THE NOVEL ENGINEERING STRATEGIES AND CLINICAL PROGRESS OF SOLID TUMOR IN CAR-T CELL THERAPY

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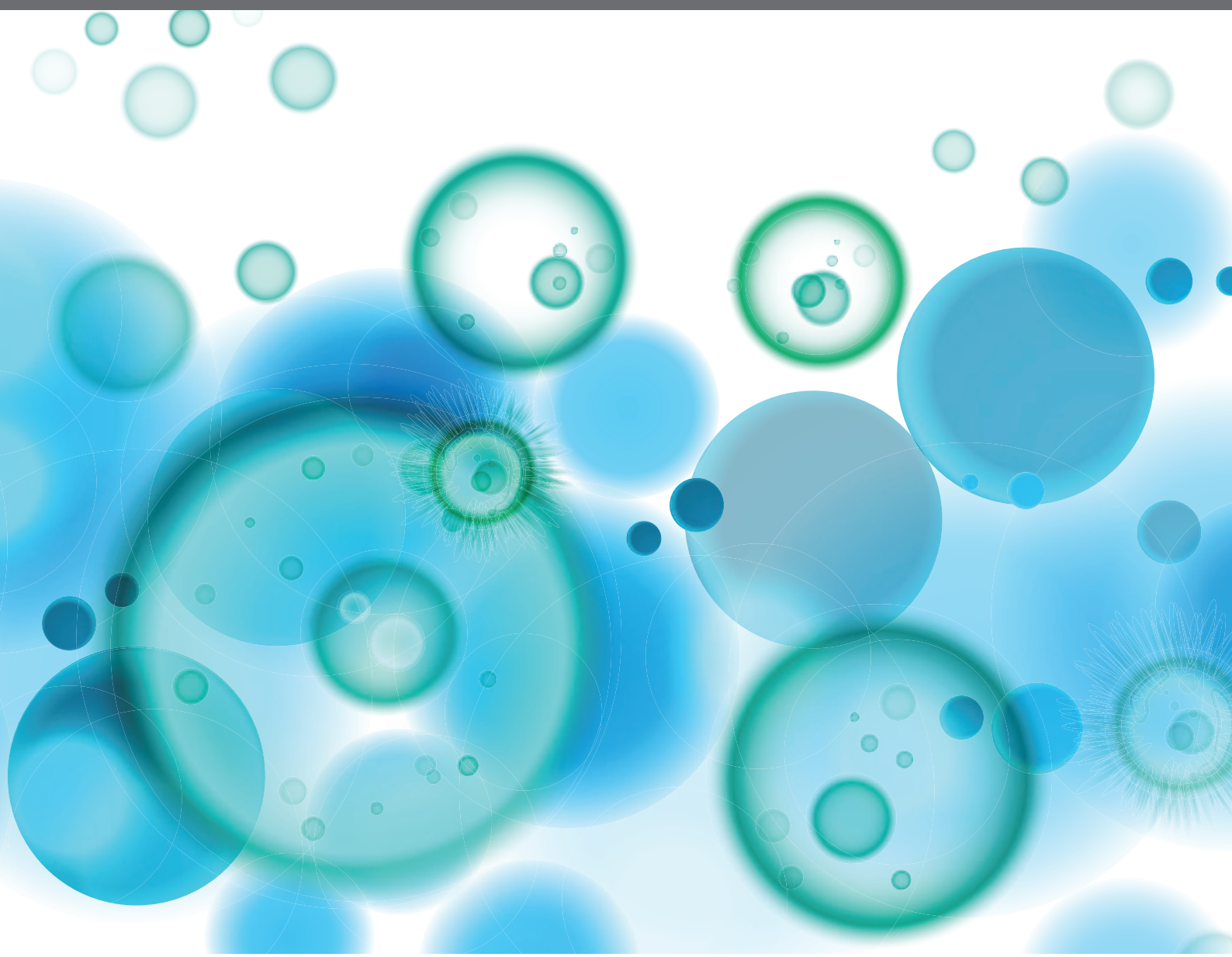

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THE NOVEL ENGINEERING STRATEGIES AND CLINICAL PROGRESS OF SOLID TUMOR IN CAR-T CELL THERAPY

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Reducing Hinge Flexibility of CAR-T Cells Prolongs Survival *In Vivo* With Low Cytokines Release

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Chimeric antigen receptor (CAR)-modified T cells targeting CD19 demonstrate unparalleled responses in B cell malignancies. However, high tumor burden limits clinical efficacy and increases the risk of cytokine release syndrome and neurotoxicity, which is associated with over-activation of the CAR-T cells. The hinge domain plays an important role in the function of CAR-T cells. We hypothesized that deletion of glycine, an amino acid with good flexibility, may reduce the flexibility of the hinge region, thereby mitigating CAR-T cell over-activation. This study involved generating a novel CAR by deletion of two consecutive glycine residues in the CD8 hinge domain of second-generation (2nd) CAR, thereafter named 2nd-GG CAR. The 2nd-GG CAR-T cells showed similar efficacy of CAR expression but lower hinge flexibility, and its protein affinity to CD19 protein was lower than that of 2nd CAR-T cells. Compared to the 2nd CAR-T cells, 2nd-GG CAR-T cells reduced proinflammatory cytokine secretion without diminishing the specific cytotoxicity toward tumor cells *in vitro*. Furthermore, 2nd-GG CAR-T cells prolonged overall survival in an immunodeficient mouse model bearing NALM-6 when tumor burden was high. This study demonstrated that a lower-flexibility of CD8α hinge improved survival under high tumor burden and reduced proinflammatory cytokines in preclinical studies. While there is potential for improved safety and efficacy, yet this needs validation with clinical trials.

Keywords: chimeric antigen receptor (CAR T), hinge region, cytokine release storm (CRS), structure optimization, cellular immunotherapy, gene modified T cell

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INTRODUCTION

Chimeric antigen receptor T cell (CAR-T) therapy for hematological malignancies has demonstrated tremendous clinical outcomes (1, 2). Four CAR-T cell products have been approved globally, including Kite's Yescarta and Tecartus, Novartis's Kymriah, and BMS's Breyanzi, all targeting CD19 antigen (2–4). However, a high tumor burden often indicates poor prognosis and significant adverse reactions after CAR-T therapy, which may be related to the over-activation of CAR-T cells (5–8). Therefore, patients with a high tumor burden have an unmet medical need for anti-CD19 CAR-T therapy.

Investigators are currently striving to improve the safety and efficacy of CAR-T cells by optimizing CAR designs to overcome their existing limitations (9). These include cytokine release syndrome (CRS) and immune-effector cell associated neurotoxicity syndrome (ICANS), both related to the excessive release of cytokines and limited persistence caused by activation-induced cell death (AICD) (10–15). The standard CAR design consists of four modular components: the antigen binding domain, hinge domain, transmembrane domain, and intracellular signaling domain, each of which has a specific function and thus the potential to be optimized (16). More attention has been paid to the improvement of signal regions, including antigen recognition and signaling argument regions such as the costimulatory domain and immunoreceptor tyrosine-based activation motif (ITAM) of CD3ξ (17, 18).

In recent years, a growing number of studies have demonstrated the significant function of non-signaling regions. The properties of the hinge and transmembrane domains also influence CAR-T cell cytokine production and AICD (19), which are related to the anti-tumor efficacy and the loss of CAR, respectively (20, 21). Ying et al. (22) constructed a new CAR design with longer extracellular and intracellular domains named CD19-BBz (86) CAR T cells, which produced a potent and durable anti-lymphoma response without causing neurotoxicity or severe CRS (greater than grade 1). The hinge provides sufficient flexibility to overcome steric hindrance, and length to facilitate access to the target antigen (23). It thus seems reasonable to down-regulate the activation of CAR-T cells by reducing the flexibility of the hinge region, thereby improving efficacy and safety. Glycine, the smallest amino acid is unique because unlike all others, it contains hydrogen as its side chain rather than a carbon (24), permitting much more conformational flexibility. $(Gly_4Ser)_n$ is often used as a linker for different polypeptides because it is not prone to misfolding errors, and Gly plays an irreplaceable role in this structure (25).

Consequently, this study entailed designing a novel CAR by deleting two consecutive glycine residues in the CD8 hinge domain of traditional second-generation (2nd) CAR and named the FMC63-CD8(Gly2-deletion)-4-1BB-CD3ζ CAR as 2nd-GG CAR. Studies were then conducted to verify the flexibility and affinity of this new CAR, and compare the functions of 2nd and 2nd-GG CAR-T cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions

Cell lines were cultured according to the manufacturers' recommendations. NALM-6 is a pre-B cell acute lymphoblastic leukemia (ALL) cell line with high expression of CD19 (German DSMZ cell collection Cat#: ACC128). NALM-6-GFP-luciferase (luc) is a stable cell line engineered to express GFP-luciferase. K562 is a chronic myelogenous leukemia cell line (ATCC; Cat#: CCL-243). K562-CD19 and K562-CD19-GFP are stable cell lines engineered to express CD19 and/or GFP. 786o is a renal cell adenocarcinoma cell line (ATCC; Cat#: CRL-1932™). CD19 was transduced using a lentivirus system into 786o to produce 786o-CD19. The method of tumor cells culture refers to our previous study (26).

Generation of CAR Constructs

Generation of lentiviral constructs and production of lentiviral particles refer to our previous study (27). The conventional second-generation 2nd CAR was constructed by the fusion of CD19 scFv, CD8 hinge and transmembrane, 4-1BB, and CD3ζ. The structure of 2nd-GG is same to the 2nd CAR except for deletion of two consecutive glycine in the CD8 hinge. Nucleotide sequence of CD8 hinge in 2nd-CAR and 2nd-GG CAR are shown in **Supplementary Figure 2**.

Selection, Activation, and Lentivector Transduction of CD3+ T Cells

Blood samples from healthy volunteers were obtained using an approved protocol by the Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital (Ethical code: Ky-2018-5-37). These studies were conducted following the Declaration of Helsinki. All subjects provided written informed consent before participation in the present study. The methods of T cell isolation and culture and gene transfer refer to our previous study (26).

Binding Assay

Briefly, through the measurement of the fluorescence intensity of different CAR T cells to CD19 protein at various concentrations, their affinity for CD19 protein can be determined. Specifically, mock-T, 2nd CAR-T, and 2nd-GG CAR-T cells were washed twice by centrifugation with PBS (1% BSA). They were treated with CD19-Fc protein (11880- H02H) at final concentrations of 180 μg/mL, 72 μg/mL, 28.8 μg/mL, 11.52 μg/mL, 4.61 μg/mL, 1.84 μg/mL, 0.74 μg/mL, 0.29 μg/mL, 0.12 μg/mL, or 0.05 μg/mL, incubated at 4° C in darkness for 45 min, and washed twice with a PBS washing solution by centrifugation. Next, the cells were treated with 10 μL goat anti-human IgG (FC)/FITC, incubated at 4°C in darkness for 20 min, washed twice with a washing solution by centrifugation, and tested utilizing flow cytometry (NovoCyte D3010).

Cytotoxicity Assay

Briefly, CFSE-labeled targets were incubated at the indicated ratios with effector T cells for 12–16 h or 6–8 h. The cells were then harvested, and Annexin V and 7-AAD were added prior to flow cytometric analysis. The residual live target cells were CFSE+

Annexin V- 7-AAD-. E:T ratios designated the ratios of the absolute number of CAR T cells to target cells. The number of T cells was the same as that in the 2nd CAR group. All experiments were carried out in triplicate.

Cytokine Production

Effector cells (5×10^4) and target cells (5×10^4) were incubated at a 1:1 ratio in RPMI (10% FBS) media with 10% human serum for 24 h. Cytokine concentration in the culture supernatant and mouse serum was measured with enzyme-linked immunosorbent assay (ELISA) kits (MultiSciences Biotech Co., Ltd., China) for human IFN-γ, TNF-α, and IL-2. E:T ratio designated the ratio of the absolute number of CAR T cells to target cells. The number of T cells was the same as that in the 2nd CAR group.

Flow Cytometry

Anti-human antibodies were purchased from Becton Dickinson, BioLegend, and Miltenyi Biotec. The Accuri C6 (Becton Dickinson, USA), FACS Calibur (Becton Dickinson, USA), and BD FACSAriaTM II cell sorter were used for the analysis of various samples. Anti-human antibodies were purchased from BioLegend, eBioscience, Acrobiosystems, or BD. Cells were isolated from *in vitro* cultures or from animals, washed once with PBS supplemented with 2% FCS, and stained on ice after blocking Fc receptors. In all analyses, the population of interest was gated based on forward vs. side scatter characteristics followed by singlet gating.

Mouse Xenograft Tumor Model

Animal experiments were conducted at the National Beijing Center for Drug Safety Evaluation and Research and at the SAFE Pharmaceutical Research Institute Co.,Ltd (IACUC-2019-001). Female NSG mice (28) aged 6–8 weeks were used. For NALM-6-acute precursor B-ALL models, 10^6 tumor cells were intravenously injected with PBS, and tumors were measured by the total bioluminescent flux using a Xenogen Imaging System (PerkinElmer-IVIS Lumina III). Peripheral blood was collected *via* the submandibular vein.

Statistical Analysis

Statistical analyses were performed using Prism version 7.0 (GraphPad). For studies comparing two groups, we utilized a Students t-test. Log rank (Mantel Cox) test was used to analyze *in vivo* survival. Survival curves were constructed using Kaplan–Meier methodology.

RESULTS

Deletion of Gly-Gly in CD8 Hinge Region of CAR Reduced the Flexibility of Hinge Without Affecting the CAR Expression Efficiency

The 2nd CAR-T cells, structured as FMC63-CD8-4-1BB-CD3ζ, have shown promising efficacy in clinical studies (1). To decrease the flexibility of the hinge region, deletion mutations were performed on two consecutive Glys in the wild-type CD8 hinge region of FMC63-CD8-4-1BB-CD3ζ CAR, and this novel CAR was named 2nd-GG CAR (**Figure 1A**). The specific nucleic acid sequences of the wild CD8 hinge region and the CD8 hinge region with deletion of 2 Gly are shown in **Supplement Figure 1**. The transduction efficiency of 2nd CAR and 2nd-GG CAR on human T cells was similar (approximately 70%) (**Figures 1B, C**). The S2 order parameters represent the restriction of movement of an atomic bond vector with respect to the molecular reference frame. The greater the value of S2, the less flexible the protein. Thus, the flexibility of the CD8-GG hinge region was less than that of the CD8 hinge region according to the index of S2 from DynaMine (29) (**Figure 1D**). Furthermore, when the two CAR-T cells were individually incubated with different concentrations of CD19 protein, the 2nd-GG CAR-T cells showed weaker binding ability to CD19 protein than 2nd CAR-T cells (**Figure 1E**).

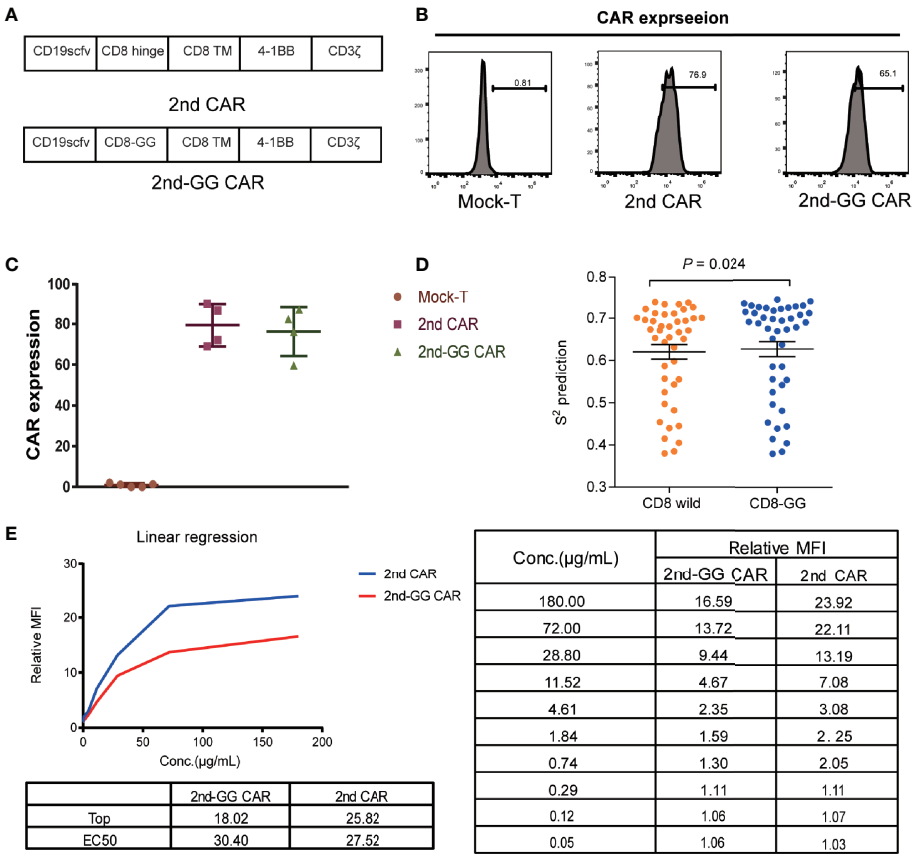
2nd-GG CAR-T Cells Showed Similar Killing Efficiency but Secreted Less Proinflammatory Cytokines Compared to 2nd-GG CAR-T Cells *In Vitro*

To evaluate the effector function of the two different CAR-T cells, a killing (cytotoxicity) and cytokine secretion assays were conducted on different cell lines. These were: NALM-6, a precursor B-cell leukemia cell line that naturally expresses CD19, plus the 786o and K562 cell lines which are CD19 negative (**Figure 2**). The two CAR-T cells showed similar cytotoxic efficacy against the CD19-positive and negative cell lines, with no statistically significant differences.

It is well known that cytokines secreted from CAR-T cells trigger an overactivation of the immune system, ultimately leading to CRS (30). We therefore examined the pro-inflammatory factors released after the incubation of CAR T cells with different tumor cells. Following incubation with CD19+ target cells, the amount of proinflammatory cytokines secreted by 2nd-GG CAR-T cells was less than that of 2nd-GG CAR-T cells ($P<0.01$). None of the CAR-T cells produced specific killing effects or proinflammatory factors against K562, a CD19- tumor cell line, demonstrating the antigen-specificity towards CD19 by the 2nd-GG CAR-T cells.

2nd-GG CAR-T Cells Exhibited Similar Antitumor Efficacy but Less Proinflammatory Cytokines Release in Mouse Model With Moderate Tumor Burden

Although 2nd-GG CAR-T cells showed a similar specific immune response to CD19+ tumor cells *in vitro* compared with 2nd CAR-T cells, their antitumor efficacy in animal models needs to be further verified. The anti-tumor efficacy of CAR-T cells in NSG immunodeficient mice bearing NALM-6-GFP-luc(luciferase) was subsequently investigated, as detailed in **Figure 3A**. Both 2nd-GG and 2nd CAR-T cells exhibited improved overall survival (OS) and reduced tumor burden compared with the mock-T cells, demonstrating improved tumor control of both CAR-T cells



	2nd-GG CAR	2nd CAR
Top	18.02	25.82
EC50	30.40	27.52

Conc.(μg/mL)	Relative MFI	
	2nd-GG CAR	2nd CAR
180.00	16.59	23.92
72.00	13.72	22.11
28.80	9.44	13.19
11.52	4.67	7.08
4.61	2.35	3.08
1.84	1.59	2. 25
0.74	1.30	2.05
0.29	1.11	1.11
0.12	1.06	1.07
0.05	1.06	1.03

FIGURE 1 | Schematic diagram and expression efficiency of 2nd and 2nd-GG CAR-T cells. **(A)** Diagrammatic model of 2nd and 2nd-GG CAR. Schematic of CAR containing scfv (FMC63), variations in the hinge, extra-membrane, and transmembrane domains. The hinge region of 2nd-GG deleted two Gly compared with that of the 2nd CAR, and the rest of the sequences were the same. **(B)** Typical flow cytometry detection of the expression efficiency of 2nd and 2nd-GG CAR on T cells. **(C)** Expression efficiency of 2nd and 2nd-GG on T cells 5-6 days after culture *in vitro* determined by flow cytometry (mean ± SD, n = 5). T cells are derived from at least three different healthy donors. **(D)**. Comparison of the flexibility between the CD8 hinge and the CD8-GG hinge. S2 order parameter (S2 RCI) values were estimated from chemical shift values using the Random Coil Index (RCI) software. S2 is inversely proportional to the hinge region flexibility. **(E)**. The affinity of CD19 protein to different CAR T cells: 2nd CAR-T cells > 2nd-GG CAR-T cells. The EC50 of 2nd and 2nd-GG CAR-T cells binding to CD19 protein was determined by flow cytometry. EC50, 50% maximal effective concentration. CAR, chimeric antigen receptor; FITC, fluorescein isothiocyanate.

