highly effective APCs, inducing specific cytotoxic T cells *in vitro* and enhancing the immune response in patients with CLL [106]. These observations support the clinical exploitation of CD40L in cancer treatment.

Agonistic CD40 monoclonal antibodies (mAbs) have produced objective anti-tumor responses in single-agent studies and in combination with other modalities, such as chemotherapy, vaccines, or other agents. Single agonistic CD40 mAb therapy will be probably effective for B cell lymphoma. However, for solid tumors, CD40 mAbs will be viable in combination with other modalities [107–109]. The anti-tumor activity of recombinant CD40L has also been tested in phase 1 trials, which showed that the infusion of autologous Ad-murine CD40L-modified CLL cells in patients resulted in a reduced leukemic burden and the activation of leukemia-specific T cells, demonstrating the capacity of recombinant CD40L to induce an endogenous anti-tumor response [110]. Furthermore, this group conducted other clinical trials of intravenous injection or intranodal direct injection of Ad-human CD40L-modified CLL cells in patients—these treatments were well tolerated and induced biological and clinical responses [111,112].

Whether CD40L can mobilize endogenous immune cells to enhance the CAR T cell anti-tumor response has been confirmed by showing that engineered CAR T cells with constitutively expressed CD40L orchestrate a sustained endogenous anti-tumor response by recruiting tumor-recognizing T cells. Brentjens *et al.* showed that the constitutive expression of CD40L by CAR19 T cells enhanced the cytotoxicity to a panel of CD19-positive tumor cell lines *in vitro* and extended the survival of xenotransplanted mice with CD19-positive systemic lymphoma [113]. DCs can prime anti-tumor T cell responses by presenting antigenic peptides to surface TCRs via the major histocompatibility complex (MHC)-I and MHC-II molecules. Kuhn *et al.* reported that CD40L⁺ CAR T cells activate DCs *in vivo*, leading to secretion of the proinflammatory cytokine IL-12 by DCs and augmenting the recruitment and cytotoxic function of endogenous tumor-specific cytotoxic T cells and transferred CAR T cells [114]. Therefore, the combination of CAR T cells with the immunostimulatory molecule CD40L is a promising strategy to ensure the recruitment and cytotoxic function of endogenous immune cells in cancer treatment.

Overcoming immune inhibition to boost immune responses in CAR T cells

Releasing the brakes of the immune inhibitory system is also an important strategy to boost the immune response

against tumors. Checkpoint and immunosuppressive cytokines are the main actors that block the immune system from acting on tumors [115].

Targeting immune checkpoints

T cells express co-inhibitory receptors, such as PD-1 [115], that decrease T cell-mediated tumor immunity and enhance tumor escape. Blocking antibodies against PD-1/PD-L1 have achieved beneficial activity in clinical practice [116–119]. PD-1 disrupts the function of tumor-infiltrating CAR T cells [120–123], indicating that inhibiting PD-1/PD-L1 can increase the anti-tumor activity of CAR T cells.

In hematologic malignancies, PD-1 blockade has been shown to potentially synergize with CAR T cells in preclinical and clinical research. Li *et al.* engineered CAR19 T cells to constitutively secrete anti-PD-1 scFvs (CAR19.aPD1 T cells), which significantly enhanced T cell proliferation. In a xenograft mouse model, these CAR19.aPD1 T cells showed significantly enhanced anti-tumor activity and prolonged overall survival. In addition, decreased level of CAR19.aPD1 T cell exhaustion was observed at the local tumor site [124]. Encouraging data have also emerged from clinical studies; for example, anti-PD1 antibodies augment the response of pediatric B-ALL patients to CAR19 T cells [125]. In another small, single-center study at the Children's Hospital of Pennsylvania, PD-1 blockade improved the persistence of CAR19 T cells in 14 children with B-ALL who failed in CAR19-T cell therapy, and B cell aplasia was reestablished in three out of six patients treated with a PD-1 inhibitor combined with CAR19-T cells, indicating CAR19-T cell function. Five patients achieved a partial response, and two achieved a complete response [124,126,127]. These studies suggest a potential strategy of combining CAR T therapy with PD-1 blockade in solid cancers.