(**Figures 3B, D**). Furthermore, compared to the 2nd CAR-T cell group, the OS in those administered 2nd-GG CAR-T cells was prolonged, although there was no statistical difference, as shown in **Figure 3C**. As expected, 2nd-GG CAR-T cells secreted less human proinflammatory cytokines, particularly IL-6 and IFN-γ, compared to the 2nd CAR-T cells *in vivo* (**Figure 3E**). In order to distinguish it from the following experiment with a higher tumor burden, this experiment was referred to as "with moderate tumor load". The 2nd-GG CAR-T cells did not show sufficient advantage compared to the 2nd CAR-T cells in experiments with moderate tumor burden, owing to the relatively lower tumor load.

2nd-GG CAR-T Cells Significantly Improved Antitumor Activity in Mouse Model With High Tumor Burden

A high tumor burden often indicates a poor prognosis and significant adverse reactions after CAR-T therapy (31). It is suggested that a high tumor burden might affect the efficacy of CAR-T cell therapy (32, 33). It was thus hypothesized that CAR T cells behave differently in mouse models with different tumor burdens. To mimic the clinical situation of a high tumor burden, NSG mice bearing NALM6-Luc tumors received delayed CAR-T cell infusion to increase the tumor load. The specific schedule is shown in **Figure 4A**. When NSG mice were challenged with high tumor burden, 2nd-GG CAR-T cells showed significantly improved overall survival compared with 2nd CAR-T cells, while the 2nd CAR-T cells showed no advantage over the mock-T cells (**Figures 4B, D**). The tumor load in group of 2nd-GG CAR-T was lower than that of 2nd CAR T (P>0.05) on day 15 and showed a downward trend (**Figure 4C**). The anergy of 2nd CAR-T cell in the mouse model with high tumor load is likely related to AICD. One mouse from each group was randomly selected on day 14, to evaluate the tumor load of peripheral blood (PB), bone marrow (BM), and spleen by flow cytometry.

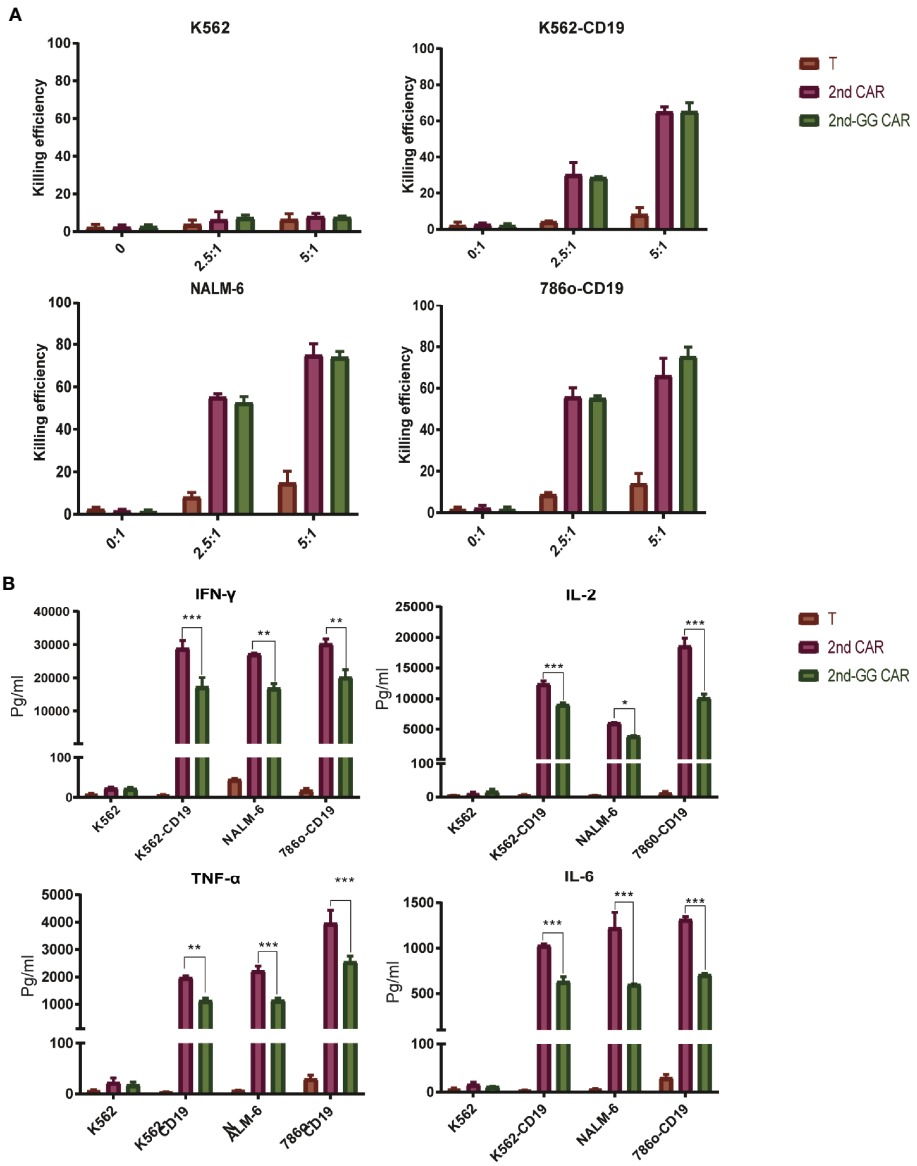


FIGURE 2 | The killing efficiency and cytokine secretion of 2nd CAR-T and 2nd-GG CAR-T cells towards tumor cells. **(A)** Cytotoxic percentages of targeted cells by mock T, 2nd and 2nd-GG CAR-T cells after 8–10 h of co-culture *in vitro*. E: T (2.5:1 and 5:1) designate the ratios of the absolute number of CAR T cells to target cells, specifically K562, NALM-6, 786o-CD19, and K562-CD19. The number of mock T cells is the same as in the 2nd CAR-T cells group. Results are representative of at least three independent experiments with T cells from different healthy donors. **(B)** Human IFNγ, TNF-α, IL-2 and IL-6 production by mock T, 2nd and 2nd-GG CAR-T cells. Cytokine concentrations in the media were measured after 24 h of co-incubation with different target cells at E: T of 1:1. Values are mean ± SD of triplicate specimens obtained with T cells isolated from one healthy donor. *P < 0.05; **P < 0.01; ***P < 0.005.

The results showed that the tumor burden of the 2nd-GG group was less than that of the other two groups after treatment (**Figure 4E**). Similarly, the amount of human proinflammatory cytokines secreted by 2nd-GG CAR T cells was lower than that of 2nd CAR T cells (**Figure 4F**).

Overall, 2nd-GG CAR-T cells exhibited stronger antitumor activity and lower cytokine release in the high tumor burden model than the 2nd CAR-T cells.

DISCUSSION

This study demonstrated that 2nd-GG CAR exhibits lower flexibility and affinity for the CD19 antigen. The 2nd-GG CAR-T cells produced lower levels of cytokines, yet showed similar cytotoxicity to CD19+ tumor cells as 2nd CAR-T cells *in vitro*. However, 2nd-GG CAR-T cells show lower cytokine release in mouse models with moderate and high tumor burden,

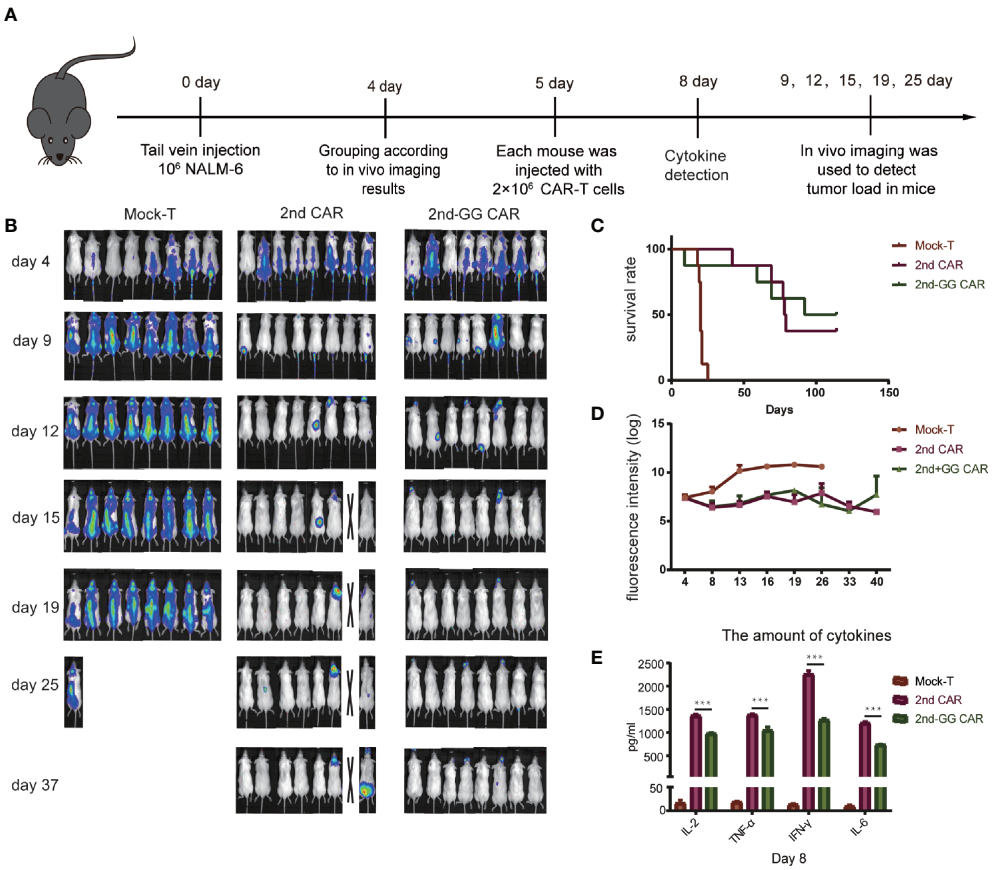


FIGURE 3 | The antitumor efficacy and cytokines release of different CAR-T cells in moderate tumor load models. **(A)** Diagrammatic representations of the experimental procedures. **(B)** Representative bioluminescent images are shown. **(C)** Overall survival curves of NALM-6 -GFP-luc challenged mice (n = 8). **(D)** Tumor burden-total flux (log) for each mouse was quantified and averaged by group. (mean ± SEM) **(E)** On day 8, approximately 1,000 μL of blood were collected from the caudal vein of each mouse mixed to detect the concentration of human IL-2, TNF-α, IFN-γ, and IL-6 using an ELISA-kit. (mean ± SD, n = 2). ***P < 0.005.

and prolong overall survival in animal models with high tumor burden.

Currently, the indication for anti-CD19 CAR T cells has been mainly for relapse and refractory B-cell malignancies, which are often insensitive to traditional radiotherapy and chemotherapy. Furthermore, an inevitable vein-to-vein time interval, typically 3-8 weeks, is required for patients preparing for CAR-T cell therapy. Pivotal trials of approved treatments have resulted in up to a third of the enrolled patients failing to receive the product. It has not been determined if bridging therapy is necessary during this gap, and which treatment regimen may be better (34). Although off-the-shelf cell therapy or Fast CAR-T cells may shorten the vein-to-vein time interval, it is still under clinical study (35). Therefore, the high tumor burden in patients before CAR-T cell therapy is an unavoidable problem. It has been reported that both the efficiency and the incidence of adverse reactions, such as CRS of the anti-CD19 second-generation CAR T cells, increased in patients with high tumor burden (36–38). Many studies have demonstrated that reduced activation of anti-CD19 CAR-T cells improves the safety and efficiency of CAR-T cells (22). This could be achieved through reducing anti-CD19 CAR T cell activation by diminishing scFv affinity (39), increasing the hinge and transmembrane region (22), replacing the co-stimulatory molecule from CD28 to 4-1BB (18), and mutation of the immunoreceptor tyrosine-based activation motif (ITAM) region of CD3ζ (40).

The hinge region has a significant impact on the function of CAR T cells, and its components are often derived from the IgG family or the co-receptor of T cells (CD4/CD8) (41), but the specific mechanism is still unclear (9). Studies have shown that the hinge region provides a spatial location for the recognition of scFv and antigens. When the epitope recognized by CAR is in a membrane proximal position, the hinge region is necessary for the recognition of CAR-T cells by antigens, such as when targeting NCAM or 5T4. Whereas if the epitope recognized by CAR is a membrane distal epitope, the hinge region is negligible for the recognition of CAR-T cells by antigens, such as when targeting CEA (42). In general, little is known about the role of the hinge domain, and strategies to optimize it need to be creatively explored.

The flexibility of the hinge region has been shown to affect the CAR T cell function. The addition of a flexible IgG hinge instead

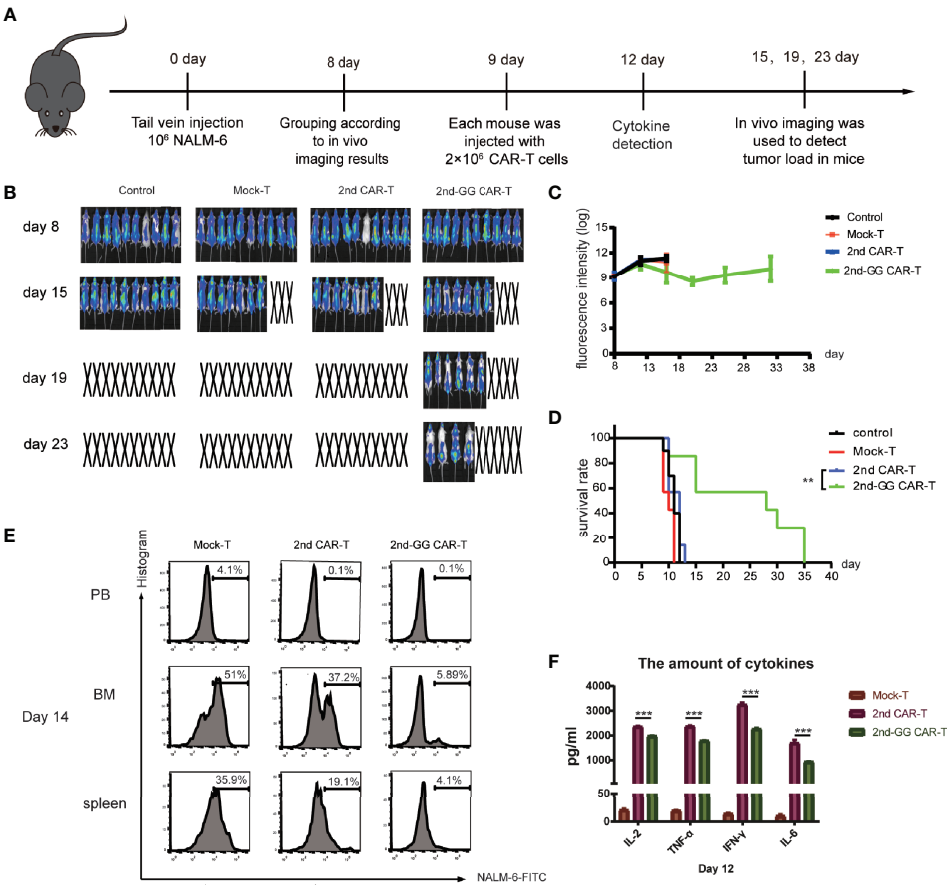


FIGURE 4 | The antitumor efficacy and cytokines release of different CAR-T cells in high tumor load models. **(A)** Diagrammatic representations of the experimental procedures. **(B)** Representative bioluminescent images are shown. **(C)** Overall survival curves of NALM-6 -GFP-luc challenged mice (n = 8). **(D)** Tumor burden-total flux (log) for each mouse was quantified and averaged by group. (mean ± SEM) **(E)** On day 14, one mouse was randomly euthanized from the Mock-T, 2nd CAR-T and 2nd-GG CAR-T groups. Cell suspensions from peripheral blood, bone marrow and spleen were collected and ground for flow cytometry detection. Since the NALM-6 cells were engineered to express GFP, the tumor load was reflected by the expression percentage of GFP+ cells. **(F)** On day 12, approximately 1,000 μL of blood were collected from the caudal vein of each mouse to detect the concentration of human IL-2, TNF-α, IFN-γ, and IL-6 using an ELISA-kit. (mean ± SD, n = 2). **P < 0.01, ***P < 0.005.

of a CD28 hinge alone (SD28ζ) led to more pro-cytokines produce and better recognition of the MUC1 epitope compared to S28ζ CAR-T cells (43). However, further verification is needed to determine whether reducing the flexibility of the hinge region can decrease CAR-T activity. We removed two consecutive glycine residues in the hinge region to reduce the flexibility of the hinge domain, thus resulting in better tumor control and lower release of inflammatory factors such as TNF-α and IL-6, which are the key molecules triggering the cytokine storm. This can be explained by the fact that reducing the flexibility of the hinge domain prevents overactivation of CAR-T cells, especially under high tumor load. However, the specific mechanism is unknown and warrants further investigation. Although studies have shown that the persistence of CAR-T cells is essential for immune surveillance of tumors, CAR gene copy numbers were unfortunately not measured (6). Studies have shown that the formation of immune synapses by CAR influences the function of CAR-T cells and changes the flexibility of the hinge region (44, 45). This may alter the formation of immune synapses in CAR, thus affecting the function of CAR-T cells, though it needs to be further explored.

Although we observed a downward trend in tumor load in the 2nd-GG group, it is a limitation of our study that the lack of evidence for enhanced anti-tumor activity of 2nd-GG CAR-T *in vivo*. Mice in the group of Mock-T, which had very low level of cytokines, had the highest mortality at day 15. Therefore, the death of mice was not caused by excessive release of cytokines. Recent study demonstrated that patients with high tumor burden had higher immune dysregulation with increased serum inflammatory markers and tumor IFN signaling. IFN signaling is associated with the expression of multiple checkpoint ligands and inferior response to CAR-T therapy (46). Therefore, we considered the direct cause of death in high tumor burden model was the increased tumor load. We hypothesized that lower levels

of inflammatory cytokine *in vivo* improved activity of 2nd-GG CAR-T through correct the immune dysregulation and reduce tumor IFN signaling, which requires further detection of phenotypes and exhaustion markers of T cells to confirm.

The present study demonstrated that a novel CD19 CAR with a less flexible hinge domain showed prolonged survival of mice under high tumor burden in preclinical studies. While there is potential for improved safety and efficacy, yet this needs validation with clinical trials.

DATA AVAILABILITY STATEMENT

All data generated and analyzed for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics committee of Fifth Medical Center of Chinese PLA General Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article The animal study was reviewed and approved by the Ethics committee of Fifth Medical Center of Chinese PLA General Hospital (ky-2018-5-37). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AZ designed the experiments. AZ and YS wrote the main body of the paper. AZ, YS, JD, YD, and HP performed the experiments and wrote the main body of the paper, with contributions from LM, SS, ZZ, MH, YY, XZ, WZ, and JP. YZ, QW, and MC supervise the experiments and revised the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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CD19 CAR-T Cells With Membrane-Bound IL-15 for B-Cell Acute Lymphoblastic Leukemia After Failure of CD19 and CD22 CAR-T Cells: Case Report

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Objectives: At present, reinfusions of chimeric antigen receptor (CAR)-T cell have exhibited limited efficacy, while their efficacy on extramedullary relapse remains to be further elucidated in B-cell acute lymphoblastic leukemia (B-ALL). Although combination with IL-15 demonstrated the potential to enhance antitumor activity of CAR-T, the efficacy of this approach remains to be validated clinically.

Methods: We reported a patient with B-ALL with extramedullary relapse after allogeneic stem cell transplantation and who was resistant to chemotherapy and radiotherapy. In total, he received four treatments with CAR-T cells repeatedly under the status of disease progression.

Results: First, the patient received autologous murine CAR19-CD28-CD3ζ-T cells and achieved full resolution of extramedullary leukemia lasting 8 months. After systemic disease relapse, he received autologous humanized CAR22-41BB-CD3ζ-tEGFR-T cells and achieved complete remission (CR) with incomplete blood count recovery (CRi) with minimal residual disease (MRD) negativity in the bone marrow and shrinkage of extramedullary leukemia. Over 2 months later, he experienced a relapse of the systemic disease and he received autologous murine CAR19-41BB-CD3ζ-mIL15-T cells and achieved CRi_{MRD-} lasting 5 months with the strongest expansion and persistence of CAR. Finally, on relapse of $CD19^{-}$ medullary disease, he received allogeneic humanized CAR22-41BB-CD3ζ-tEGFR-T cells but only achieved a transient decrease in the number of blasts. No CAR-T-cell-related encephalopathy syndrome was observed, and all side effects were manageable.

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Conclusion: Our report hints the feasibility and safety of CD19 CAR-T cell expressing membrane-bound IL-15 for patient with B-ALL even if relapsed after multiple CAR-T-cell therapies.