Combination with anti-PD-1 antibodies

John *et al.* first showed that anti-PD-1 antibodies can significantly enhance the therapeutic efficacy of CAR T cells in a mouse solid tumor model. They also discovered that anti-PD-1 antibodies increased Her2 CAR T cell activation and proliferation *in vitro*, significantly inhibiting the growth in two different Her2 positive tumors. Strikingly, the percentage of myeloid-derived suppressor cells (MDSCs) significantly decreased in mice treated with the combination therapy [128]. A small phase 1 trial on relapsed or refractory neuroblastoma patients by Heczey [49] reported that CAR T cells administered with an anti-PD-1 antibody (pembrolizumab) were safe.

However, unlike the above experience in preclinical experiments, pembrolizumab causes no effect on CAR T cell expansion and persistence in patients with neuroblastoma [129], possibly because of the doses and schedules of

PD-1 antibodies affecting their synergy with CAR T cells; this hypothesis has been further verified, that is, high-dose PD-1 antibodies enhanced anti-tumor activity of CAR T cells in a syngeneic breast cancer model [128]; by contrast, low-dose antibodies showed no effect [124]. These results suggest that optimal doses and schedules of PD-1 blockade will be a promising strategy to maximize the synergy of PD-1 blockade and CAR T cells. In addition, Suarez *et al.* engineered carbonic anhydrase IX-targeted CAR T cells to locally deliver PD-L1 antibodies [130], leading to NK cell recruitments and a fivefold reduction in tumor growth. This result was further verified in a human lung carcinoma xenograft mouse model by Li *et al.*, who also engineered CAR T cells to secrete anti-PD-1 antibodies [124]. They demonstrated that the secreted anti-PD-1 antibodies efficiently reversed the inhibitory effect, diminished T cell exhaustion, and improved CAR T cell anti-tumor activity.

Engineering of PD-1/PD-L1

Genetic strategies, such as knockdown or knockout of PD-1 by suicide gene “safety switch” systems or shRNAs and the incorporation of a PD-1 dominant-negative receptor (DNR), have been used in modified CAR T cells to induce PD-1/PD-L1 blocking.

The PD1:CD28 chimera that converts PD-1 inhibitory signals to the CD28 costimulatory receptor has been used to modify CAR T cells, and this chimera resulted in enhanced cytotoxic T-lymphocyte (CTL) functionality by increasing cytokine secretion and proliferation and enhancing the expression of effector molecules without leading to CTL exhaustion when engaged with PD-L1-positive tumors [131]. Another genetic strategy to counteract PD-1 inhibitory signaling in CAR T cells is to knockout or knockdown PD-1. Cherkassky's group generated a PD-1 DNR and shRNA targeting PD-1 in mesothelin-specific CAR T cells, and they observed that this genetic blockade of PD-1 restored the functions of exhausted CAR T cells [24].

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system emerged as an efficient way to strengthen CAR T cell anti-tumor activities. Ren *et al.* utilized this CRISPR/Cas9 system to generate PD-1-deficient CAR prostate stem cell antigen (PSCA) T cells [132]. They observed that CD137 was upregulated, and CAR T cell anti-tumor activity was enhanced. A similar result was achieved by Rupp *et al.*, who developed PD-1-deficient CAR19 T cells [133] and observed the augmented CAR T cell killing of tumor cells. This CRISPR/Cas9 approach has also been utilized to disrupt the PD-1 gene locus in mesothelin-targeted CAR T cells to enhance tumor control and relapse prevention [134].

In conclusion, this part demonstrates the therapeutic promise of integrated immune checkpoint blockade in CAR T cells in boosting immune responses and enhancing the anti-tumor activity of CAR T cells. Table 2 summarizes the ongoing clinical trials investigating the combination of CAR T cells with immune checkpoint blockade in cancer immunotherapy.

Inhibition of immunosuppressive cytokines

Many cancers produce an immunosuppressive environment by secreting or aberrantly expressing cytokines and their ligands, including TGF- β , IL-4, and colony-stimulating factor-1 (CSF-1), which protect malignant cells from immune destruction and promote tumor formation and

Table 2 Summary of ongoing clinical trials investigating the combination of CAR T cells with immune checkpoint blockade in cancer immunotherapy

Module	Antigen	Phase	Clinical trial ID	Disease condition	Status	Sponsor
Expressing PD-1 antibody	EGFR family	I/II	NCT02873390	Advanced malignancies	Unknown	Ningbo Cancer Hospital
Expressing PD-1 antibody	EGFR family	I/II	NCT02862028	Advanced solid tumor (lung, liver, and stomach)	Unknown	Shanghai International Medical Center
Cytoplasmic activated PD-1	CD19	I	NCT03540303	Refractory/relapsed B cell lymphoma	Recruiting	Henan Cancer Hospital
PD-1 knockout	MUC1	I/II	NCT03525782	Lung cancer, NSCLC	Recruiting	The First Affiliated Hospital of Guangdong Pharmaceutical University
Expressing PD-1 antibody	Mesothelin	I/II	NCT03030001	Solid tumor, adult advanced cancer	Unknown	Ningbo Cancer Hospital
PD-1 knockout	MUC1	I/II	NCT03706326	Esophageal cancer	Recruiting	The First Affiliated Hospital of Guangdong Pharmaceutical University
PD-1 and TCR knocked out	Mesothelin	I	NCT03545815	Solid tumor	Recruiting	Chinese PLA General Hospital
PD-1 knockout	CD19	I	NCT03298828	ALL, Burkitt lymphoma	Not yet recruiting	Third Military Medical University
Expressing CTLA-4 and PD-1 antibodies	EGFR	I/II	NCT03182816	Advanced solid tumor	Unknown	Shanghai Cell Therapy Research Institute
Expressing CTLA-4 and PD-1 antibodies	MUC1	I/II	NCT03179007	Advanced solid tumor	Unknown	Shanghai Cell Therapy Research Institute
Expressing CTLA-4 and PD-1 antibodies	Mesothelin	I/II	NCT03182803	Advanced solid tumor	Unknown	Shanghai Cell Therapy Research Institute
PD-1 knockout	Mesothelin	I	NCT03747965	Solid tumor	Recruiting	Chinese PLA General Hospital
Transduced with a PD-1/CD28 chimera 1 antiviral vector	CD19	I	NCT03932955	Lymphoma	Recruiting	Peking University
Combination with anti-PD-1 antibody (Pembrolizumab)	CD19 and CD22	I/II	NCT03287817	DLBCL, relapsed or refractory DLBCL	Recruiting	Autolus Limited
Expressing PD-1 antibody	Mesothelin	I/II	NCT03615313	Advanced solid tumor	Recruiting	Shanghai Cell Therapy Research Institute
Combination with anti-PD-1 antibody (Pembrolizumab)	EGFRvIII	I	NCT03726515	Glioblastoma	Recruiting	University of Pennsylvania
Incorporation of a PD-1shRNA expressing cassette	CD19	I	NCT03208556	Relapsed or refractory B cell lymphoma	Recruiting	Peking University

Abbreviations: NSCLC, non-small-cell lung carcinoma; ALL, acute lymphoblastic leukemia; DLBCL, diffuse large B cell lymphoma; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; PD-1, programmed cell death protein 1.

progression [135–137]. Thus, to provide a clinical benefit to patients with solid tumors, additional engineering is required to protect CAR T cells from the inhibitory effects of immunosuppressive cytokines.