Keywords: chimeric antigen receptor-T cells, IL-15, B-ALL, extramedullary relapse, CD19, CD22

INTRODUCTION

Chimeric antigen receptor (CAR)-T cells have been remarkably successful in treating B-cell acute lymphoblastic leukemia (B-ALL) (1–4). The high 12-month relapse rate is the major cause of treatment failure, with an early relapse of antigen-positive disease and subsequent relapses associated with antigen loss or decrease of antigen density (5, 6). Although reinfusions of CAR-T cells seem a reasonable approach to conquer antigen-positive relapse with loss of CAR-T cell persistence, this strategy has exhibited limited efficacy (7–9). Relapse of leukemia can be categorized into medullary and extramedullary (EM) relapse depending on the site of leukemia recurrence, with the latter accounting for approximately 30% of ALL cases after allo-HSCT (10). In previous trials, patients with EM involvement were frequently excluded from the study population (7, 11). Several small studies have demonstrated that CAR-T-cell traffic to sites of EM disease and exhibit clinical efficacy (12–16) although further investigation is needed.

IL-15 promotes $CD8^+$ T and natural killer (NK) cell activation, proliferation, cytotoxicity, and survival—enhancing both specific and nonspecific antitumor activity (17). High serum IL-15 levels were associated with the effectiveness of CD19 CAR-T-cell treatment (18). Furthermore, transgenic expression of IL-15 in CAR-T cells exhibited improved proliferative capacity, persistence, and cytokine production in a preclinical study of glioblastoma (19). To our knowledge, no clinical study on CAR-T with mIL-15 expression has been reported. Herein, we report a case of B-ALL experiencing EM relapse after allo-HSCT with resistance to multiple chemotherapy and radiotherapy regimens. The patient totally received four times of single-dose intravenous infusions of CAR-T cells under disease progression, which were CAR19-CD28-CD3ζ, CAR22-41BB-CD3ζ-tEGFR, CAR19-41BB-CD3ζ-mIL15, and CAR22-41BB-CD3ζ-tEGFR-T cells, respectively.

Case Report

A 39-year-old male patient complained of chest pain and abdominal distension for 1 month. He had been diagnosed with B-ALL 4 years prior. Leukemia blasts accounted for 94.1% in bone marrow (BM) and were immunophenotypically characterized as $CD19^+/CD34^+/CD123^+/c\text{-}IgM^+/cCD79a^+$ and partially $CD10^+/CD20^{dim}/HLA\text{-}DR^{dim}/cTDT^+$. Positron emission tomography-computed tomography (PET-CT) revealed multiple high metabolic lesions in bilateral neck, axilla, mediastinum, abdominal, and retroperitoneal cavities. A biopsy of the right cervical lymph node and the immunohistochemical phenotype profiles indicated diffused TdT, CD43, BCL-2, Pax-5, and CD10, scattered CD3, CD79a, CD45RO, MPO, and CD15, focalized CD20 and CD21, individual CD1a, and 60% $Ki\text{-}67^+$ cells. The expression of Bcl-6 and Mum-1 was negative. He achieved complete remission (CR) after one cycle of standard vincristine, daunomycin, cyclophosphamide, l-asparaginase, prednisone (VDCLP) induction. The postremission treatment included one cycle of cyclophosphamide, cytarabine, and 6-mercaptopurine (CAM), one cycle of hyper-CVAD B (methotrexate, leucovorin, sodium bicarbonate, cytarabine), and one cycle of vincristine, daunomycin, cyclophosphamide, prednisone (VDCP). The patient maintained CR but never achieved minimal residual disease (MRD) negativity in the BM by flow cytometry prior to transplantation. He underwent an HLA-identical sibling donor allo-HSCT subsequent to bis-chloroethyltrosourea, cyclophosphamide, and total body irradiation as the preconditioning regimen followed by cyclosporine A and short-term methotrexate for prophylaxis of graft *versus* host disease. The treatment progress is shown in **Figure 1A**. The characteristics of each CAR-T-cell infusion are summarized in **Table 1**. He achieved a CR with MRD negativity (CR_{MRD-}) 1 month after transplantation. Unfortunately, the patient underwent EM relapse in the mediastinum 2 years after allo-HSCT (**Figure 1B**). The blasts of mediastinal mass expressed B cell markers (PAX5 and CD19) and naive cell markers (CD34, CD10, and TdT) by immunohistochemistry. Next, he received one cycle of L-asparaginase, cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone (L-CHOP), two cycles of vincristine, mitoxantrone, L-asparaginase, prednisone (VMLP), one cycle of vincristine, idarubicin, L-asparaginase, prednisone (VDLP) combined with dendritic cells and cytokine-induced killer cells (DC-CIK) treatment, two cycles of VDLP. The EM disease did not regress and MRD tested positive (0.02%). After one cycle of hyperCVAD B+Teniposide, and tested MRD negative, but the EM leukemia progressed. PET-CT revealed multiple lesions in the mediastinum and abdominal cavity involved EM leukemia, with further evidence of gastric wall involvement (**Figure 1C**).

After providing informed consent, he was enrolled in a clinical trial (NCT02186860) and was admitted for CAR-T-cell treatment. He underwent leukapheresis in the preparation of CAR-T-cell infusions. The excess cells were cryopreserved and used for the next two CAR-T-cell preparation. He received four cycles of intra-abdominal lymph node intensity-modulated radiotherapy and EM disease remained in the lymph nodes in left lower pulmonary aorta, bilateral axillary, and mediastinal on contrast-enhanced CT. One month later, he received FC (cyclophosphamide, at day −4; fludarabine, at days −4 to −2) preconditioning and autologous murine CAR19-CD28-CD3ζ-T-cell infusion administered (day 0). EM leukemia full resolution was confirmed 1 month later by PET/CT (**Figure 1D**). However, the MRD positivity in the BM increased to 0.02% at 4 months with EM leukemia free

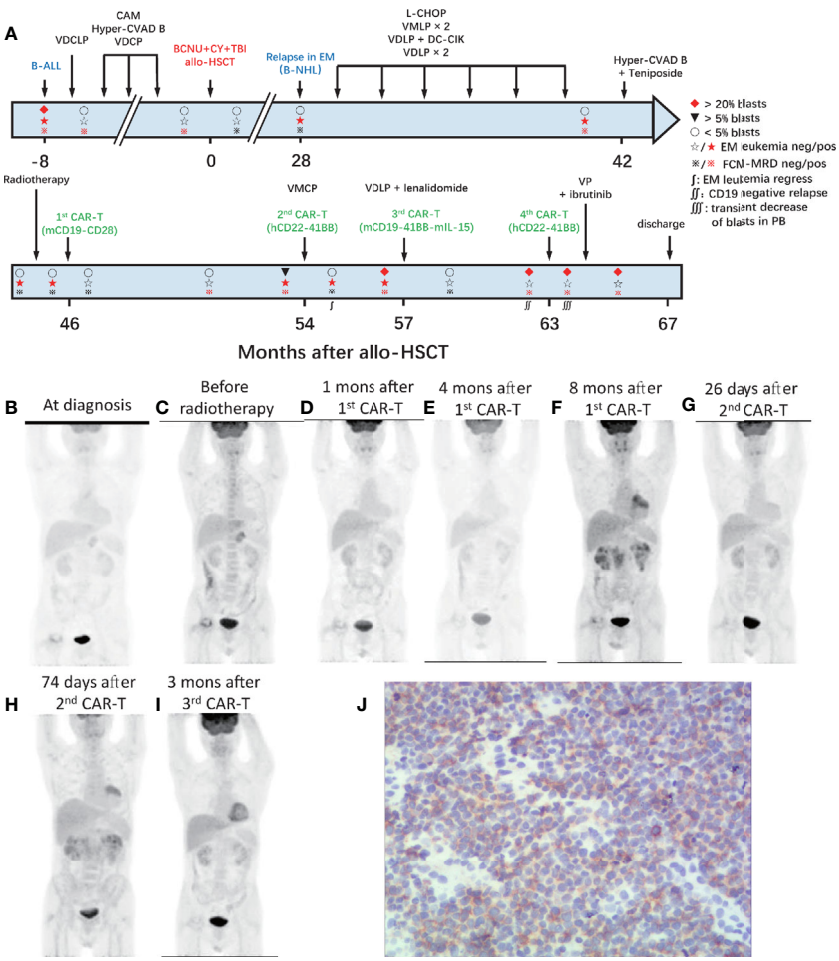

FIGURE 1 | Process of treatment with CAR-T cells and clinical response. **(A)** The process of CAR-T-cell treatment and clinical response in the BM. The results of PET-CT are shown in **(B–I)**. **(B)** At diagnosis, multiple high metabolic lesions were detected in bilateral neck, axilla, mediastinum, abdominal, and retroperitoneal cavity. **(C)** Before radiotherapy, multiple lesions in the mediastinum and abdominal cavity were involved by EM leukemia, as well as the gastric wall. **(D)** One month after the 1st CAR-T-cell infusion, extramedullary leukemia achieved full resolution. **(E)** Four months after the 1st CAR-T infusion, extramedullary leukemia remained at full resolution. **(F)** Eight months after the 1st CAR-T infusion, the EM leukemia recurred in multiple lesions, including lymph nodes in the mediastinum and right inner mammary region, intrapericardial, bilateral kidney, retroperitoneal, perirenal, and pelvic peritoneum. **(G)** Twenty-six days after the 2nd CAR-T cell infusion, EM leukemia regressed. **(H)** Seventy-four days after the 2nd CAR-T cell infusion and 10 days before the 3rd CAR-T infusion, EM leukemia progressed. **(I)** Three months after the 3rd CAR-T cell infusion, EM leukemia achieved full resolution again. BM, bone marrow; EM, extramedullary; CAR, chimeric antigen receptor. **(J)** Immunohistochemical results of renal tissue showed weak positive CD22 in tumor cells.

(**Figure 1E**) followed by blasts in the BM and MRD increased to 5.5% ($CD19^+CD22^+$) and 6.08% at 8 months after CAR-T-cell infusion. PET/CT revealed the EM leukemia recurred in multiple lesions, including lymph nodes in the mediastinum and right inner mammary region, intrapericardial, bilateral kidney, retroperitoneal, perirenal, and pelvic peritoneum (**Figure 1F**). Renal biopsy revealed the immunophenotype of leukemia cells was $CD19^+$, with scattered $CD20^+/CD22^+/CD34^+/PAX\text{-}8^+/CD79a^+/TdT^+/CD10^+/CD3^-/CD33^-/CD99^-$, and $CD1a^-$ (**Figure 1J**; **Supplementary Figure S1**).

After one cycle of vincristine, melphalan, cyclophosphamide, prednisone (VMCP) to reduce tumor burden, the patient received FC preconditioning and autologous humanized CAR22-41BB-CD3ζ-tEGFR-T-cell infusion (NCT03262298). He achieved CR_{MRD-} in the BM with transfusion-independent and EM leukemia burden marked decreased on PET/CT 1 month later (**Figure 1G**). Nonetheless, $CD19^+CD22^+$ blasts quickly rose to 96% in the BM along and EM leukemia recurred more than 1 month later (**Figure 1H**).

After one cycle of VDLP and lenalidomide to reduce tumor burden, the patient received FC preconditioning and the autologous murine CAR19-41BB-CD3ζ-mIL15-T-cell infusion for compassionate use. He achieved transfusion-independent CRi_{MRD-} in the BM, which was 1 month later and achieved full resolution of EM leukemia 3 months later on PET/CT (**Figure 1I**). Five months after CAR-T-cell infusion, the blasts count rose to 86.38% in the BM and were $CD19^-CD22^+$. No evidence of EM leukemia was found on computed

TABLE 1 | Characterization of the four infusions of CAR-T cells.

	1st	2nd	3rd	4th
Clinical trial number	NCT02186860	NCT03262298	Compassionate use	NCT03262298
Vector[c]	Retroviral	Retroviral	Retroviral	Retroviral
CAR structure[c]	Anti-CD19 scFv (FMC63)-CD8-CD28-CD3ζ	Anti-CD22 scFv (m971)-CD8-4-1BB-CD3ζ-tEGFR	Anti-CD19 scFv (FMC63)-CD8-4-1BB-CD3ζ-mIL15	Anti-CD22 scFv (m971)-CD8-4-1BB-CD3ζ-tEGFR
scFv	Murine	Humanized	Murine	Humanized
Derived	Autologous	Autologous	Autologous	Allogenic
Classification	$CD3^+$ 98.8% $CD8^+$ 16.7% $CD4^+$ 66.9%	$CD3^+$ 98.5% $CD8^+$ 37.5% $CD4^+$ 57.9%	$CD3^+$ 99.8% $CD8^+$ 76.4% $CD4^+$ 20.8%	$CD3^+$ 99.1% $CD8^+$ 34.2% $CD4^+$ 57.5%
CD4/CD8	4.01	1.54	0.27	1.68
Memory T cells[b]	21.49%	35.28%	35.6%	39.83%
Total cells dose	3.79×10^7	1.24×10^8	5.25×10^8	1.576×10^8
Efficiency	66.4%	50.1%	12.4%	79.1%
Total CAR^+ cells dose	2.49×10^7	6.12×10^7	6.50×10^7	1.24×10^8
CAR^+ cells (kg)	0.5×10^6	1×10^6	1×10^6	2×10^6
Pretreatment	–	VMCP	Lenalidomide + VDLP	–
Blasts% pre-CAR-T cell infusion	EM^+ and MRD^-	EM^+ and 5.5% in BM	EM^+ and 96% in BM	EM free; 96.5% in BM
ECOG score pre-CAR-T cell infusion	1	1	2	2
Immunotype pre-CAR-T-cell infusion	Positive for CD19, CD38, CD34, TdT, CD81dim, CD9, CD22, negative for CD10, CD13, CD33, CD20, CD138	Positive for CD19, cCD79a, CD38, TdT, CD22, partial positive for CD34, CD10, negative for CD20	Positive for CD38, cCD79a, TDT, CD81, CD22, partial positive for CD10, CD34	Positive for HLA-DR, CD34, cCD79a, CD38, cTdT dim, CD81, CD22, CD24, partial positive for CD10, negative for CD19, CD20
Maximum of CAR copy (copies/μg gDNA)	2.64×10^3	4.95×10^4	5.81×10^5	2.53×10^3
Precondition	CyFlu[a]	CyFlu	CyFlu	CyFlu
Response in BM	MRD^- CR (8 months)	MRD^- CR (1 months)	MRD^- CRi (5 months)	Transient blasts decrease in PB
Response in extramedullary	Full resolution	Regress	Full resolution	–
Expression of CD19/CD22 before treatment	$CD19^+CD22^+$	$CD19^+CD22^+$	$CD19^+CD22^+$	$CD19^-CD22^+$
Reasons of relapse	The loss of CAR	The loss of CAR	CD19 antigen escape	The loss of CAR
CAR detected when relapse	No	No	Yes	No
CRS	Yes	Yes	Yes	Yes
Severe CRS	No	No	No	Yes
Glucocorticoid	No	No	No	Methylprednisolone

CAR, chimeric antigen receptor; scFv, single-chain fragment variable; tEGFR, truncated human epidermal growth factor receptor (EGFR) polypeptide; MRD, minimal residual disease; VMCP, vincristine, melphalan, cyclophosphamide, prednisone; VDLP, vincristine, melphalan, cyclophosphamide, prednisone; CyFlu, cyclophosphamide, fludarabine; CR, complete remission; CRS, cytokine release syndrome; EM, extramedullary.

[a]*Cy, cyclophosphamide; Flu, fludarabine; Cy, 1,000 mg/m^2/day, d1; Flu, 30 mg/m^2/day, d1-3; dose of the 1st Flu in d2 is 80 mg, the others are 50 mg.*

[b]*Immunophenotyping of memory T cells is $CD45RA^+CD62L^+$ and $CD45RA^-CD62L^+$.*

[c]*See the Materials and Methods section in the* ***Supplementary Material***.

tomography (CT) or ultrasound examination. CARs design is shown in **Figure 2**.

At relapse, the patient is in a state of incomplete hematopoietic recovery and the cryopreserved cells are depleted. Failing to meet the conditions for preparation of autologous CAR-T cells, he received predonor-derived humanized CAR22-41BB-CD3ζ-tEGFR-T-cell infusion (NCT03262298) after FC preconditioning and achieved a transient decrease in blasts in the peripheral blood (PB). Unfortunately, due to loss of CAR following methylprednisolone, the disease progressed within 28 days. The patient then received one cycle of vincristine, prednisone (VP) as reinduction chemotherapy with ibrutinib. After more than 3 months, the patient did not achieve remission. He abandoned treatment and was discharged for financial reasons.

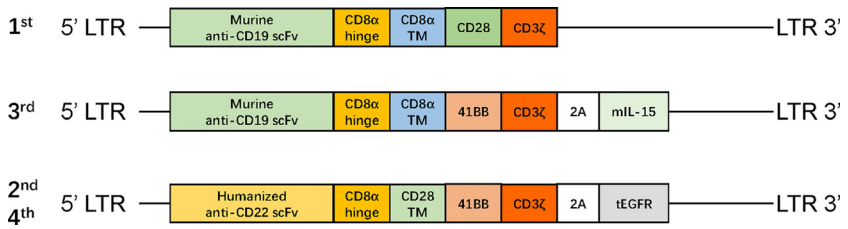

FIGURE 2 | The structure of retroviral vectors encoding CARs. The CAR19-CD28-CD3ζ consisted of murine anti-CD19 scFv, a CD8 hinge region, CD28 transmembrane and cytoplasmic domain, and a CD3ζ cytoplasmic region. The CAR19-41BB-CD3ζ-mIL15 consisted of humanized anti-CD19 scFv, a CD8 hinge and transmembrane region, 4-1BB costimulatory domain, and a CD3ζ cytoplasmic region; mIL-15 is connected with CAR gene *via* a P2A peptide. The CAR22-41BB-CD3ζ-tEGFR consisted of humanized anti-CD22 scFv, a CD8 hinge and transmembrane region, 4-1BB costimulatory domain, and CD3ζ cytoplasmic region; tEGFR is integrated with CAR gene through a P2A peptide. mIL-15, membrane IL-15; EGFR, human epidermal growth factor receptor; tEGFR, truncated EGFR polypeptide; scFv, single-chain fragment variable.

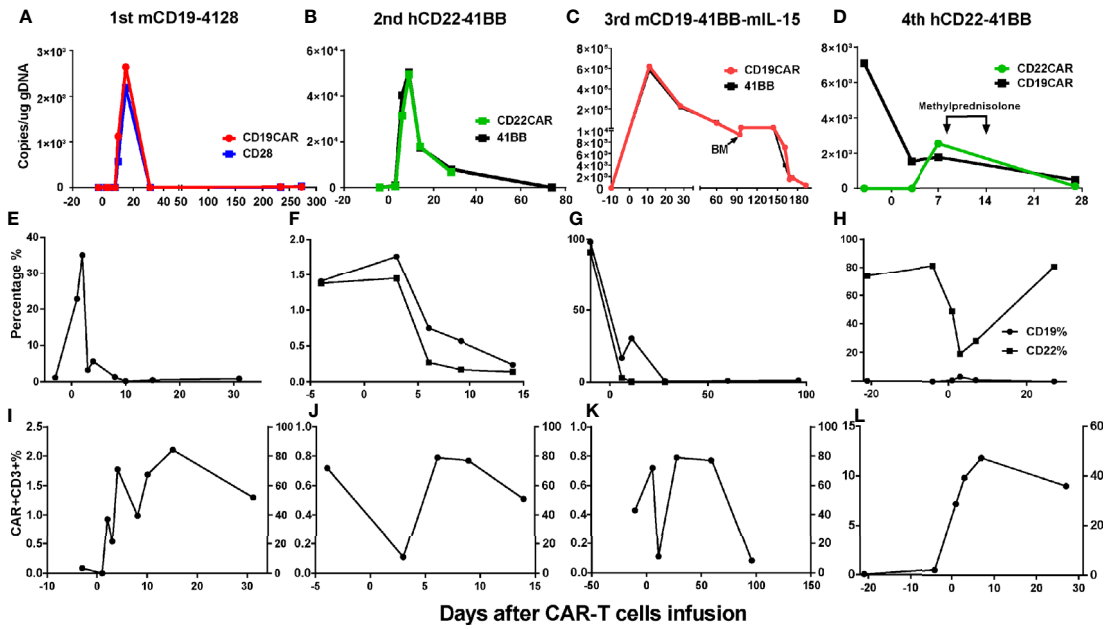

FIGURE 3 | Changes of CAR% and blasts% in PB after CAR-T cell infusions. **(A, E, I)** After the 1st infusion, the copies of CAR reached the expansion peak on d15 and fell to undetectable on d31 and remained almost undetectable during relapse 8 months later. The expansion of CAR copies was coincided with the drop of $CD19^+$ blasts in PB. **(B, F, J)** After 2nd infusion, the expansion peak was within 14 days and coincided with the drop of $CD19^+CD22^+$ blasts in PB. After about 2 months, the CAR copies gradually fell to being undetectable. **(C, G, K)** After the 3rd infusion, the copies of CAR ascended to expansion peak about 10 days later in keep with the drop of $CD19^+CD22^+$ blasts in PB and could be detected after $CD19^-$ relapse lasting more than 5 months. **(D, H, L)** The nadir of $CD22^+$ blasts in PB were corresponding with the peak of CAR expansion. The drop of CART22 was in keeping with CART19, eliminated by methylprednisolone (40 mg d1-3, 20 mg d 4-6).