TGF- β

TGF- β exhibits antagonistic effects on various immune cells [138–140]. For example, TGF- β inhibits CD4 and CD8 T cell activation or cytolytic activity, regulates the polarization of macrophages and inhibits their chemotactic function, inhibits the maturation of DC and NK cells, and induces the apoptosis of B cells [141–143]. Inhibition of the negative effects of TGF- β signaling in the cancer microenvironment effectively promotes the anti-tumor effects. Recently, TGF- β signal inhibition has gained great interest in combination with CAR T cells for tumor treatment.

Morgan’s group in 2013 reported TGF- β signal inhibition in CAR T cells; they genetically modified CAR T cells to secrete soluble TGF- β receptors or TGF- β DNR II (DNRII); TGF- β DNR-modified CAR T cells dramatically improved T cell anti-tumor activity in a melanoma tumor model [144]. However, soluble TGF- β receptors expressed by CAR T cells were weak at blocking the TGF- β activity, and the reason may be the low concentration of expressed receptor or incapability of the soluble receptors to bind with the TGF- β presented on the T cells [145]. Recently, CAR prostate-specific membrane antigen (PSMA) T cells engineered to co-express TGF- β DNR demonstrated enhanced innate and acquired immunity, showing increased proliferation and infiltration, sustained survival and resistance to exhaustion, and enhanced secretion of proinflammatory cytokines. In aggressive human prostate cancer mouse models, TGF- β -inhibited CAR T cells exhibited augmented anti-tumor activity [146]. The above studies suggest that TGF- β DNR expression is an effective way to improve the therapeutic effect of CAR T cells.

Similar to the previously reported PD1:CD28 chimera, Chang *et al.* also generated a CAR composed of a TGF- β binding domain and CD28- ζ stimulatory signaling, thereby effectively converting TGF- β from an inhibitor to a stimulator of T cell growth. TGF- β /CD28 CAR T cells not only retained the cytotoxicity of the T cell itself but also enhanced the anti-tumor efficacy of adjacent T cells and caused no effect on their anti-tumor function by mixing with Treg cells [147]. Based on these results, we believe that CAR T cells expressing TGF- β /CD28 have a promising future in the treatment of solid tumors.

In addition, TGF- β is used to overcome the immunosuppressive environment, and its capability to enhance the tumor-selective effects of CAR T cells has been explored. Recently, Juan and colleagues designed tumor-specific

molecular pattern-activated and regulated T cells, named SmarT-cells, which are T cells engineered with three individual receptors that recognize PSCA, TGF- β , and IL4 and contain intracellular domains important for T cell activation, co-stimulation, and cytokine support. These SmarT-cells selectively and safely enhance the anti-tumor effects [148]. Overall, these data indicate that the mechanisms targeting TGF- β signaling can be combined in various ways with CAR T cells to boost their anti-tumor activity.

IL-4

As a cancer immunotherapeutic agent, IL-4 is highly expressed in a variety of tumors, where it has been reported to promote tumor cell growth and protect tumor cells from apoptosis [136,149,150]. Under physiologic conditions, IL4 inhibits the tumor-directed Th1 polarization response and induces the Th2 immune response [151,152]. IL-4 exposure also reduces the cytotoxicity of NK cells in *in vitro* and *in vivo* models [153,154]. Based on this evidence, IL-4 provides a selective growth signal for regulating CAR T cell expansion and tumor cytolytic activity.

Wilkie and colleagues created 4 $\alpha\beta$ (with the ectodomain of IL-4R fused to the endodomain of the shared IL-2/15 β c subunit)-expressing MUC1-targeted CAR T cells and showed that IL-4 drove the antigen-targeted expansion and potent anti-tumor activity of these CAR T cells. Furthermore, 4 $\alpha\beta$ has been co-expressed with a CAR recognizing two additional tumor-associated targets (PSMA and ErbB), and similar results were observed [155]. This finding was further verified by Mohammed and colleagues [156], who co-expressed a IL-4/IL-7-inverted cytokine receptor (4/7 ICR) generated by fusing the exodomain of the IL-4 receptor with the endodomain of the IL-7 receptor on CAR-PSCA cells; these 4/7 ICR co-expressing CAR-PSCA T cells exhibited enhanced anti-tumor effects in pancreatic cancer (as characterized by elevated IL-4 levels and PSCA overexpression) [157,158]. Collectively, these findings provide proof of principle for the development of IL-4-enhanced CAR T cells for cancer immunotherapy.