Toxicities, Persistence of CAR-T Cells, and Tumor Antigen Expression

Grades (G) of treatment-emergent adverse events (AEs) after each infusion are shown in **Supplementary Table S1**. No >G2 AE were observed after the first and second infusions. After the first infusion of CAR-T cells, the patient developed G1 cytokine-release syndrome (CRS) (**Figures 4A, E**). The copies of CAR reached the expansion peak at day 14 and fell to undetectable levels within 1 month (**Figure 3A**). After the second infusion of CAR-T cells, the patient developed G1 CRS as well, which improved after symptomatic treatment (**Figures 4B, F**). The CAR copies peaked within 14 days and gradually became undetectable after about 2 months (**Figure 3B**). After the third infusion of CAR-T cells, the patient was evaluated as G2 CRS on day 6 and was cured by anti-infection and symptomatic supportive treatment (**Figures 4C, G**). The copies of CD19 CAR reached an expansion peak approximately 10 days later and were detected even after $CD19^-$ relapse (**Figure 3C**). The final infusion

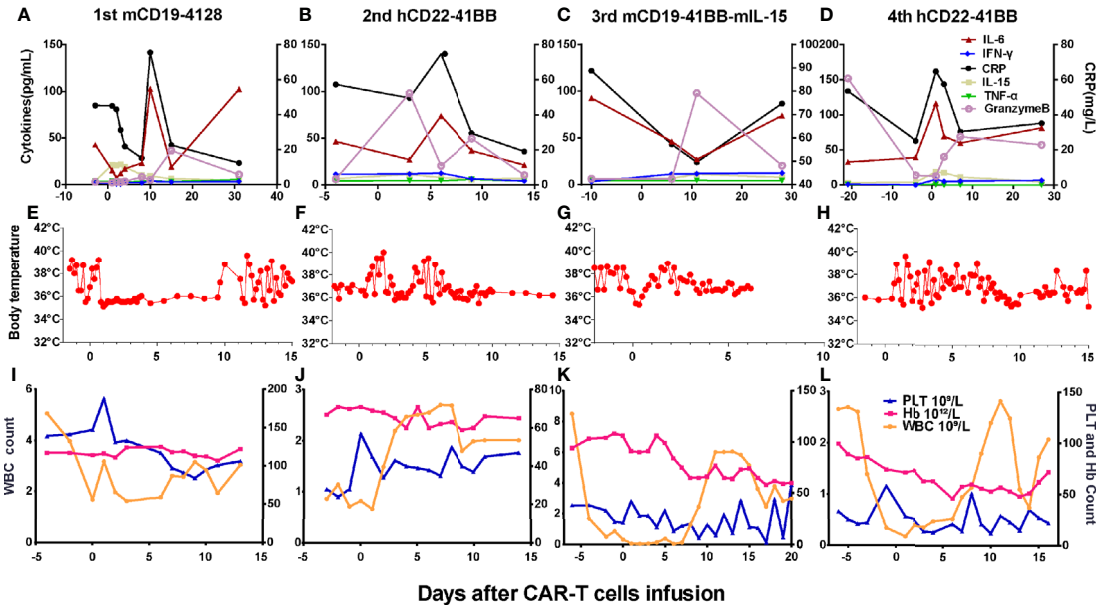

FIGURE 4 | Changes of cytokines, CRP, body temperatures, and hemograms after CAR-T cell infusions. **(A, E)** After the 1st infusion, the patient developed fever, elevation of IL-6, granzyme B, and CRP on day 9. **(B, F)** After the 2nd infusion, the patient developed fever, chill on the day 1. The level of IL-6, granzyme B, and CRP elevated on days 3 and 6, respectively, and then dropped to baseline on day 14. **(C, G)** After the 3rd infusion, the patient developed fever, chill quickly, then elevation of IL-6, granzyme B, and CRP successively 1 week later. **(D, H)** The last infusion caused fever, elevation of granzyme B, and CRP on day 1 and IL-6 thereafter. **(I–L)** Changes of hemogram after the CAR-T-cell infusions.

of CAR-T cells caused fever, and rapid elevation of granzyme B, CRP, and IL-6, evaluated as G4 CRS on day 10 (**Figures 4D, H**). He underwent treatment with methylprednisolone, which caused rapid loss of CAR copies (**Figure 3D**). No nervous system disorder was observed during any infusion. After each infusion of CAR-T cells, the expansion of CAR copies coincided with the drop in PB blasts (**Figures 3E–L**). After each relapse, CD22 site density remained almost unchanged (**Supplementary Figure S2**). Hemogram changes during treatments are shown in **Figures 4I–L**.

DISCUSSION AND CONCLUSIONS

Herein, we report a refractory B-ALL patient with EM relapse after allo-HSCT who received repeat CAR-T-cell therapies. Both CAR19-CD28-CD3ζ-T and CAR22-41BB-CD3ζ-tEGFR-T cell only achieved short persistence following by $CD19^+$ relapse. By contrast, after systemic relapse, CAR19-41BB-CD3ζ-mIL15-T cell achieved CRi_{MRD-} and full resolution of EM leukemia lasting 5 months with transfusion independence, followed by high CAR persistence and $CD19^-$ relapse.

The results of clinical trials indicate that CAR containing CD28 persists shorter than CAR with 4-1BB (20, 21). Preclinical and clinical data confirm that CAR-T cells with 4-1BB tend to expand in patients at later time points compared with those with CD28 (22–24). The short persistence of the first CAR-T-cell infusion was possibly associated with CD28 costimulatory domain. CAR copies fell to undetectable levels 1 month after infusion on achieving CR_{MRD-}. Although minimal CAR expansion and persistence after the first infusion was maintained, CAR19-CD28-CD3ζ-T cells exerted a sustained control of leukemia, possibly related to prior radiotherapy. In preclinical studies, radiotherapy enhanced the efficacy of CAR-T cells by promoting their migration to the tumor site and increased effector functions in glioblastoma models (25) and by enhancing the sensitivity of CAR-T cell to antigen-negative tumor cells to reduce immune escape (26). Radiotherapy as a bridging strategy for CART19 in high-risk lymphoma has been proven to be clinically safe (27). Our report shows that radiotherapy bridging to CAR-T cells is feasible, but whether radiotherapy enhances the infiltration and activity of CAR-T cells in extramedullary lesions needs further investigation.

Recent studies have indicated that humanized CART19 could induce remission in patients with relapsed/refractory B-ALL, especially in patients who received a reinfusion of murine CAR-T cells (28). Thus, considering the loss of CAR possibly caused by murine CAR, humanized CAR22-41BB-CD3ζ-tEGFR-T cells have also been used. CD22 CAR-T cells exhibited high response rates in B-ALL patients after failure of CD19 CAR-T cells (29). However, the patient progressed rapidly after first CART22 infusion with loss of CAR without achieving full resolution of EM leukemia. Diminished CD22 site density is sufficient to permit escape of leukemia from CD22-directed CAR therapy rather than total loss of surface expression of CD19 (29) but did not diminish on relapse following CART22 infusions in our study. CD19 CAR-T-cell efficacy of CD22 CAR-T cells may be related to the low level of CD22 expression in EM sites and the

affinity of the single-chain fragment variable, which needs further investigation.

For the first time, we used CD19 CAR-T cells with transgenic expression of membrane IL-15 clinically. Although the tumor burden before infusion was extremely high, the patient achieved CRi_{MRD-} with the highest expansion and longest persistence of CAR copies and revisable toxicity after CAR19-41BB-CD3ζ-mIL15-T-cell infusion. Moreover, remission induced by subsequently murine CART19 demonstrated that endogenous factors or immunosuppression but not the production of human antimouse antibodies resulted in relapse after the first murine CART19 infusion. The serum level of IL-15 was detected after each infusion of CAR-T and maintained at low level without significant change. Since CAR-T cells express mIL-15, it is understandable that the serum level of IL-15 has not changed. It is our limitation that mIL-15 expression on CAR-T cells was not detected in our clinical study. However, transgenic expression of IL-15 in CAR-T cells exhibited improved proliferative capacity and persistence preclinically (19). Thus, continuous expansion of CAR-T cells may indirectly reflects the function of IL-15 in our study. Furthermore, excessive expansion of CAR19-41BB-CD3ζ-mIL15-T cells possibly cause sustained inflammation and cytokine production, which could explain the incomplete hematopoietic recovery. The patient underwent CD19-negative relapse with CAR-T-cell persistence, illustrating CAR19-41BB-CD3ζ-mIL15-T cells possibly exerted a stronger immunopressure. Although antigen-negative relapses are not uncommon in B-ALL with CAR-T therapy, previous report has also highlighted the presence of antigen loss variants following infusions of anti-IL13Rα2 CAR-T cells with IL-15 expression, although the mechanisms remain unknown (19). Coexpression of mIL-15 enhanced effector functions, engraftment, and tumor control of CAR-T cells and remodeled the tumor microenvironment (TME) to favor tumor control, including NK cell activation and reduced presence of M2 macrophages (30). In our case, after treatment with CD19 CAR-T cells with mIL-15 expression, the patient relapsed without EM leukemia, which may relate to TME reprogramming, and thus requires further investigation.

The final infusion of CD22 CAR-T cell achieved inferior leukemia responses and expansion of CAR. High tumor burden in the BM, high dosage of CAR-T cells, and thrombocytopenia before lymphodepletion might contribute to the development of severe CRS after the fourth infusion of CAR-T cells (31). Drugs including alemtuzumab and corticosteroids may abrogate CAR-T cell expansion and persistence (32, 33). Thus, treatment with glucocorticoids caused the rapid reduction of CAR-T cells.

This study provides a rare perspective that compares the outcomes of different CAR-T cells in the same patient. Our report hints that it is feasible and safe to infuse CD19 CAR-T-cell-expressing membrane-bound IL-15 for patient with B-ALL even if relapsed after multiple CAR-T-cell therapies.

MATERIALS AND METHODS

See the *Materials and Methods* section in the **Supplementary Material**.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at the Fifth Medical Center of Chinese PLA General Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LH designed the trial and experiments and revised the manuscript. YaS performed experiments, analyzed the data, and wrote the paper. YoS, HN, and YW managed the patients. NL, YL, JC, JN, JH, ZQ, and BZ analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.728962/full#supplementary-material

Supplementary Figure 1 | Immunohistochemical results of renal tissue showed positive CD19, CD34, and TdT in tumor cells.

Supplementary Figure 2 | CD22 site density when relapse after CAR T cells.

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Anti-BCMA CAR-T Cell Therapy in Relapsed/Refractory Multiple Myeloma Patients With Extramedullary Disease: A Single Center Analysis of Two Clinical Trials

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Background: The prognosis of relapsed/refractory multiple myeloma (RRMM) patients with the extramedullary disease was significantly poor. Extramedullary multiple myeloma (EMM) patients gained limited benefits from traditional drugs. Anti-B cell maturation antigen (BCMA) chimeric antigen receptor (CAR) T-cell therapy seems to be a promising approach to treat RRMM patients. However, very few clinical studies are designed for EMM. Our study aimed to compare and assess the safety, efficacy, and pharmacokinetics of anti-BCMA CAR-T cell therapy in EMM and non-EMM.

Methods: The results from published anti-BCMA CAR-T clinical trials, in which raw data of EMM patients were available, were reviewed and summarized. Two trials conducted in our clinical centers were analyzed and presented with detailed data.

Results: According to published anti-BCMA CAR-T clinical trials, the ORR of EMM ranged from 57% to 100%, with the complete remission (CR) rate of 29% to 60%. Between February 22, 2017, and September 26, 2019, a total of 61 subjects (EMM 25; non-EMM 36) received anti-BCMA CAR-T cell infusion. The data-cutoff date was April 1, 2021. There were no statistical differences between EMM and non-EMM groups in adverse events (AEs), including cytokine release syndrome (CRS). The most common AEs of grade ≥ 3 in both groups were hematologic toxicities. There was no significant

difference in the objective response rate (ORR) and ≥ complete remission (CR) rate between both groups. However, the ≥ CR rate of the EMM group was lower than the non-EMM group receiving the fully human anti-BCMA CAR-T cell therapy ($p = 0.026$). The median progression-free survival (PFS) for EMM and the non-EMM group was 121 days and 361 days, respectively ($p = 0.001$). The median overall survival (OS) for EMM and the non-EMM group was 248 days and 1024 days, respectively ($p = 0.005$). The C_{max} and $AUC_{0\text{-}28d}$ for EMM group were lower than non-EMM group (C_{max}, $p = 0.016$; $AUC_{0\text{-}28d}$, $p = 0.016$). Extramedullary disease was an independent prognostic risk factor for PFS (hazard ratio, 2.576; 95% CI, 1.343 to 4.941; $p = 0.004$) and OS (hazard ratio, 2.312; 95% CI, 1.165 to 4.592; $p = 0.017$) in RRMM patients receiving anti-BCMA CAR-T cell therapy.

Conclusions: Based on our results, EMM patients could benefit from the two anti-BCMA CAR products, although they had a shorter PFS and OS compared with non-EMM patients.

Clinical Trial Registration: http://www.chictr.org.cn, identifier ChiCTR-OPC-16009113 and ChiCTR1800018137.

Keywords: BCMA (TNFRSF17), car-t, relapsed, refractory, extramedullary, multiple myeloma

INTRODUCTION

Extramedullary multiple myeloma (EMM), one of the natural courses of advanced multiple myeloma (MM), is an aggressive subentity. It is characterized by the involvement of multiple organs such as the central nervous system, liver, pleura, lymphatic system, skin, etc. (1, 2). EMM may be found in newly diagnosed MM or at the time of relapse (secondary EMM). Plasma cell leukemia (PCL), characterized by drug resistance, rapid progression, and short survival, is classified as a variant of aggressive EMM (3). With the development of imaging technology and the prolonged lifespan by new drugs, the diagnostic rate of EMM is increasing (4). At the disease progression stage, the incidence of EMM ranges from 10% to 30% (5, 6). Novel drugs such as monoclonal antibodies (mAbs), immunomodulatory drugs (IMiD), and proteasome inhibitors (PI) have improved the survival of MM patients. However, EMM, including PCL patients, have limited benefits from the existing strategies (3, 7–10).

Anti-BCMA CAR-T therapy achieved the most prominent responses in RRMM, with a high objective response rate (ORR) (11–17). We reviewed the published clinical trials with raw data available and found that several of these studies have enrolled EMM patients, but no analysis was performed on this specific subgroup (10, 17).Therefore, we firstly reported the differences in clinical response, adverse events, and pharmacokinetics between EMM and non-EMM patients receiving anti-BCMA CAR-T cell therapy in our center.

MATERIALS AND METHODS

Study Conduct and Patients

We reviewed and summarized the results from published anti-BCMA CAR-T clinical trials in which raw data of EMM patients was available. We then focused on the two trials conducted in our clinical centers. The phase I study of murine anti-BCMA CAR-T cell therapy was registered at Chinese Clinical Trial Registry as ChiCTR-OPC-16009113, and the phase II study of a fully human anti-BCMA CAR (CT103A) was registered at Chinese Clinical Trial Registry as ChiCTR1800018137. The murine anti-BCMA CAR product was composed of a murine anti-BCMA single-chain variable fragment (scFv), a CD8a hinge, the CD28 co-stimulatory domain (including CD28 transmembrane, and intracellular domains), and the CD3ζ activation domain. The fully human anti-BCMA CAR product (CT103A) was composed of a fully human scFv, a CD8a hinge, and transmembrane domain, 4-1BB co-stimulatory, and CD3ζ activation domains. Between February 22, 2017, and September 26, 2019, a total of 73 (murine 44; fully human 29) consecutive adult subjects with BCMA positive RRMM were screened according to the study protocols, and 12 (murine 6; fully human 6) patients were excluded (**Figure 1**).

Assessments Criteria

Most studies agree that EM could be divided into two groups: the first group comprises tumors that are extending directly from osteolytic bone lesions (EM-B, extramedullary-bone related), while the second results from plasma cell infiltration into soft tissues, with no relationship to the bone (EM-E, extramedullary-extraosseous) (7, 8). The EMM in our study included EM-E and PCL. The PCL in our research included primary PCL and secondary PCL (7). Cytokine release syndrome (CRS) and symptoms of immune effector cell-associated neurotoxicity syndrome (ICANS) were graded according to the criteria of Lee et al. (18, 19). All other adverse events (AEs) and severe adverse events (SAEs) are evaluated by the National Cancer Institute Common

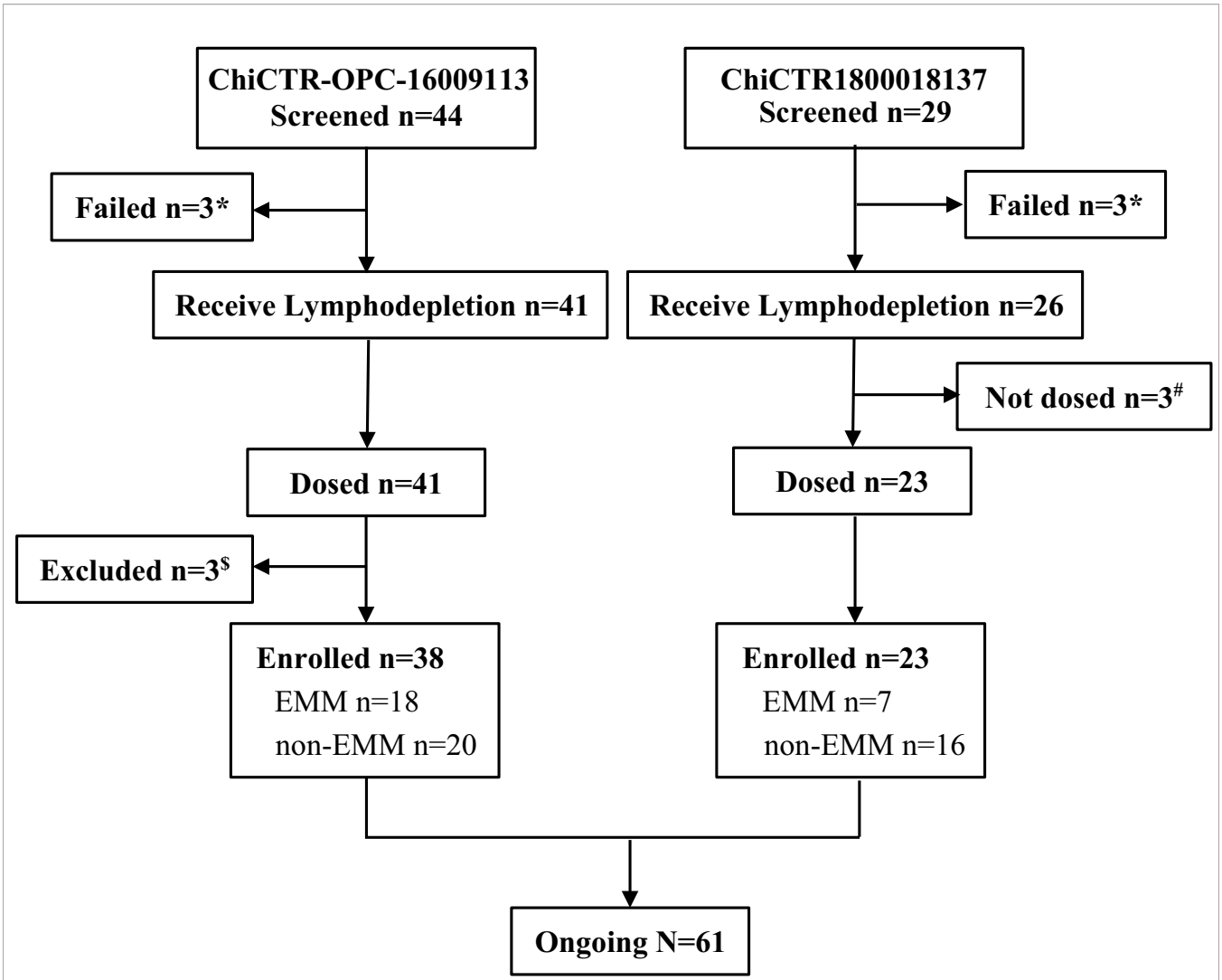

FIGURE 1 | Flowchart. *Three patients failed in the screening of trail ChiCTR-OPC-16009113, and three patients failed in screening ChiCTR1800018137, respectively, because of not meet inclusion criteria or rapid progression. #Three patients who received lymphodepletion were not dosed because of heart failure, severe liver function damage, and severe infection, respectively. $A patient who had POEMS rather than MM or EMM was excluded, and two patients were excluded because of early death.

Terminology Criteria for Adverse Events (CTCAE) Version 5.0 (20, 21). The diagnose, clinical response, and disease progression was assessed according to the IMWG consensus criteria at serial time points after CAR-T infusion. The data-cutoff date was April 1, 2021. CAR transgene copies in the patients' peripheral blood monocytes were monitored by digital droplet polymerase chain reaction (ddPCR).

Study Approval

These study protocols were approved by the institutional review board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Details of the protocols, CAR-T cell preparation, and assessments criteria were as described in our previous studies (12, 14). Both trials were conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from each participant.

Statistical Analysis

Continuous variables were described using median and range. Categorical variables were reported in number and percentage. The analysis of categorical variables was performed by the the chi-square test, Fisher's exact chi-square test, or Pearson's chi squared test. For continuous variables, Wilcoxon rank-sum test was used. Kaplan-Meier method was employed to estimate the probabilities of overall survival (OS) and progression-free survival (PFS). Estimations of risk were performed by Cox regression. Statistical analyses were performed by SPSS 22 and Graphpad Prism 8. P values less than 0.05 (two-tailed) were considered statistically significant.

TABLE 1 | Anti-BCMA CAR-T cell products in clinical trials included EMM patients.

No. of EMMpatients	Name	Clinical trial information	Major response	Reference
1 (16)	Anti-BCMA CAR-T cell therapy	NCT02215967	the ORR of EMM is 100% (1/1), with 1 (100%) VGPR (at 51 weeks); the ORR of non-EMM is 80% (12/15) with 2 (13%) sCR/CR and 9 (60%) ≥VGPR.	Brudno, J N et al. (13)
9 (33)	bb2121	Phase Ib NCT02658929	the ORR of EMM is 89% (8/9), with 4 (44%) sCR/CR and 6 (66%) ≥ VGPR; ORR of non-EMM is 83% (20/24) with 11 (33%) sCR/CR and 18 (75%) ≥VGPR.	Raje, Noopur et al. (15)
5 (17)	LCAR-B38M	Phase I/II NCT03090659	the ORR of EMM is 80% (4/5), with 3 (60%) sCR/CR and 4 (80%)≥ VGPR; ORR of non-EMM is 92% (11/12) with 9 (75%) sCR/CR and 11 (92%) ≥VGPR.	Xu, Jie et al. (11)
7 (25)	Anti-BCMA CAR-T cell therapy	Phase I NCT02546167	the ORR of EMM is 57% (4/7), with 2 (29%) sCR/CR and 4 (57%)≥ VGPR.	Cohen, Adam D et al. (16)
50 (128)	ide-cel (bb2121)	Phase II NCT03361748	CR rate: non-EMM>EMM (no accurate values); ORR: non-EMM>EMM (no accurate values).	Munshi, N C et al. (10)
7 (23)	CT103A	Phase I ChiCTR1800018137	the ORR of EMM was 100% (7/7), with 2 (29%) sCR/CR and 5 (71%) ≥ VGPR; the ORR of non-EMM was 100% (16/16), with 13 (81%) sCR/CR and 14 (88%) ≥ VGPR.	Wang, D et al. (14)
18 (38)	Murine BCMA CAR-T cell therapy	Phase I ChiCTR-OPC-16009113	the ORR of EMM was 77.78% (14/18), with 7 (39%) sCR/CR and 10 (56%) ≥ VGPR; the ORR of non-EMM was 90% (18/20), with 9 (45%) sCR/CR and 11 (55%) ≥ VGPR.	Li, C et al. (12)

EMM, extramedullary myeloma; BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor; ORR, objective response rate; OS, overall survival; sCR, stringent complete response; VGPR, very good partial response.

TABLE 2 | Comparison of baseline characteristics between EMM patients and non-EMM patients in the two clinical trials conducted in our center.

Characteristics	EMM (n = 25)	non-EMM (n = 36)	p-Value
Age, yr, median (range)	55 (34 - 70)	53 (34 - 69)	0.509
Sex, n (%)			0.353
Male	13 (52.0)	23 (63.9)	
Female	12 (48.0)	13 (36.1)	
ECOG performance-status score			1.000
0-1	23 (92.0)	34 (94.4)	
2-3	2 (8.0)	2 (5.6)	
Time since diagnosis, yr, median (range)	3.2 (0.8 - 12.6)	2.9 (0.7 - 11.3)	0.363
Prior lines of therapy, median (range)	4 (3 - 11)	4 (3 - 10)	0.114
Durie-Salmon stage, n (%)			0.145
I	1 (4.0)	0	
II	0	3 (8.3)	
III	22 (95.0)	33 (91.7)	
ISS stage, n (%)			0.288
I	7 (30.0)	16 (44.4)	
II	11 (48.0)	10 (27.8)	
III	5 (22.0)	10 (27.8)	
Myeloma type, n (%)			0.104
IgG κ	5 (20.0)	8 (22.2)	
IgG λ	9 (36.0)	10 (27.8)	
IgA κ	2 (8.0)	3 (8.3)	
IgA λ	0	2 (5.6)	
IgD λ	1 (4.0)	3 (8.3)	
Light chain κ	0	6 (16.7)	
Light chain λ	7 (28.0)	2 (5.6)	
Non-secretor	1 (4.0)	2 (5.6)	
High risk cytogenetics, n (%)[&]	6 (24.0)	18 (50.0)	0.041
TP 53 mutations, n (%)	3 (13.6)	3 (9.1)	0.674
BCMA MFI on plasma cells, median (range)	2417 (840 - 12516)	1586 (303 - 51023)	0.133
CAR-T structure, n (%)			0.192
Murine	18 (72.0)	20 (55.6)	
Fully human	7 (28.0)	16 (44.4)	

EMM, extramedullary myeloma; BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor; ECOG, Eastern Cooperative Oncology Group; DS, Durie Salmon; ISS, International Staging System; MFI, Mean Fluorescence Intensity.

[&]Cytogenetic features were measured using florescence in situ hybridization. The probes include t(4;14), Del(17p), 1q21, t(14;16), Del (13q), t(11;14) and. High risk cytogenetic features (any t(4;14), Del(17p), and t(14;16)) evaluated with conventional cytogenetics or fluorescence in-situ hybridization (FISH).

TABLE 3 | Comparison of adverse events occurred in 10% or more patients during the first eight weeks post-infusion between EMM and non-EMM patients in two clinical trials conducted in our center.

Adverse event	EMM (n = 25)				non-EMM (n = 36)				*p*-Value
	Grade 1-2n (%)	Grade 3n (%)	Grade 4n (%)	Any Graden (%)	Grade 1-2n (%)	Grade 3n (%)	Grade 4n (%)	Any Graden (%)	
Hematologic									
Leukopenia	1 (4.0)	1 (4.0)	23 (92.0)	25 (100.0)	1 (2.8)	1 (2.8)	34 (94.4)	36 (100.0)	1.000
Neutropenia	1 (4.0)	2 (8.0)	22 (88.0)	25 (100.0)	0 (0.0)	1 (2.8)	35 (97.2)	36 (100.0)	0.410
Lymphopenia	0 (0.0)	0 (0.0)	25 (100.0)	25 (100.0)	0 (0.0)	0 (0.0)	36 (100.0)	36 (100.0)	–
Anemia	6 (24.0)	15 (60.0)	2 (8.0)	23 (92.0)	4 (11.1)	28 (77.8)	4 (11.1)	36 (100.0)	0.056
Thrombocytopenia	6 (24.0)	3 (12.0)	16 (64.0)	25 (100.0)	4 (11.1)	3 (8.3)	29 (80.6)	36 (100.0)	0.292
Coagulative									
Prolonged APTT	17 (68.0)	0 (0.0)	0 (0.0)	17 (68.0)	28 (77.8)	1 (2.8)	0 (0.0)	29 (80.6)	1.000
Fibrogenopenia	4 (16.0)	1 (4.0)	0 (0.0)	5 (20.0)	15 (41.7)	2 (5.6)	0 (0.0)	17 (47.2)	1.000
Metabolic									
Hypokalemia	18 (72.0)	0 (0.0)	0 (0.0)	18 (72.0)	19 (52.8)	3 (8.3)	0 (0.0)	22 (61.1)	0.262
Hyponatremia	8 (32.0)	2 (8.0)	0 (0.0)	10 (40.0)	12 (33.3)	1 (2.8)	1 (2.8)	14 (38.9)	1.000
Hypocalcemia	14 (56.0)	1 (4.0)	0 (0.0)	15 (60.0)	20 (55.6)	0 (0.0)	0 (0.0)	20 (55.6)	0.410
Elevated ALT	7 (28.0)	2 (8.0)	0 (0.0)	9 (36.0)	11 (30.6)	1 (2.8)	0 (0.0)	12 (33.3)	0.562
Elevated AST	6 (24.0)	2 (8.0)	0 (0.0)	8 (32.0)	13 (36.1)	3 (8.3)	0 (0.0)	16 (50.0)	1.000
Heart failure	4 (16.0)	0 (0.0)	2 (8.0)	6 (24.0)	8 (22.2)	0 (0.0)	1 (2.8)	9 (25.0)	0.562
Arrhythmia	5 (20.0)	3 (12.0)	0 (0.0)	8 (32.0)	9 (25.0)	0 (0.0)	0 (0.0)	9 (25.0)	0.064
Creatinine increased	3 (12.0)	2 (8.0)	0 (0.0)	5 (20.0)	3 (8.3)	1 (2.8)	0 (0.0)	4 (11.1)	0.562
Others									
Fever	16 (64.0)	3 (12.0)	0 (0.0)	19 (76.0)	26 (72.2)	6 (16.7)	0 (0.0)	32 (88.9)	0.725
Lung infection	0 (0.0)	9 (36.0)	0 (0.0)	9 (36.0)	3 (8.3)	17 (47.2)	1 (2.8)	21 (58.3)	0.307
Upper respiratory infection	3 (12.0)	1 (4.0)	0 (0.0)	4 (16.0)	3 (8.3)	0 (0.0)	0 (0.0)	3 (8.3)	0.410

APTT, activated partial thrombin time; ALT, alanine aminotransferase; AST, aspartate aminotransferase. The grading of AE was according to the CTCAE 4.03. The P value is based on Fisher's exact test, or Pearson's chi-squared test. P values less than 0.05 (two-tailed) were considered statistically significant.

RESULTS

Anti-BCMA CAR-T Clinical Trials and Patient Characteristics

Eight anti-BCMA CAR-T clinical trials had enrolled EMM patients (10–17,) and seven of them presented the preliminary efficacy of EMM patients. We summarized their results in **Table 1**. The ORR of EMM ranged from 57% to 100%, with the complete remission (CR) rate of 29% to 60%.

We compared the baseline characteristics of 25 EMM patients and 36 non-EMM patients in the two trials conducted in our clinical centers (**Table 2**). The analysis showed no statistical differences between EMM and non-EMM patients in baseline characteristics except for high-risk cytogenetics. The median age of EMM and non-EMM patients was 55 (range, 34 - 70) years and 53 (range, 34 - 69) years, respectively. The median time from diagnosis to infusion was 3.2 (range, 0.8 - 12.6) years for EMM patients, and 2.9 (range, 0.7 - 11.3) years for non-EMM patients. 6 (24%) EMM patients and 18 (50%) non-EMM patients had high-risk cytogenetic profile.

Safety

A total of 73 different types of adverse events were recorded, and those with incidence ≥ 10% were summarized in **Table 3**. No statistical differences were observed between EMM and non-EMM patients in all AEs. The most common AEs of grade ≥ 3 in both groups was hematologic toxicities, including leukopenia, lymphopenia, neutropenia, anemia, and thrombocytopenia. The median recovery times of neutropenia for EMM and non-EMM patients were 9 (range, 0 to 45) days and 10 (range, 0 to 58) days post-infusion, respectively (**Figure 2A**). Delayed recovery of neutropenia (4.00% in EMM and 8.57% in non-EMM) was observed in both groups.

Patients with EMM tended to have lower cytokine release syndrome (CRS) grade than individuals without EMD in both trials, although the difference was not statistically significant (**Figure 2B**). The incidence of ≥ grade 3 CRS was 12% in EMM patients and 27.78% in non-EMM patients, respectively. Only one of the non-EMM patients that received murine anti-BCMA CAR-T therapy experienced ICANS. No differences were observed in serum ferritin and IL-6 levels between the two groups (**Figures 2C, D**).

Efficacy

There was no significant difference in the ORR and ≥ CR rate between EMM and non-EMM patients. The ORR of EMM and non-EMM patients was 84.00% (21/25) and 94.44% (34/36), respectively (**Figure 3A**; $p = 0.363$). The ≥ CR rate of EMM and non-EMM patients was 36.00% (9/25) and 61.11% (22/36), respectively (**Figure 3A**; $p = 0.054$). However, for patients receiving the fully human anti-BCMA CAR-T cell therapy, the ≥ CR rate of EMM patients was lower than non-EMM patients (**Figure 3A**; 28.57% vs. 81.25%; $p = 0.026$).

The median follow-up time was 873 days. The Kaplan-Meier method showed that there were significant differences in PFS (121 days vs. 361 days, $p = 0.001$) and OS (248 days and 1024 days, $p = 0.005$) for all the EMM and non-EMM patients

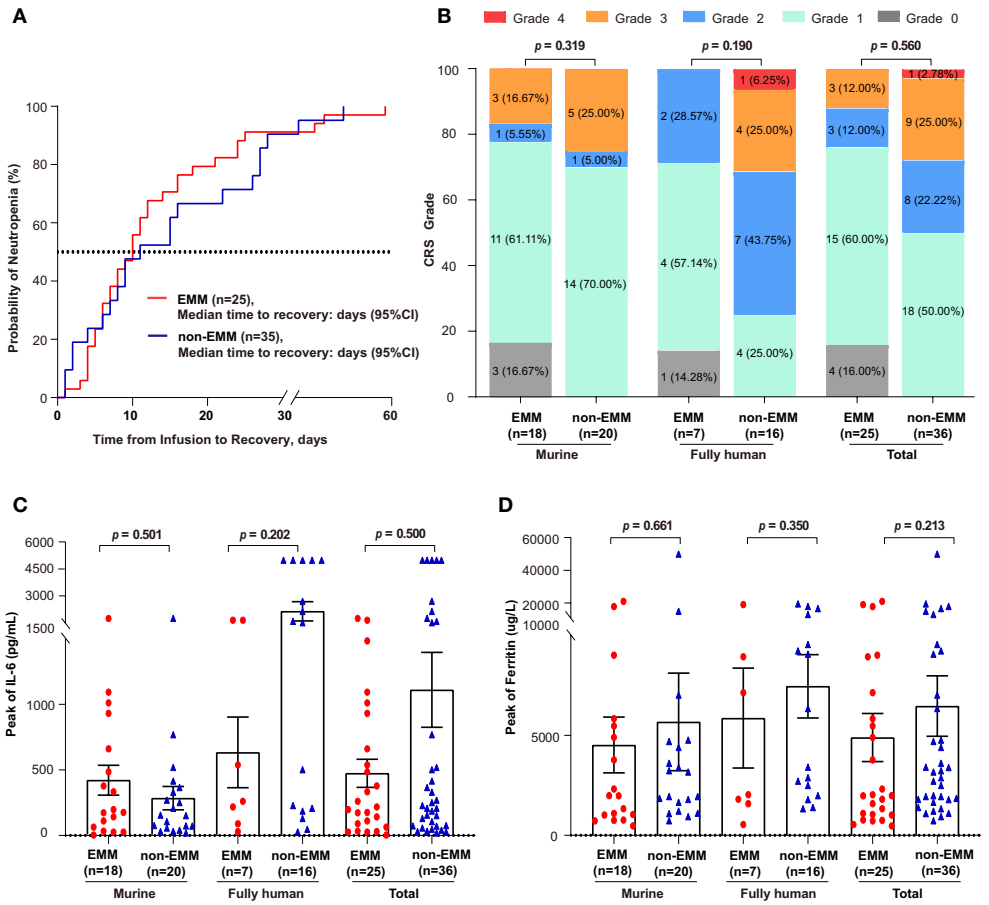

FIGURE 2 | Comparison of time to recovery of neutrophil, cytokine release syndrome (CRS), and inflammatory factors between EMM patients and non-EMM patients receiving anti-BCMA CAR-T cell therapy in two clinical trials conducted in our center **(A)** Time to the recovery of patients with grade 3/4 neutropenia is shown. One non-EMM patient was excluded because the neutrophil count had not recovered until death (OS = 20 days). Time to recovery, which is defined as absolute neutrophil count ≥1000 cells/μL from infusion till the first time of recovery. **(B–D)** Comparison of the CRS grade, the peak levels of IL-6 and Ferritin.

(**Figures 3B, C**). Interestingly, for the patients that received murine CAR, the difference was only observed in OS (248 vs. 640 days, p = 0.007) other than PFS (120 vs. 181 days, p = 0.099). While for patients that received fully human CAR, the difference was only observed in PFS (121 days vs. not reach (NR); p = 0.005) other than OS (both NR; p = 0.400). The rates of OS for EMM and non-EMM patients that received fully human CAR were 57.14% (4/7) and 75% (12/16) at one year, respectively.

Pharmacokinetics

After infusion, the peak value of CAR copies (C_{max}) and the area under the curve of the transgene level from infusion to 28 days (AUC_{0-28d}) in EMM patients was lower than in non-EMM patients (C_{max}, p = 0.016; AUC_{0-28d}, p = 0.016) (**Figures 4A, C**). There was no difference in the T_{max} for EMM and non-EMM patients (**Figure 4B**). As shown in **Figure 4D**, the CAR copies of EMM patients were lower than non-EMM patients from the infusion to the last follow-up. CAR-T cells tended to have lower expansion in EMM patients than in non-EMM patients.

Risk Factors

We further analyzed the factors that may impact the OS and PFS of patients receiving anti-BCMA CAR-T therapy in a Cox model (**Figure 5**). Univariate Cox regression analysis showed that previous lines, best response, and extramedullary diseases were significantly associated with OS and PFS ($p < 0.05$). Multivariate Cox regression analysis revealed that extramedullary disease was also an independent prognostic risk factor in RRMM patients receiving anti-BCMA CAR-T cell therapy (hazard ratio, 2.576; 95% CI, 1.343 to 4.941; p = 0.004; OS hazard ratio, 2.312; 95% CI, 1.165 to 4.592; p = 0.017).

DISCUSSION

In general, the prognosis of EMM, including PCL patients, is poor. There is currently no consensus on the standard regimen

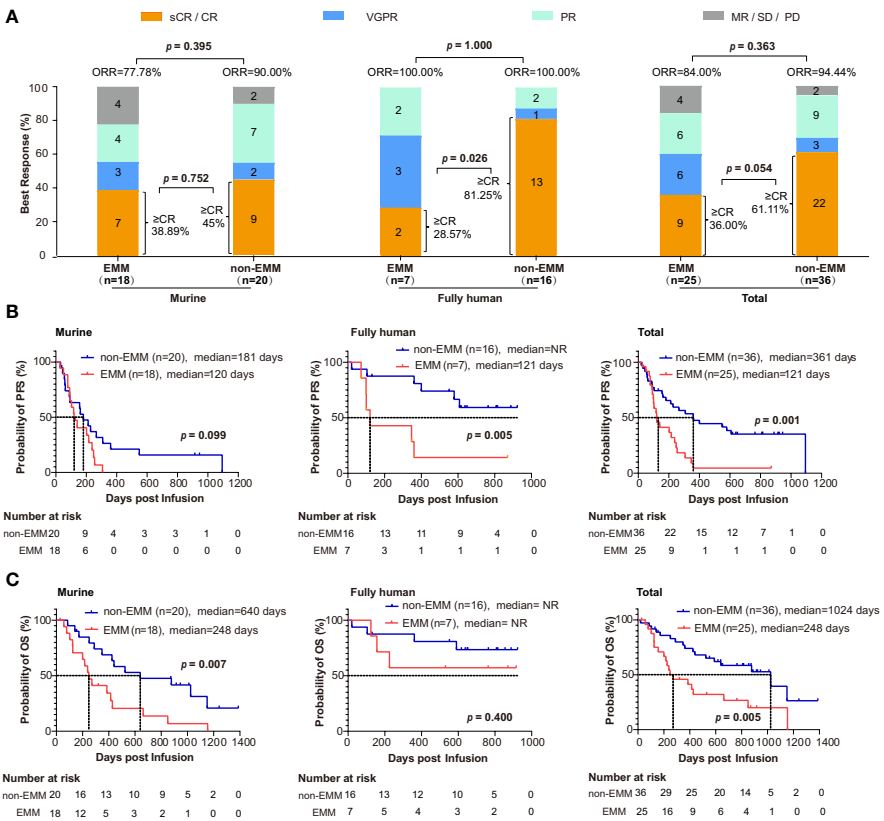

FIGURE 3 | Comparison of clinical response and survival analysis between EMM patients and non-EMM patients receiving anti-BCMA CART cell therapy in two clinical trials conducted in our center **(A)** ORR was analyzed between EMM and non-EMM. The *p* value is based on Fisher's exact test. **(B, C)** Analysis of PFS and OS for EMM and non-EMM patients using the Kaplan-Meier method. P values less than 0.05 (two-tailed) were considered statistically significant. CR, complete response; EMM, extramedullary myeloma; ORR, objective response rate; MR, minimal response; NR, not reach; OS, overall survival; PD, relapse/progressive disease. PFS, progression-free survival; PR, partial response; sCR, stringent complete response; SD, stable disease; VGPR, very good partial response.

for EMM patients, and few clinical studies are designed for them. The efficacy of conventional chemotherapeutic drugs and novel agents, either alone or combined, was limited in EMM patients (7, 8, 22). The ORR of secondary EMM patients receiving novel agents, such as Carfilzomib, Daratumumab, Lenalidomide, etc., was mostly reported no more than 50% (23–26). What's more, the median PFS and OS of daratumumab-based therapy for EMM patients were only 69 days and 198 days, respectively, in one study (26). In another study that included 357 MM (24 secondary EMM) patients, the median PFS and OS for these secondary EMM patients was about two months and seven months, respectively (27).