CSF-1

CSF-1, also known as macrophage CSF, is a cancer-associated cytokine that is overexpressed in many cancers, such as ovarian cancer, breast cancer, and classical HL, and is correlated with high grade and poor prognosis [159,160]. CSF-1 regulates the proliferation and differentiation of monocytic lineage cells [161] through its cell surface receptor (CSF-1R). However, CSF-1 shows no direct effects upon T cells lacking CSF-1R expression. Therefore, engineering T cells to respond positively to

CSF-1 might provide a useful means to amplify the anti-tumor immune response. To examine the CSF-1 responsiveness, Maher and colleagues [162] expressed CSF-1R in human T cells. Soluble CSF-1 synergistically enhanced T cell proliferation and activation in response to IL-2. In further examinations of whether activation of CSF-1R could enhance the function of CAR T cells, PSMA-specific CAR T cells co-expressing CSF-1R significantly augmented the PSMA-dependent expansion of T cells and preserved tumor cytolytic activity. These data support the potential of CAR T cells to be genetically modified to acquire responsiveness to CSF-1 and provide proof of principle for a novel strategy to enhance the effectiveness of CAR T cell immunotherapy.

Overall, the data presented here establish that CAR T cells can combine with checkpoint and tumor-derived cytokines to obtain potent co-stimulation of the immune response and enhance their anti-tumor activity in solid tumors.

Combination with oncolytic virotherapy

OVs mediate their anti-tumor effects through a dual mechanism: selectively lysing cancer cells [163] and providing viral risk signals to enhance effector cell expansion and function [164,165]. Several multi-oncolytic virotherapies have received the FDA approval [166]. Currently, renewed attention is focused on selectively expressing OVs in the TME to boost the anti-tumor immune response [167–171]. The combination of OVs with immune checkpoint inhibitors has produced high response rates in patients with advanced melanoma [172–175].

OVs also possess the potential to synergize with CAR T cells to mitigate the challenges in the treatment of solid tumors. First, OVs facilitate the trafficking, proliferation, and persistence of CAR T cells in the TME. Second, OVs promote the release of TAAs to induce an adaptive immune response. Moreover, OVs directly enhance the anti-tumor functions of CAR T cells harboring therapeutic transgenes, including those encoding cytokines [176,177], chemokines

[178], and immune checkpoint inhibitors [179–181]. Here, we discuss recent strategies to explore the synergy between CAR T cells and OVs in cancer treatment.

OVs were modified to deliver intratumoral cytokines or chemokines to enhance CAR T cell therapy. June's group [182] reported that oncolytic adenovirus expressing TNF- α and IL-2 robustly increased meso-CAR T cell and host T cell infiltration into the tumor, overcame the immunosuppressive TME, and increased the DC maturation; these results indicate that combining with cytokine-expressing OVs can enhance the efficacy of CAR T cell therapy in solid tumors. Studies have also examined the ability of chemokine-expressing OVs in CAR T cells; a modified oncolytic vaccinia virus expressing CXCL11 successfully recruited T cells and significantly enhanced the anti-tumor efficacy of meso-CAR T cells [183]. This finding was further demonstrated by Dotti *et al.*, who combined CAR GD2-T cells with OVs expressing the chemokine RANTES and cytokine IL-15, facilitating the migration and survival of CAR T cells and increasing their overall anti-tumor activity in tumor-bearing mice. Checkpoint inhibitors are a natural choice for combination with OVs in cancer therapy [184]. An oncolytic adenovirus expressing a PD-L1 minibody enhanced the anti-tumor effect of HER2 CAR T cells against human solid cancer cells [185]. A similar study created an oncolytic adenovirus construct encoding anti-PD-L1 antibodies and IL-12 and showed augmented therapeutic efficacy of CAR HER2 T cells in primary and metastasized head and neck squamous cell tumors [186].