Compared to the drugs' limited effect mentioned above, anti-BCMA CAR-T therapy is a promising strategy for RRMM patients with EMM (10–16). In our study, the ORR of EMM patients reached 100% with ≥ CR rate of 28.57% in the fully human trial, which was significantly higher than existing regimens, and the median PFS and OS were also longer. Similar results were reported by other studies (28–32). These encouraging results showed that anti-BCMA CAR-T therapy has obvious predominance over the existing drugs in response rate, depth of remission, and survival. But in subgroup analysis, the treatment efficacy in EMM patients was not as satisfactory as in non-EMM patients, as we observed lower ≥ CR rate and shorter PFS/OS in EMM patients. As we analyzed the murine and fully human CAR separately, the difference between EMM and non-EMM patients was only observed in PFS for patients receiving fully human CAR. Meanwhile the difference in OS was only observed in patients receiving murine CAR. These findings may result from the advantage in remission depth of our fully human CAR over our murine CAR (12, 14), as increased depth of response is often associated with improved response durability (10). Moreover, our study demonstrated that extramedullary disease was an independent prognostic risk factor for RRMM patients receiving anti-BCMA CAR-T therapy.

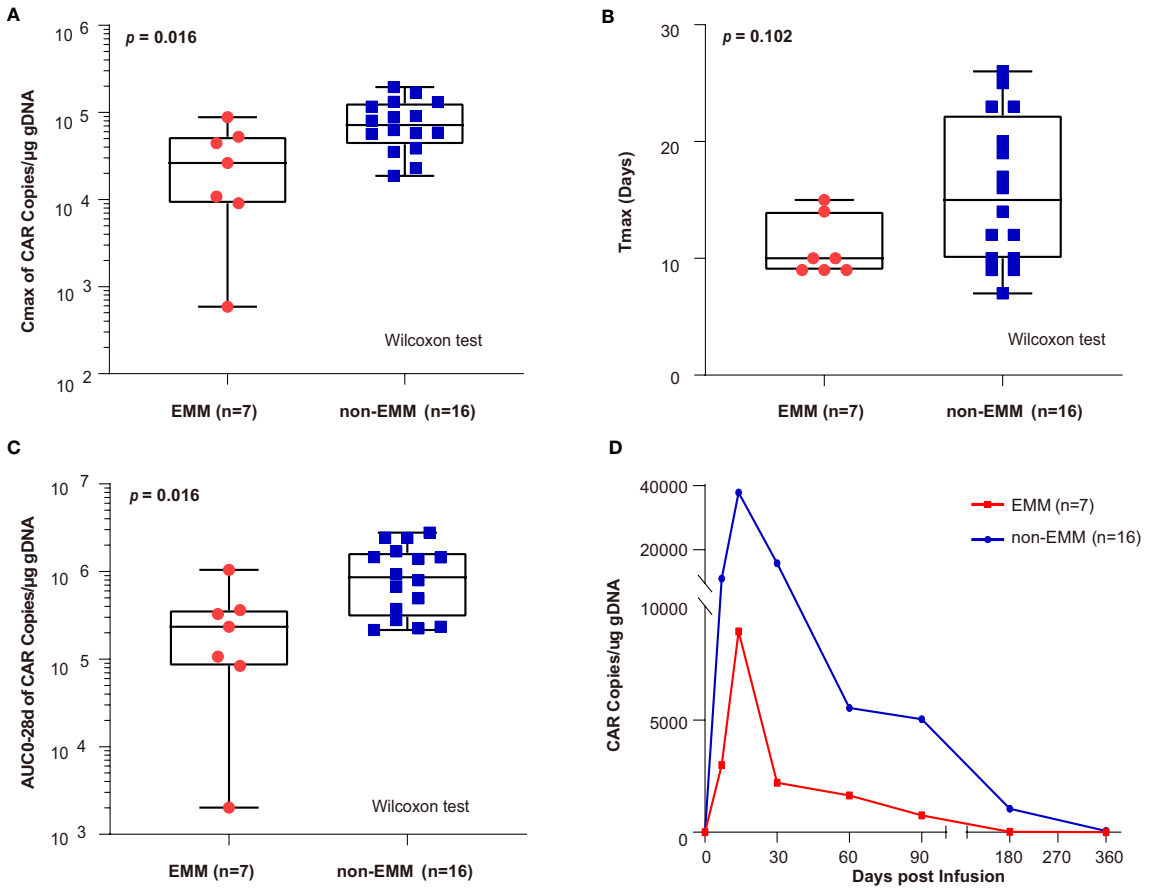

FIGURE 4 | Comparison of CAR transgene kinetics between EMM patients and non-EMM patients receiving novel fully human anti-BCMA CAR-T infusion conducted in our center **(A–C)** the comparison of C_{max}, T_{max}, and area under the curve of the transgene level from infusion to 28 days (AUC_{0-28d}) between EMM and non-EMM patients that received novel fully human anti-BCMA CAR-T therapy. The *p* value is based on a twosided Wilcoxon ranksum test and *p* values less than 0.05 (two-tailed) was considered statistically significant. **(D)** The Car copies from infusion to the last follow-up were compared between EMM and non-EMM patients. Data presented as a geometric mean. AUC_{028d}, area under the curve during the first 28 days after the infusion; Cmax, peak value of CAR copies; Tmax, the time elapsed till peak value appeared.

Most published studies have demonstrated the safety of anti-BCMA CAR-T cell therapy to RRMM. However, there is limited data available for EMM patients (10, 17). In our study, we demonstrated that anti-BCMA CAR-T cell therapy is safe for EMM patients. In addition, we found that EMM patients tended to have a lower grade of CRS than non-EMM patients. The low expansion of CAR-T cells in EMM patients may be one of the reasons for lower CRS grade and poorer efficacy. Although ORR was over 90% in both trials, our fully human CAR has significantly longer persistency than our murine CAR (12, 14). The mechanism for the poor persistency of CAR T-cells is complicated. T-cell exhaustion and senescence, immune escape, costimulatory domain selection, generation of anti-drug antibody, and other mechanisms may contribute to the low expansion of CAR T-cells (33–36). EMM subclones are highly heterogenic, which can more easily generate clones with escape mutations of BCMA (7). Moreover, subclones of EMM could thrive and grow independent of the bone marrow microenvironment, resulting in a relatively high-risk and more 'hostile' microenvironment for the penetration and persistence of CAR T-cells (37). How EMM negatively impacts CAR-T efficacy is still unknown and needs further investigation.

Taken together, this work described the efficacy and safety of anti-BCMA CAR-T cell therapy in EMM patients from the two clinical trials conducted in our center. According to our studies, although it holds great promise for those patients, the duration and depth of remission seems to be limited compared with non-EMM patients. Further trials are needed to combine CAR-T cell therapy with other new agents, or stem cell transplant, to achieve a better result in EMM patients.

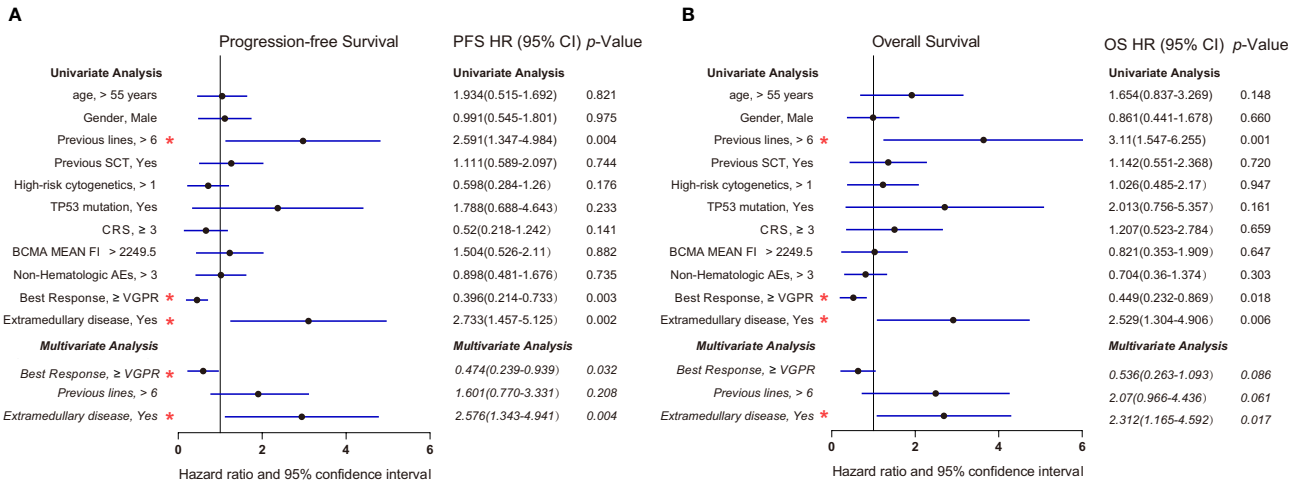

FIGURE 5 | Forest plots showing the association between risk factors and survival in our center **(A)** Progression-free survival (Cox regression, HR, and 95% CI). **(B)** Overall survival (Cox regression, HR, and 95% CI). Multivariate Cox regression analysis was performed by using the significant prognostic factors identified in univariate analysis. Multivariate analysis was indicated in italics. *p* values less than 0.05 (two-tailed) were considered statistically significant (highlighted with red "*"). AEs, adverse events; CRS, cytokine release syndrome; HR, hazard ratio; MFI, Mean Fluorescence Intensity; SCT, stem cell transplantation; VGPR, very good partial response.

DATA AVAILABILITY STATEMENT

For original data, please contact the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ, CL, and DW designed the clinical study, screened and recruited participants, examined patients and analyzed the clinical data. Moreover, they also supervised the CAR-T cell production for preclinical quality control. MX collected clinical data and was responsible for patient follow-up. YQ checked, extracted, and analyzed data and performed statistical analyses. What's more, YQ also interpreted data and wrote the manuscript. YQ and YX edited and formatted the document. All authors contributed to the article and approved the submitted version.

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REVIEW
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Chimeric Antigen Receptor T-Cell Therapy in Lung Cancer: Potential and Challenges

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Chimeric antigen receptor T (CAR-T) cell therapy has exhibited a substantial clinical response in hematological malignancies, including B-cell leukemia, lymphoma, and multiple myeloma. Therefore, the feasibility of using CAR-T cells to treat solid tumors is actively evaluated. Currently, multiple basic research projects and clinical trials are being conducted to treat lung cancer with CAR-T cell therapy. Although numerous advances in CAR-T cell therapy have been made in hematological tumors, the technology still entails considerable challenges in treating lung cancer, such as on–target, of–tumor toxicity, paucity of tumor-specific antigen targets, T cell exhaustion in the tumor microenvironment, and low infiltration level of immune cells into solid tumor niches, which are even more complicated than their application in hematological tumors. Thus, progress in the scientific understanding of tumor immunology and improvements in the manufacture of cell products are advancing the clinical translation of these important cellular immunotherapies. This review focused on the latest research progress of CAR-T cell therapy in lung cancer treatment and for the first time, demonstrated the underlying challenges and future engineering strategies for the clinical application of CAR-T cell therapy against lung cancer.

Keywords: chimeric antigen receptor, T cell, immunotherapy, lung cancer, engineering strategy

1 INTRODUCTION

Lung cancer is one of the most frequently occurring malignant tumors worldwide and is characterized by a substantially high malignancy and poor prognosis (1). According to the latest global cancer statistics, lung cancer remains the leading cause of cancer-related deaths worldwide (2). Lung cancer can be histologically classified into two main subtypes: small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC) (3). NSCLC accounts for approximately 85% of diagnosed lung cancer cases and can be further divided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (4, 5).

The present therapeutic measures for NSCLC primarily include surgical resection, chemoradiation, molecular-targeted therapy, and immunotherapy (6). The surgical resection procedure was based on the TNM stage of NSCLC patients. Conventional or stereotactic radiotherapy is applicable to patients with surgically unresectable NSCLC (7). Platinum-based double-agent combination chemotherapy is generally accepted as the standard chemotherapy regimen for NSCLC (8). Neoadjuvant chemotherapy is applied preoperatively to downgrade the cancer stage, whereas adjuvant chemotherapy is administered postoperatively, primarily involving cisplatin-based combination regimens (7). The primary molecular-targeted therapies include epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), anti-EGFR monoclonal antibodies, fusion gene ALK and ROS1 inhibitors, and anti-vascular endothelial growth factor receptor monoclonal antibodies (9–12). Combined therapy with multiple immune checkpoint inhibitors, such as a combination of nivolumab and ipilimumab, has been shown to achieve better response rates than monotherapy (13, 14).

Non-surgical treatment involving systemic chemotherapy plus radiotherapy is the mainstream procedure for SCLC patients because metastases occur when SCLC is newly diagnosed. Etoposide-platinum and topotecan are the standard first-line and second-line regimens for SCLC patients, respectively (15, 16). Although SCLC is very sensitive to chemotherapy, many SCLC patients relapse due to the clinical development of chemoresistance. Moreover, nivolumab was the first FDA-approved immunotherapy agent for SCLC treatment (17). Several small molecular inhibitors, including PARP inhibitors, have also been demonstrated to exert anti-tumor activity in SCLC in clinical trials (18, 19). However, due to the heterogeneity of tumors, it is imperative to explore effective novel therapies.

Chimeric antigen receptors (CARs) are engineered receptors that can enable modified T cells to recognize and kill tumor cells expressing a tumor-specific antigen (20). CAR-T cells contain two sections: autologous T cells separated from the peripheral blood of patients and integration of CARs into T cells through genetic engineering in the laboratory. Patient's T cells are extracted, isolated, and genetically engineered to express a CAR on their surface, targeting tumor-specific antigens of cancer cells. The modified CAR-T cells are amplified *in vitro* and then infused back into the patients **(Figure 1)** (21). Subsequently, CARs can identify and bind to specific antigens expressed on cancer cells and consequently eliminate and kill cancer cells (22, 23).

CAR-T cell therapy is an emerging method against hematological malignancies and has demonstrated satisfactory curative effects, which is a substantial breakthrough in adoptive cell therapy (24, 25). CAR-T cells targeting CD19 have become a leading engineered T-cell therapy strategy against relapsed or refractory acute lymphocytic leukemia and B-cell non-Hodgkin lymphoma (26, 27). Yescarta (axicabtagene ciloleucel) and Kymriah (tisagenlecleucel) are currently approved to treat B-cell-derived malignancies, with response rates greater than 80% (28, 29). Recently, Tecartus (brexucabtagene autoleucel) has also been approved for the treatment of adult mantle cell lymphoma (30, 31). However, only targeting CD19 did not show considerable efficacy in most refractory multiple myeloma (MM) patients, partly due to the lower expression of CD19 on the cell surface of myeloma, and there is no FDA-approved CAR-T cell therapy against it (22, 32, 33). Clinical trials have indicated that CD269 (B cell maturation antigen, BCMA) and CD138 (also known as syndecan 1) molecules, which are mostly expressed in mature B cells or plasma cell surfaces, could exert substantial anti-MM activity (34–36). The unprecedented achievements of CAR-T cell therapy in hematological malignancies have also improved the use of CAR-T cells in various solid tumors.

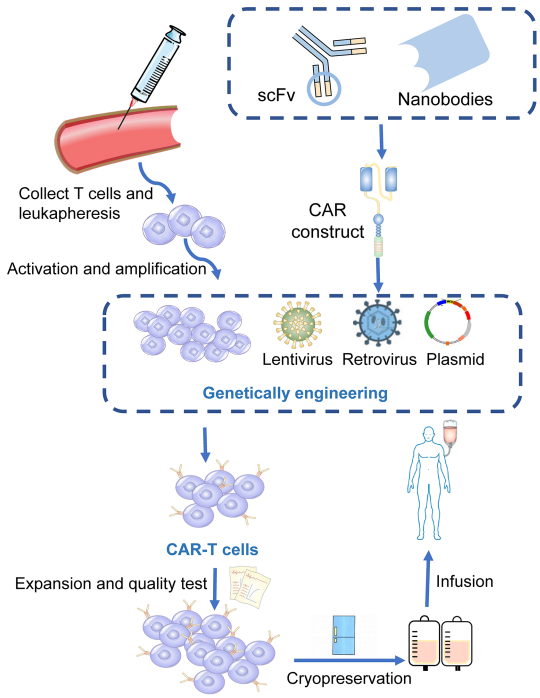

FIGURE 1 | Manufacturing procedures of CAR-T cells. T cells are firstly collected from the peripheral blood of the patients. The activated and amplified T cells are genetically engineered with CAR structure *via* retroviral, lentivirus or other vectors. CAR-T cells are then expanded *ex vivo* and a quality control procedure is applied. Finally, those modified T cells were infused back into the patients.

1.1 The Design and Development of CAR Structure

CARs are artificial fusion proteins that comprise four major parts: extracellular antigen recognition and binding domains, spacer/hinge domains, transmembrane domains, and intracellular signaling domains (37, 38). Every component of the CAR structure has unique properties and has evolved to optimize the CAR function (39). The extracellular domains are responsible for recognizing and binding the targeted tumor-specific antigens, whereas intracellular signal domains

primarily induce T-cell proliferation and corresponding signal transduction **(Figure 2)** (40). Recently, armored CAR-T cells have been engineered to overcome immunosuppressive tumor microenvironment (TME) (41). Engineered CAR-T cells can secrete various cytokines such as IL-12, chemokines, or co-expressing immunomodulatory ligands to alter the inhibitory microenvironment in the TME and support CAR-T cell function (20).

1.1.1 Antigen Recognition and Binding Domains

The single-chain variable fragment (scFv) is derived from the variable heavy and variable light chains of a monoclonal antibody connected by a flexible linker (42). It is the major component of the extracellular antigen recognition and binding moieties, which can effectively recognize tumor antigen targets in a major histocompatibility complex (MHC)-independent manner and trigger CAR downstream signaling and CAR-T cells (43). The scFv sequences determine the specificity and binding affinity of the targeted antigens of the CAR (44). The high affinity of scFv has been reported to result in on-target, off-tumor toxicity, and severe cytokine release syndrome (45). Moreover, scFv can be designed to bind to soluble ligands, such as transforming growth factor-beta (TGF-β), contributing to the conversion of the immunosuppressive role of TGF-β (46). Single-domain antibodies (known as nanobodies or VHHs), whose variable regions only contain heavy chains instead of light chains, are stable camelid-derived single-domain antibodies (47). They are smaller in size and have a similar affinity to traditional scFv; however, they avoid the shortcomings of traditional scFv, such as low folding efficiency and tendency to aggregate (48, 49). In addition, cytokines (50), ligands (51–54) and antigen recognition peptides (adnectins and designed ankyrin repeat proteins) could be applied as an option for antigen recognition and binding regions of CARs (55, 56).

1.1.2 Hinge Domains

The length of the hinge regions can be adjusted to optimize the distance between CAR-T cells and targeted tumor cells, ensuring the folding efficiency of CAR scFv and providing a flexible and persistent connection for CAR signal transduction (57). In addition, the domains also augment the binding affinity of CAR-T cells and targeted cells (38). Hinge domains play a crucial role in regulating the expression and transport efficiency of CAR and the definition of the CAR signaling threshold (57). The spacer domains enable the CAR to access target epitopes that are otherwise sterically inaccessible (58). They can also be used to modulate synaptic cleft distances, as distal membrane antigen epitopes commonly require shorter spacers, whereas proximal membrane antigen epitopes require longer spacers (58, 59). Non-IgG-based spacers, including CD8 and CD28, and IgG-based spacers, such as IgG1 or IgG4, have been proven to be equally effective and are utilized in the construction of CAR hinge domains (58, 60). The spacers containing Fc domains must be changed after recognizing the targeted antigens, in case of *in vivo* interactions with cells expressing Fc gamma receptors that result in off-target activation of CAR-modified T cells or impaired antitumor efficacy (61).