Moreover, CAR T cells can augment OV anti-tumor function. CAR T cells have been used as a carrier to deliver OVs to the tumor site to protect the viruses from neutralizing antibodies. Various studies have been performed to determine whether CAR T cells could be loaded with OVs and effectively create a dual-pronged anti-tumor agent [187,188]. Bramson *et al.* [189] demonstrated that murine and human CAR T cells can deposit viruses into the TME and effectively enhance the anti-tumor responses of both approaches. Table 3 summarizes the ongoing clinical trials investigating the combination of CAR T cells with OVs in cancer immunotherapy.

Table 3 Summary of ongoing clinical trials investigating the combination of CART cells with OVs in cancer immunotherapy

Module	Antigen	Phase	Clinical trial ID	Disease condition	Status	Sponsor
Oncolytic adenovirus	HER2	I	NCT03740256	Bladder cancer, HNSCC, salivary gland cancer, lung cancer, breast cancer, gastric cancer, ESCA, colorectal cancer, pancreatic adenocarcinoma	Not yet recruiting	Baylor College of Medicine

Abbreviations: HNSCC, head and neck squamous cell carcinoma; ESCA, esophageal cancer.

Combination with chemotherapy

In addition to reducing the tumor burden, chemotherapeutic agents perform considerable immunomodulatory functions. On the one hand, chemotherapeutic agents can stimulate tumor cells to release ATP or damage-associated molecular patterns, which increase the recruitment of DCs and T lymphocytes to infiltrate into tumor sites and promote the maturation and activation of DCs. On the other hand, chemotherapeutic agents may remodel the TME by selectively inhibiting the activity of immunosuppressive cells (such as Treg cells and MDSCs). Therefore, combining CAR T cells with chemotherapeutic agents may be a promising strategy to boost the anti-tumor efficacy of CAR T cell immunotherapy.

Numerous studies have confirmed this effect. For example, a preclinical study by Maher's group demonstrated that noncytotoxic doses of carboplatin sensitized tumor cells to ErbB-targeted CAR T cells and enhanced the efficacy of the anti-tumor response [190]. Pretreatment chemotherapy can counteract the potential immunogenicity of T cells and prolong the persistence of T cells [191]. In addition, chemotherapeutic agents, such as cyclophosphamide (CTX) and fludarabine, have been frequently utilized as a preconditioning regimen to deplete lymphocytes before infusion of CAR T cells in various clinical trials [192–197]. Brentjens' group revealed that the persistence of CAR19 T cells was significantly enhanced in patients with prior administration (2 days) of a relatively high dose of CTX [198]. Recent studies by Curran's group suggest that minimal pretreatment with CTX positively affects the CAR19 T cell response without negatively affecting the toxicity [192].

In conclusion, chemotherapeutic agents may be a feasible adjuvant for use with CAR T cell immunotherapy in the treatment of solid tumors. Further research on this combination strategy may provide an opportunity to harness the full potential of CAR T cells in the treatment of solid tumors.

Combination with radiotherapy

Recently, radiotherapy has been proposed as a popular adjunct therapy to improve the efficacy of tumor immunotherapy [199]. Radiotherapy not only can kill tumor cells directly but can also partially induce tumor-specific immune responses by heightening CTL recognition mediated through increased cell surface expression of MHC class I molecules and improved presentation of new peptides. In addition, radiotherapy leads to the release of proinflammatory cytokines and chemokines to moderate the TME. Thus, combining CAR T cell therapy with radiotherapy could lead to further improvements in CAR T cell therapy [200].