1.1.3 Transmembrane Domains

The transmembrane domains serve as anchors to connect the extracellular antigen-binding domain to the cell membrane and transduce extracellular antigen-recognition signals to the intracellular domains (38, 58). They primarily originate from type I transmembrane proteins, including CD3ζ, CD8-alpha, CD4, or CD28 (20, 62). The stability and function of CARs are associated with transmembrane domains (38). Bridgeman et al. reported that CARs containing the CD3ζ transmembrane domain can form a complex with

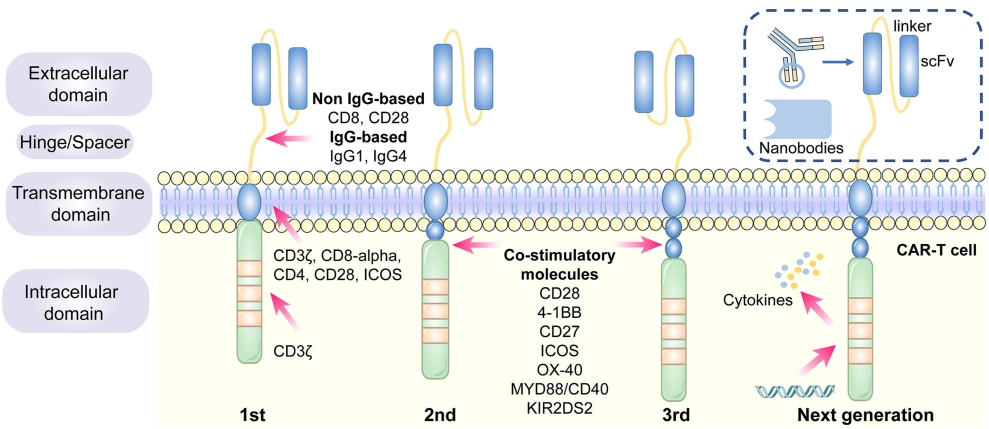

FIGURE 2 | The structure and evolution of CAR-T cells from the first generation to the fourth generation. The CAR-T cells are consisted of extracellular tumor antigen binding domains (scFv, nanobodies), hinge regions, transmembrane regions and intracellular signaling domains. Different generations of CAR structures are primarily characterized by distinct intracellular signaling domains. The first generation of CAR-T cells only contain a CD3ζ intracellular signaling domain, with less persistence and efficacy in clinical practice. The second or third generation of CAR-T cells include one or more costimulatory molecules, and the next generation of CAR-T cells are engineered to express cytokines, which greatly improve their competence to eliminate the tumor cells.

endogenous T cell receptor (TCR), and subsequently, may induce T cell activation (63). *In vivo* studies indicated that CD8-alpha resulted in lower levels of inflammatory cytokines and T-cell activation-induced death than CD28 (64). CD28 is currently the most stable transmembrane domain (39). Third-generation CAR T cells carry a B7-family inducible costimulator (ICOS) transmembrane domain (65). The persistence and anti-tumor activity of CAR-T cells is substantially promoted when the ICOS transmembrane domain is connected to an ICOS intracellular domain (62).

1.1.4 Intracellular Signaling Domains

The endodomains normally comprise a CD3ζ transducer, and one or more co-stimulatory signaling molecules such as CD28, 4-1BB (CD137), CD27, ICOS, OX-40, MYD88/CD40, and KIR2DS2 (66). This design pattern further prolongs the survival time and promotes the proliferation and antitumor activities of CAR-T cells (38, 67, 68). CD28 and 4-1BB, fused to the intracellular CD3ζ domain, are the most extensively studied and intensively applied co-stimulatory molecules (69). However, their clinical efficacy is far from each other. CAR-T cell therapy based on 4-1BB costimulatory domain is generally admitted to have more superior clinical efficacy, because 4-1BB costimulatory domain could ameliorate the exhaustion mediated by CAR signaling (70, 71). CAR-T cell product based on CD28 costimulatory domain initiates faster antitumor property, while compared with 4–1BB costimulatory domain, it is less persistent since fewer central memory T cells are formed (72) (**Table 1**). Additionally, CAR-T cells, incorporated two costimulatory molecules, such as ICOS and 4-1BB, have showed tremendous efficacy in preclinical mouse models (62, 73).The other co-stimulatory signaling molecules, including CD27 (74, 75), OX-40 (76, 77), MYD88/CD40 (78) and KIR2DS2 (79) have demonstrated promising efficacy in preclinical models but have not been tested in clinical trials.

1.2 The Generation of CAR–T Cells

Different generations of CAR structures, characterized by distinct intracellular signaling domains, have been designed to improve the safety and efficacy of CAR-T cell therapy against various cancers (80). First-generation CAR-T cells only contain one intracellular signaling domain, CD3ζ, with less impressive clinical efficacy for the lack of persistence and proliferative activity (38). Inclusion of the costimulatory molecules equipped with second-generation CAR-T cells with the necessary signals for activation considerably prolonged the survival time of CAR-T cells and improved clinical outcomes in cancer patients (81). Third-generation CAR-T cells aggrandize a costimulatory molecule compared with second-generation CAR-T cells, consisting of CD3ζ and two costimulatory molecules (CD27, CD28, 41BB, ICOS, OX-40, etc.), further augmenting and enhancing their competence to clear tumor cells (82, 83). In particular, the fourth generation of CAR-T cells known as T cells redirected for universal cytokine-mediated killing (TRUCK), which can recruit nuclear factor of activated T cells (NFAT) to induce the release of cytokines IL-12 IL-15 and granulocyte–macrophage colony-stimulating factor (84). The anti-tumor activity of the fourth generation of CAR-T cells is enhanced by overcoming the immunosuppressive effect of the TME (**Figure 2**). The fifth-generation CAR-T cells, which is proposed to remove the TCR alpha and beta chains through gene editing technology, avert the risk of graft-vs.-host disease, and manufacture "off the shelf" products, are still under investigation (85).

Although the structure of CARs is constantly evolving to promote efficacy and diminish the cytotoxic effects of CAR-T cell therapy, second-generation CAR-T cells still remain the mainstay of clinical application (86).

1.3 NSCLC and SCLC–Associated Antigens for CAR–T Cell Therapy in Preclinical Studies

CAR-T cell therapy has emerged as a novel approach to adoptive cell immunotherapy in recent decades. In solid cancers, it is more complex to construct CAR-T cells because it is difficult to identify tumor-specific antigens to be targeted. Several surface antigens have already been evaluated in preclinical studies as potential CAR-T cell therapy targets. Thereafter, we provide detailed descriptions of several novel targets.

1.3.1 Mesothelin (MSLN)

MSLN, a tumor differentiation antigen with the low expression on normal mesothelial cells, is overexpressed in a wide range of solid cancers, including lung cancer, mesothelioma, and pancreatic carcinoma; therefore, it could be used as a potential target (87, 88). High expression of MSLN is commonly correlated with negative clinical outcomes in NSCLC (67). In *ex vivo* experiments, MSLN-targeted CAR-T cells exerted substantial inhibitory effects on cancer cell proliferation and invasion (89). The efficiency of MSLN-targeted CAR-T cell therapy has been assessed in subcutaneous mouse lung cancer models (90). A slower growth rate of tumor size was observed in the tail vein injection of MSLN-targeted CAR-T cells (89). In

TABLE 1 | Comparison of properties of different costimulation 4-1BB versus CD28 in CAR-T cell.

Property	4-1BB	CD28
Expansion ability	Low	High
Anti-tumor response	Persistent	Rapid
Susceptibility to exhaustion	Low	High
Phenotype formation	Memory phenotype	Effector phenotype
Metabolic type	Fatty acid oxidative metabolism	Glycolytic metabolism
Overall efficacy	Superior	Inferior

summary, MSLN-targeted CAR-T cells could be feasible for MSLN-positive cancers, such as NSCLC.

1.3.2 EGFR

EGFR belongs to the HER/ErbB family of receptor tyrosine kinases that transduces extracellular growth signaling into the cells (91). More than 60% of NSCLC patients harbor activating EGFR mutations, contributing to the overexpression of EGFR, making it possible to target EGFR as a treatment for CAR-T cell therapy against NSCLC (91). EGFR-CAR T cells were found to exhibit greater cytotoxic activity *in vitro* (92). In nude mouse subcutaneous xenografts, EGFR-CAR T cells dramatically decreased tumor size and volume (93). The above results indicate that EGFR-targeted CAR-T cell therapy could be applied to NSCLC patients in the future (94).

1.3.3 Receptor Tyrosine Kinase-Like Orphan Receptor 1 (ROR1)

ROR1 is a crucial oncofetal glycoprotein that can sustain pro-survival and pro-apoptotic signaling in lung adenocarcinomas (95, 96). It has been proposed as a targeted antigen in CAR-T cell therapy as the overexpression of ROR1 protein has been observed in various malignancies, including lung cancer (97, 98). ROR1-CAR T cells maintained their anti-tumor activity, cytokine secretion, and proliferation in NSCLC models *in vitro and in vivo* (97, 99). Carolina et al. demonstrated the safety and function of second-generation ROR1 CAR-T cells in macaques (100).

1.3.4 Mucin-1 (MUC1) and Prostate Stem Cell Antigen (PSCA)

Aberrant high expression of MUC1 regulates the expression of programmed death-ligand 1 (PD-L1) in cancer cells, which could prevent cancer cells from being cleared by the immune system (101, 102). PSCA, a glycosylphosphatidylinositol (GPI)-anchored cell surface protein, belongs to the Thy-1/Ly-6 family (103). MUC-CAR T cells and PSCA-CAR T cells identify and eliminate PSCA+ or MUC1+ NSCLC cells, respectively, *in vitro* (104). PDX mouse subcutaneous models generated from NSCLC patients whose tumors only express PSCA or both PSCA and MUC1 were applied to explore the efficacy of PSCA and MUC1 CAR-T cells against NSCLC. Tumor growth was substantially inhibited in CAR-PSCA T cells. Thereafter, a combination of PSCA and MUC1 CAR-T cells exerted a synergistic effect on tumor survival (104). Therefore, MUC1 and PSCA could be promising CAR-T cell therapy targets for the treatment of NSCLC.

1.3.5 Human Epidermal Growth Factor Receptor 2 (HER2)

HER2 belongs to the HER/ErbB family of receptor tyrosine kinases involved in cell proliferation and angiogenesis (105). The anti-tumor effect of HER2 CAR-T cells against two NSCLC cell lines, A549 and H1650, was observed in a 96-h co-culture assay (106). Moreover, in orthotopic or subcutaneous A549 NSCLC mouse xenograft models, HER2 CAR-T cell therapy decreased tumor growth and could not completely eliminate tumors (106, 107).

1.3.6 Carcinoembryonic Antigen (CEA)

CEA is an oncofetal glycoprotein generally expressed during fetal development; however, its expression declines after birth (108). CEA levels increase rapidly in the tumorigenesis and development of lung cancer (109). Therefore, preclinical studies of CAR-T cell therapy targeting CEA have been conducted. CEA-targeted CAR-T cells have been found to eradicate advanced lung carcinomas (110).

1.3.7 PD-L1

Immunotherapy targeting programmed death-1(PD-1)/PD-L1 signaling has achieved substantial progress in NSCLC treatment. Accumulating evidence shows that PD-L1, both in tumor cells and in the TME, suppresses T cell proliferation and mediates anti-tumor immunity (111). PD-L1-targeted CAR-T cells exhibited robust cytotoxic effects against NSCLC cells *in vitro* and *in vivo* (112, 113). Therefore, PD-L1-targeted CAR-T cells could be a novel curative approach for PD-L1-positive NSCLC patients.

1.3.8 Fibroblast Activation Protein (FAP)

FAP is a marker expressed on cancer- associated fibroblasts (CAFs) in a majority of human malignancies (114). FAP molecule itself and FAP-positive cells in TME could contribute to cancer cell proliferation, invasion, angiogenesis and extracellular matrix (ECM) remodeling (115).

FAP targeted CAR-T cells inhibited the proliferation of TC1 and A549 lung cancer cells by eliminating FAP-positive stromal cells in mice models (114, 116). In contrast, another study claimed that FAP targeted CAR-T cell achieved limited antitumor efficacy and severe side effects for bone marrow stromal cells (BMSCs) were also being killed (117). Therefore, the feasibility of targeting FAP as a specific antigen in CAR-T therapy remains to be verified.

1.3.9 Other Targeted Antigens

Several tumor antigens, such as lung-specific X (LUNX), variant domain 6 of CD44 gene, melanoma-associated antigen-A1 (MAGE-A1), erythropoietin-producing hepatocellular carcinoma A2 (EphA2), and glypican-3 (GPC3), are under active investigation for application as targeted antigens of CAR-T cell therapy against NSCLC (118–122). For SCLC, CD56-and Delta-like ligand 3 (DLL-3)-targeted CAR-T cells are being explored (123, 124). Bivalent tandem CAR-T cells are equipped with two targeted antigens. CD70, B7-H3, MUC1, PSCA, PD-L1, and CD80/CD86, have exhibited enhanced antitumor efficacy in lung cancer (104, 125). B7-H3 is one of inhibitory ligands, which belongs to B7 immunoglobulin family. Although its corresponding immune checkpoint receptors remain undetermined, the inhibitory role of B7-H3 has been confirmed in preclinical studies (126). The expression of B7-H3 is aberrantly augmented in a wide range of solid tumor tissues, compared with normal tissues, which supports the possibility of targeting B7-H3 in CAR-T cell therapy against lung cancer (125, 127). CD80/CD86 are immune checkpoint ligands shared by inhibitory CTLA-4 and costimulatory CD28. CD80/CD86-targeted CAR-T cells have been generated to reverse the

inhibitory CTLA4-CD86/CD86 signals and prevent the survival of B cell malignancies and other tumors including NSCLC (128). The efficacy of CAR-T cell therapy, which targets both tumor cells and tumor-associated macrophages in the TME, has also been validated in NSCLC (129) (**Figure 3**).

1.4 NSCLC and SCLC–Associated Antigens for CAR–T Cell Therapy in Clinical Trials

CAR-T cell treatment has achieved substantial success against several hematological malignancies. At present, the primary task is to broaden the applications of CAR-T cell therapy from merely hematologic tumors to multiple solid tumors. Thus, its safety and efficacy in solid cancers are under intensive investigation. The feasibility of CAR-T therapy against solid tumors is currently being evaluated in approximately one-third of CAR-T clinical trials (130). Among them, the majority are on CAR-T therapy for the treatment of lung cancer. The extraordinary progress of CAR-T therapy for lung cancer is promising; however, many challenges and hurdles exist. Therefore, the clinical application of CAR-T in NSCLC and SCLC treatment is still under intensive exploration. The optimal target for CAR-T cell therapy is specifically expressed or generally overexpressed in tumor cells, whereas it is expressed at very low or limited levels in normal peripheral cells or tissues (131). Current clinical trials of CAR-T therapy against NSCLC and SCLC primarily focus on MSLN, MUC1, GPC3, PSCA, EGFR, CEA, HER2, PD-L1, ROR1, and other promising targets (**Table 2**).

2 CHALLENGES AND ENGINEERING STRATEGIES

Over the past few years, there has been a rapid increase in the use of CAR-T cell therapy to treat hematological malignancies and solid tumors. Many clinical trials have made substantial achievements; however, severe therapeutic responses to CAR-T cell therapy and unsatisfactory treatment efficacy hinder rapid development. In 2010, a patient with multiple metastases of colon cancer died after administering CAR-T cells targeting ERBB2. The patient experienced respiratory distress within 15 min after CAR-T cell transfusion and died five days after the treatment (132). Compared with hematological malignancies, solid tumors face a unique set of challenges, including issues confusing hematological malignancies, more severe and complicated related toxicities, the lack of a strongly expressed tumor-associated antigen target, low infiltration of T cells in tumor tissue, CAR-T cell exhaustion, and a highly immunosuppressive and metabolically challenging TME, which limit the safety and effectiveness of treatment (133–135). Future studies to develop practical engineering strategies to enhance the efficacy of CAR-T cell therapy and minimize adverse reactions should be conducted.

2.1 Overcoming Treatment-Related Toxicities

CAR-T cell therapy can result in a range of toxicity events. The major treatment-related toxicities include cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity (ICANS), which particularly peak in the first or second week of CAR-T cell administration, respectively (133). Patients with CRS mostly have common manifestations such as fever, tachycardia, hypoxia, dyspnea, hypertension, coagulopathy, and elevated serum cytokines, including interleukin-6 (IL-6) (136, 137). ICANS is characterized by tremor, encephalopathy, cerebellar alteration, or seizures (138). Both CRS and ICANS are caused by the activation of CAR-T cells and cytokines secreted by the associated immune cells. CAR-T cells can release pro-inflammatory cytokines, including IL-2, IL-6, and IFN-γ, and then activate more immune cells to secrete IL−1RA, IL−10, IL−6, IL−8, IFNα, and other cytokines, which eventually could lead to massive cytokine release (139). Hemophagocytic lymphohistiocytosis/macrophage activation

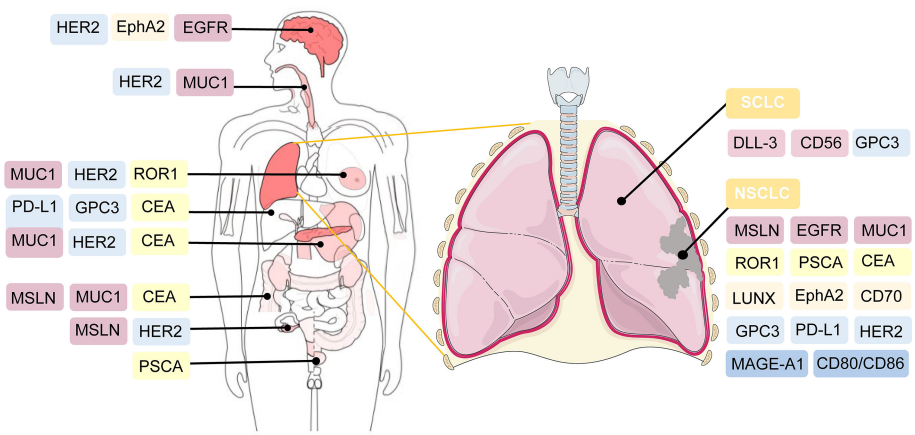

FIGURE 3 | Potential targeted antigens for CAR-T cell therapy in preclinical and clinical trials. In the right, antigen targets are listed against SCLC and NSCLC. As shown in the left of the figure, these antigens are also broadly applied in CAR-T cell therapy against other solid tumors.

TABLE 2 | Underlying targeting antigens of NSCLC and SCLC for CAR-T cell therapy in clinical trials.

Clinical Trial	Cancer type	Targeting antigen	Sponsor	Estimated Enrollment	Phases	Status
NCT03054298	NSCLC	Mesothelin	University of Pennsylvania	18	Phase 1	Recruiting
NCT03330834	NSCLC	PD-L1	Sun Yat-sen University	1	Phase 1	Terminated
NCT04489862	NSCLC	αPD1, MSLN	Wuhan Union Hospital, China	10	Early Phase 1	Recruiting
NCT03392064	SCLC	delta-like protein 3 (DLL3)	Amgen	6	Phase 1	Suspended
NCT03198546	SCLC	GPC3	Second Affiliated Hospital of Guangzhou Medical University	30	Phase 1	Recruiting
NCT04348643	Lung cancer	CEA	Chongqing Precision Biotech Co., Ltd	40	Phase1/2	Recruiting
NCT04864821	Lung cancer	CD276 (B7-H3)	PersonGen BioTherapeutics (Suzhou) Co., Ltd.	24	Early Phase 1	Not yet recruiting
NCT03740256	Advanced HER2 Positive lung cancer	HER2	Baylor College of Medicine	45	Phase 1	Recruiting
NCT02706392	NSCLC	ROR1	Fred Hutchinson Cancer Research Center	60	Phase 1	Recruiting
NCT03525782	NSCLC	MUC1, PD-L1	The First Affiliated Hospital of Guangdong Pharmaceutical University	60	Phase1/2	Recruiting
NCT02587689	NSCLC	MUC1	PersonGen BioTherapeutics (Suzhou) Co., Ltd.	20	Phase1/2	Unknown
NCT04025216	NSCLC	TnMUC1	Tmunity Therapeutics	112	Phase 1	Recruiting
NCT03198052	Lung cancer	HER2, Mesothelin, PSCA, MUC1, Lewis-Y, GPC3, AXL, EGFR, Claudin18.2, or B7-H3	Second Affiliated Hospital of Guangzhou Medical University	30	Phase 1	Recruiting
NCT03060343	NSCLC	PD-L1, CD80/CD86	Yu Fenglei	10	Phase 1	Unknown

syndrome has also been reported following CAR-T cell therapy. It is characterized by hyperinflammatory syndrome and multiple organ dysfunction (140). IL−6/IL−6R antagonists and corticosteroid usage can interrupt the inflammatory process and play a substantial role in symptom remission (141). It is critical to detect these treatment-related toxicities early and provide appropriate treatment based on the toxicity grade as soon as possible.

Selecting co-stimulatory signaling molecules and transmembrane domains could have an impact on cytokine production and CAR-T cell function. Compared with CD28/CD3ζ CAR T cells, 4–1BB/CD3ζ CAR T cells amplified more slowly, persisted for a longer time, and secreted less cytokines (142). CAR-T cells with CD8-alpha transmembrane domains have been shown to release less cytokines than those with CD28 domains (64). In addition, the inclusion of inducible caspase-9 safety switches to CARs has been verified to control the expansion of CAR-T cells and the load of cytokines (143). In summary, genetic modification of CAR designs might help reduce the generation of cytokines and the incidence of treatment-related toxicities (**Figure 4**).