Preclinical studies by Weiss and colleagues [201] showed that radiotherapy resulted in synergistic activity in an orthotopic mouse model of glioblastoma by promoting the migration of NK group 2 member D (NKG2D)-targeted CAR T cells to the tumor site and increasing the effector functions. They also observed that radiation significantly induced the expression of NKG2D ligands on malignant cells, possibly explaining the synergy between the two methods described above. DeSelm and colleagues [202] further confirmed that low-dose radiation conditioning sensitizes tumor cells (not only sLeA⁺ but also sLeA[−]) to CARsLeA T cells in a model of heterogeneous pancreatic adenocarcinoma.

This finding suggests that the combination of radiotherapy and CAR T cell therapy can overcome antigen-negative tumor escape and effectively eliminate heterogeneous tumors. The above studies provide a rationale for translating this combined immunotherapeutic strategy into clinical trials. Qu and colleagues [203] pretreated patients with radiotherapy or chemotherapy before infusion of CAR T cells for relapsed or refractory diffuse large B cell lymphoma and noted that radiotherapy is a safe and promising approach to enhance the efficacy of CAR T cell therapy. In conclusion, radiotherapy is an effective agent in combination with CAR T cells in solid tumors.

Combination with biomaterials

Given the success of immunotherapy, increasing research has focused on combining biomaterials with immunotherapy to enhance the efficacy against solid malignancies. Biomaterials have previously been designed as carriers to deliver patient-specific irradiated tumor cells, tumor lysates, or other stimulating signals to the local regions [204–206]. Currently, biomaterials are used to address the shortcomings of CAR T cells in cancer immunotherapy. Biomaterials not only improve the expansion of T cells *in vitro* but also enhance their *in vivo* efficacy. One of the important methods is the delivery of immunomodulating agents to recruit and facilitate the immune response against tumors. Nanoparticles are attractive biomaterials used by researchers to help in CAR T cell immunotherapy.

Nanoparticles were used to transport immunoagonists to the tumor site and stimulate anti-tumor effector cells prior to CAR T cell therapy in a breast cancer model [207,208]. Stephan *et al.* used lipid nanoparticles containing a potent drug cocktail to create a therapeutic window, and infusing CAR T cells during this time frame enhanced the infiltration and robust expansion of CAR T cells and ultimately augmented the anti-tumor effect [209]. These findings were further confirmed in an immunotherapy-resistant mouse model, in which nanoparticle preconditioning was conducive to CAR T cell therapy [209–212]. Smith's group further demonstrated that codelivery of

STING agonists with CAR T cells in an alginate-based scaffold approach rendered CAR T cells with the ability to eradicate tumors effectively and simultaneously stimulated the endogenous immune response to eliminate tumor cells not recognized by CAR T cells [204].

Outlook

Utilizing CAR T cells to fight against malignancies marks the beginning of a new era of cancer treatment, and the remarkable success against hematologic malignancies is encouraging, supporting a proof-of-principle system to extend this treatment paradigm to other cancers. However, given the complexity of solid tumorigenesis, optimized CAR T cells are insufficient to overcome all the obstacles presented by solid tumors. Therefore, the combination of a variety of modifications to compensate for the shortcomings of CAR T cells and resolution of the challenges posed by solid tumors are necessary. In addition, mobilizing endogenous immune cells is necessary for CAR T cells to achieve the best therapeutic effect in solid tumors.

In this regard, the combinatorial approaches of CAR T cells have demonstrated tremendous potential in various preclinical solid models, several of which are already being transformed into clinical therapies. This promising combinatorial method shows the potential to stimulate immune cell activation and proliferation while counteracting immunosuppressive signals. However, the vigorous CAR T cells might seriously damage normal host tissues and lead to severe toxicities. Recently, different strategies have been developed to improve the safety of CAR T cells, including the identification of predictive biomarkers, design of safety switches, and exploration of novel ways to mitigate toxic drugs.

CAR T cells have matured from experimental studies to effective treatment options for patients with poor prognosis. However, this field is still in its infancy, and further research is needed to fully launch the potential of CAR T cells as a successful cancer treatment.

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Compliance with ethics guidelines

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