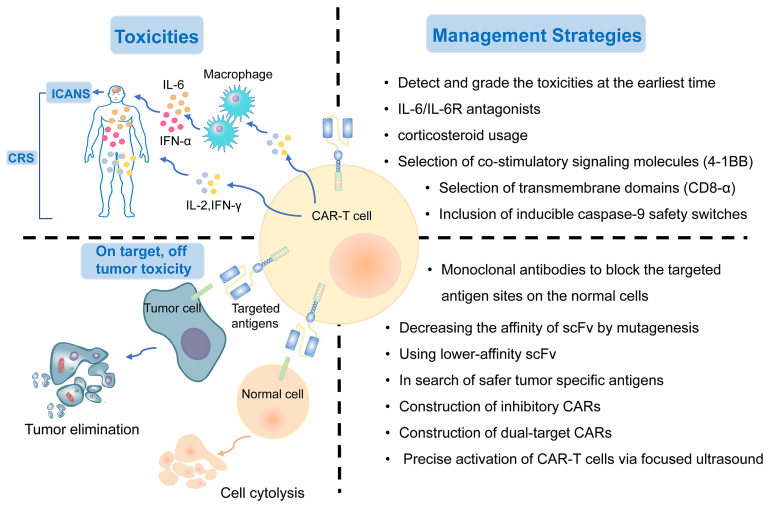

FIGURE 4 | The treatment-related and on-target, off-tumor toxicities and corresponding management strategies of CAR-T cell therapy.

2.2 On-Target, Off-Tumor Toxicity

Although the targeted tumor-associated antigens are carefully screened, many normal cells still suffer from the attack of T cells because they express the same or similar antigens. On-target, off-tumor toxicity, manifesting multiple organ injury and failure, is an issue impeding the development of CAR-T cell treatment. Thus, there is an urgent need to explore safer targeted tumor-associated antigens for lung cancer treatment. To date, MSLN, EGFR, ROR1, MUC1, PSCA, and HER2, as described previously, are the most targeted antigens in CAR-T cell therapy for NSCLC. Several other tumor antigens, including LUNX and B7-H3, also exhibit great potential as targeted antigens in CAR-T cell therapy because they are aberrantly expressed in lung cancer tissues, with a relatively low expression in normal tissues (118, 144).

The on-target toxicity is antigen-oriented, and shielding of a CAR-targeted antigen expressed on normal tissues could minimize toxicity and optimize the efficacy of CAR-T cell therapy. Some renal cell carcinoma patients developed hepatic enzyme disorders that required discontinuation of therapy after receiving anti-carbonic anhydrase IX (CAIX) CAR-T cell therapy. This on-target toxicity can be overcome by pre-administration of parental anti-CAIX monoclonal antibodies to block the CAIX antigen sites in the liver (145). In addition, decreasing the affinity of scFv by mutagenesis or using lower-affinity scFv as a replacement could also substantially reduce on-target, off-tumor reactivity without affecting the antitumor activity (45). Other attempts include the construction of inhibitory CARs, which could protect the normal cells from being attacked by targeted CAR-T cells, and dual-target CAR-T cells, which require two signals to be full activated (146). Recently, an inducible CAR-T cell, was developed to be activated *via* focused ultrasound within specific tumor sites, which could dramatically mitigate the on-target, off-tumor toxicity, in comparison to conventional CAR-T cells (147) (**Figure 4**).

2.3 Evasion of Antitumor Immune Responses

A common mechanism for tumor cells to evade immune surveillance in CAR-T cell therapy is the downregulation or even loss of targeted antigens, whose expression level could exert a direct impact on the therapeutic efficacy (148). Targeting CD19/CD20 CAR-T cell therapies have led to promising achievements in treating B-cell malignancies in recent years (149). Tumor-associated antigens in hematologic malignancies are highly expressed and easier to target, whereas antigens in solid tumors have greater heterogeneity and lower expression levels, making it difficult to eliminate solid tumor cells (150). Intratumor heterogeneity might be a key factor contributing to the evasion of antitumor immune responses (151). In lung cancer, common targets such as MSLN, MUC1, PSCA, and epithelial cell adhesion molecule, have intratumoral heterogeneity, leading to an unsatisfactory outcome of CAT-T cell therapy in lung cancer (21). Many clinical studies have shown that when tumors relapse after treatment, tumors are found to undergo antigen loss or become antigen-negative (50, 152). This phenomenon may be mediated by the selective pressure applied by CAR-T cells to tumor cells, leading to the progressive selection of antigen-negative cells (82).

To overcome the evasion of antitumor immune responses, one approach is to engineer CARs with dual-specificity (i.e., simultaneously targeting two antigens) (153). Bispecific T cell-engagers (BiTEs), consisted of two scFvs, are produced by genetically engineered CAR-T cells to redirect both T cells and CAR-T cells against specific tumor cells (154, 155). EGFRvIII-specific CAR-T cells secreting BiTE have shown to circumvent antigen escape in glioblastoma, and its effect on lung cancers remains to be further investigated (154). Tandem CAR-T cells can mitigate antigen escape and translate into superior antitumor activity (156, 157) (**Figure 5**). Armored CAR-T cells secreting pro-inflammatory cytokines, such as IL-18, have also been shown to elicit an enhanced antitumor immune response in preclinical models (158).

2.4 Physical Barriers

Cancer-associated fibroblasts (CAFs) and fibrotic environment contribute to the formation of physical barrier, preventing the CAR-T cells from being trafficked into tumor sites. Less infiltration of CAR-T cells into tumor tissues is another reason why the efficacy of CAR-T cell therapy in NSCLC is not as ideal as that in hematological malignancies.

2.4.1 CAFs

CAFs are the predominant component of stromal cells in the TME and cannot be cleared by apoptosis (159). Owing to the heterogeneity of CAFs, they could play a dual role in pro-tumorigenicity and anti-tumorigenicity (160). They could regulate the growth, invasion, and angiogenesis of tumor cells by reshaping the ECM and secreting soluble growth factors (160). Moreover, growth factors, cytokines and chemokines, including fibroblast growth factor (FGF), TGF-β, C-X-C motif chemokine ligand 12 (CXCL12), and IL-6, are also secreted by CAFs to mediate immunosuppressive responses (161). Hence, they can be applied as potential targets for anticancer treatment. However, many challenges still prevail in modulating CAFs as an ideal target for CAR-T cell therapy. As previously mentioned, FAP-targeted CAR-T cell therapy induced lethal adverse effects because CAR-T cells attacked FAP-positive BMSCs (117). In addition, CAFs have been shown to contribute to the development of therapeutic resistance because the ECM produced by CAFs could serve as a thick barrier to block the penetration of drugs (162). Accordingly, we hypothesized that the physical barrier formed by CAFs could also hinder the delivery of CAR-T cells into tumor tissues, thus diminishing the effectiveness and efficacy of CAR-T cell therapy (**Figure 6**).

Several studies have been made to deplete or remodel the CAFs in the TME. One potential strategy is to apply FAP-redirected synthetic Notch CAR T cells or heparanase-modified CAR-T cells to deliver CAF remodeling molecules to suppress the expression profile of CAFs (163).

2.4.2 Fibrotic Environment

In contrast to hematological tumors, the infiltrative ability of CAR-T cells in lung cancer tissues is greatly restrained by the

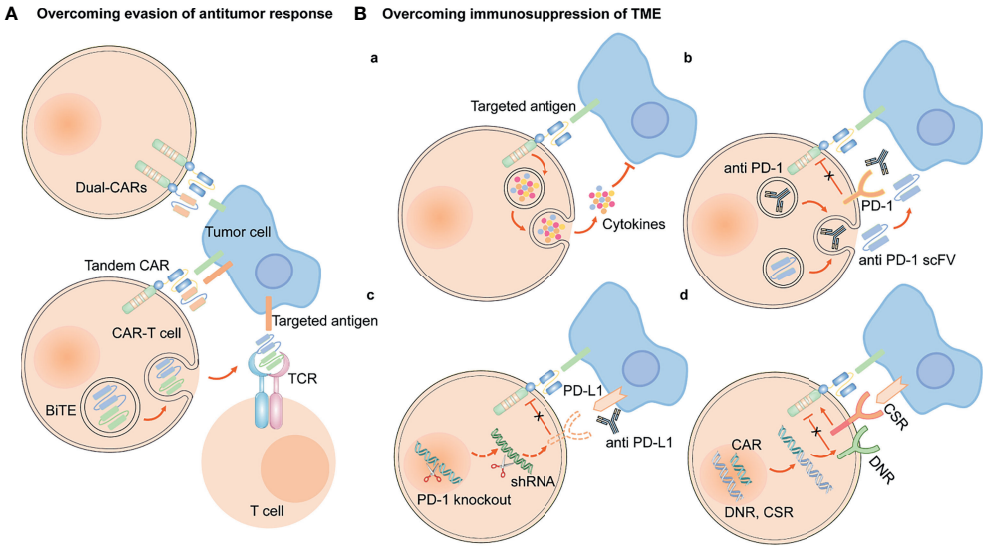

FIGURE 5 | Engineering strategies to overcome evasion of antitumor response and immunosuppression of TME. **(A)** CAR-T cells are engineered to simultaneously target two antigens (dual CAR-T cells), and secret BiTE to redirect both T cells and CAR-T cells against specific tumor cells and circumvent antigen escape. Tandem CAR-T cells have bispecific receptors, which could target two different antigens. **(B) (a)** Armored CAR-T cells expressed immunostimulatory cytokines. Approaches to overcoming the immunosuppression of immune checkpoints in TME are as follows, **(b)** CAR-anti-PD-1/PD-L1 antibodies or scFv, **(c)** PD-1 gene knockout or downregulation of PD-1 expression by shRNA, **(d)** express a PD-1 DNR or a PD-1 CSR.

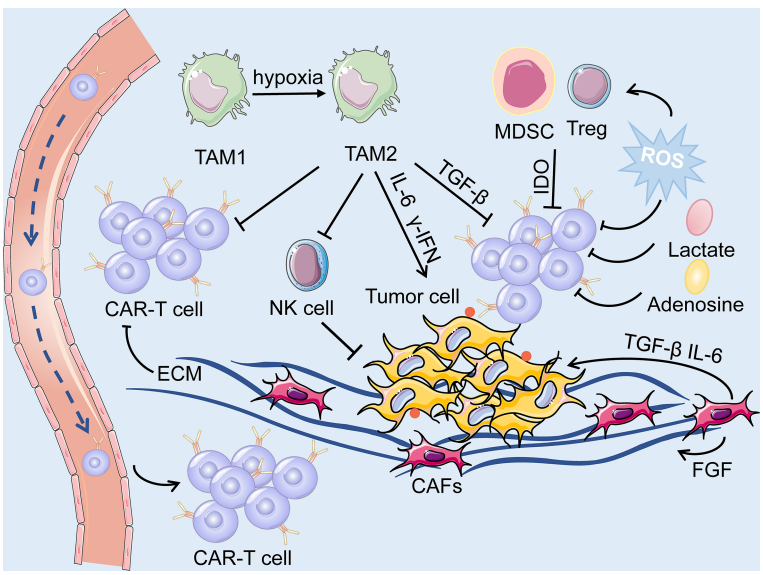

FIGURE 6 | The TME is primarily composed of tumor cells, immune cells, immunosuppressive cells (including TAM, MDSC and Treg) and cytokines, CAFs, ECM and dysregulated tumor vasculatures. On the one hand, ECM produced by CAFs forms a physical barrier, impairing the infiltration of the CAR-T cells. On the other hand, the soluble cytokines secreted by the CAFs mediate immuno-suppressive responses, and consequently, facilitate the survival of tumor cells. The hypoxic and acidic environment directly deteriorate the metabolism of T cells while activating suppressive Tregs, leading to immunosuppression of CAR-T cells.

presence of a physical barrier. CAF activation, abnormal dense collagen, and ECM deposition contribute to developing a dense and fibrotic environment, altering the localization and migration of effector immune cells in NSCLC, which hinders immune cell infiltration and influences the efficacy of immunotherapy (164, 165). In addition, the extensive fibrotic environment mostly lacks blood vessels, which creates a hypoxic TME and further impairs immune function (166).

The binding of chemokines and their corresponding receptors can mediate the trafficking of CAR-T cells through fibrotic environment. Hence, one approach to enhance the infiltration level of CAR-T cells is to engineer them to express chemokines or transgenic chemokine receptors (167). The CAR-T cells engineered to express IL-7 and CCL19 have been validated to increase the infiltration of peripheral CAR-T cells and dendritic cells and into tumor tissues and enhance the anti-tumor immune responses (168). Another engineering strategy is to construct enzyme-modified CAR-T cells to express heparanase, which accelerates the degradation of ECM and facilitates CAR-T cell trafficking to tumor sites (169). In addition, local injection of CAR-T cells is under investigation.

2.5 Immune Suppression in the TME

The TME of lung cancer has an immunosuppressive effect, as T cell activity is suppressed due to anti-inflammatory cytokines and upregulated immune checkpoint ligands. Additionally, the immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), tumor associated macrophages, and tumor associated neutrophils are broadly present in the TME (**Figure 6**). CAR-T cell therapy against lung cancer is less efficient because of immune suppression of the TME and loss of CAR-T cell function.

One engineering approach to overcome the immunosuppressive role of TME is to establish armored CAR-T cells that secrete pro-inflammatory cytokines or chemokines, such as IL-12, IL-15, and IL-18 (20). These cells can recruit and activate innate immune cells such as natural killer (NK) cells and macrophages, and reprogram the immunosuppressive TME, which subsequently supports the proliferative and antitumor activity of CAR-T cells (170). In addition, based on blocking immune checkpoints, genetic knockdown of immune checkpoint receptors in CAR-T cells, such as PD-1, was demonstrated to enhance the anti-tumor effect. The clinical outcomes are being actively assessed in clinical trials on lung cancer (171). Other strategies include engineering CAR-T cells to secrete immune-checkpoint inhibitors, including anti–PD-1 scFv and anti–PD-L1 antibodies, to express PD-1 dominant-negative receptors (DNR) or PD-1 chimeric switch receptors (CSR) (113, 172, 173) (**Figure 5**).

2.6 Metabolic Profile of the TME

Cumulating evidence supports that metabolism plays an essential role in the immune response because it could regulate the function and activity of T cells. The inhibition of T cell metabolism may directly deteriorate the activity of T cells while activating suppressive Tregs, resulting in immuno-suppression (174). The proliferation of CAR-T cells, secretion of cytokines, and elimination of tumor cells are all energy-demanding processes. However, tumor cells mostly consume a large proportion of energy and nutrients, while generate a mass of immunosuppressive metabolites, such as adenosine, lactate, and kynurenine (135, 174). Moreover, indolamine-2,3-dioxygenase (IDO) secreted by tumor cells and MDSC could catalyze tryptophan into kynurenine, leading to the inactivation of CAR-T cells and the proliferation of Tregs (175) (**Figure 6**). On the other hand, the dysregulated vasculatures also result in an extremely hypoxic and acidic TME. All of the above elements contribute to the formation of the metabolically hostile TME, which further impairs the function of CAR-T cells.

Reprogramming the CAR-T cells to adjust their metabolic properties through genetic or pharmacological inhibition of adenosine receptors A_1 and $A_{2A}R$ substantially elevated CAR T cell efficacy in breast cancer, which appears to be a promising method to enhance CAR-T cell function in the TME (176). Additionally, ROS generated by MDSC exerts a negative impact on CAR-T cells, and therefore, the reduction of ROS might be a potential strategy to overcome the metabolic profile of TME. Furthermore, CD28 and 4-1BB, the co-stimulatory domains of CAR-T cells, respectively, improved the metabolic fitness of CAR-T cells in melanoma by upregulating the intake of glucose and the expression of glycolytic enzymes, and enhancing mitochondrial biogenesis and oxidative metabolism (177, 178). However, limited data are available on the metabolic reprogramming of CAR-T cells in lung cancer.

2.7 CAR-T Cell Exhaustion

The existence of inhibitory ligands in the TME and endogenous TCRs leads to the gradual exhaustion of CAR-T cells (134). Clinical evidence has confirmed that CAR-T cell exhaustion markedly limits the efficacy of CAR-T cell therapy; therefore, it is imperative to prevent or reduce CAR-T cell exhaustion. However, it is difficult to reverse the cell exhaustion process directly by dedifferentiating T cells for exhaustion, which is a transcriptional and epigenetic forced differentiation state (179). Therefore, less differentiated T cell populations, such as naive T cells, whose proliferative activity is more robust, are selected for CAR-T cell manufacture (180). The negative regulators inducing T-cell exhaustion include PD-1, CTLA4, T-cell immunoglobulin and mucin domain 3, and lymphocyte-activation gene 3, which could restrain the activity of T cells while promoting the suppressive function of Tregs (181–183).

The above research advancements may shed light on new strategies to increase CAR-T cell persistence. Engineering strategies to inhibit these negative regulators primarily involve: (1) immune checkpoint blockades, (2) genetic knockdown of negative regulators in CAR-T cells, (3) PD-1 DNR, and (4) autocrine secretion of anti–PD-1 scFv and anti–PD-L1 antibodies from CAR T cells (20, 73, 182, 184). At present, combination therapy of CAR-T cells and immune checkpoint blockades has been utilized to overcome CAR-T cell exhaustion in clinical trials of NSCLC (185). CRISPR/Cas9-mediated knockdown of negative regulators in CAR-T cells may become a novel therapeutic approach to increase the persistence of CAR-T cells (182). CAR-T cells targeting PD-L1z, equipped with CAR-T cells with intrinsic blockade properties of PD-1, demonstrated efficacious antitumor activity in NSCLC models (113). CAR-T cells secreting anti–PD-L1 antibodies have been demonstrated to combat T cell exhaustion in a renal cell carcinoma mouse model (172) (**Figure 5**). In addition, transient cessation of CAR signaling, 4-1BB and CD28

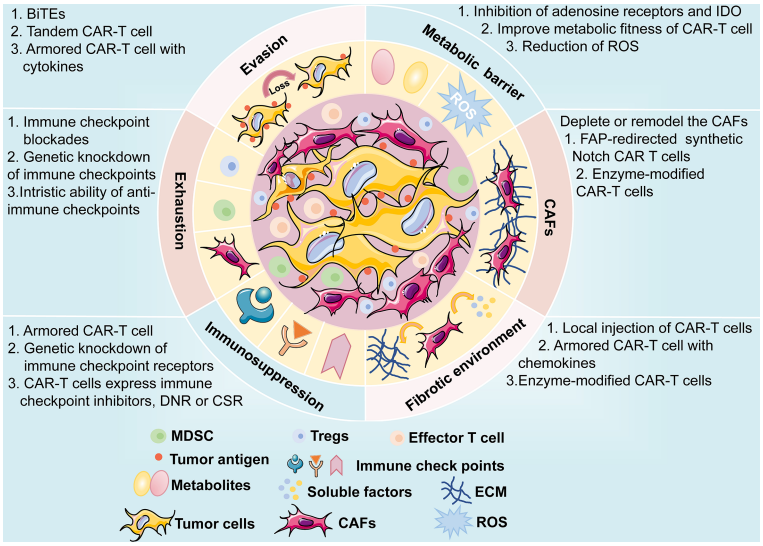

FIGURE 7 | A brief overview of potential challenges faced by CAR-T cell therapy, including antigen evasion, metabolic barrier, CAFs, fibrotic environment, immunosuppression of TME, and exhaustion of CAR-T cells. The possible mechanisms and engineering strategies are also presented.

costimulatory signaling, c-Jun, and transcription factors, such as nuclear receptor subfamily 4 group A, NFAT, and thymocyte selection-associated high mobility group box protein have also been shown to regulate T cell exhaustion (179, 184, 186). Further studies are required to apply these findings to enhance CAR-T cell resistance to exhaustion.

3 FUTURE OUTLOOK

CAR-T cell therapy has emerged as a novel and effective immunotherapy against multiple cancers, especially hematological malignancies. The same issues, such as CAR-T therapy-related toxicities, on-target, off-tumor toxicity, and evasion of antitumor responses, have plagued the treatment of hematologic malignancies; the treatment of solid tumors encounters even greater challenges. Moreover, the physical barrier impedes the infiltration of CAR-T cells to tumor sites, and the TME is immunosuppressive. In recent years, the successful improvements in the safety and efficacy of the therapy have facilitated the application of CAR-T therapy in solid tumors, including lung cancer. CAR structures persistently undergo evolution to enhance efficacy and reduce the cytotoxic effects of CAR-T cell therapy. In addition, the engineering solutions mentioned above are in their early stages and are being progressively developed towards the clinical application phase, and further investigations are expected **(Figure 7)**. Among these engineering strategies, gene editing technology is one of powerful tools to improve the efficacy and safety of CAR-T cell therapy and is driving the application of this novel cancer therapy. The manufacture of "off the shelf" CAR-T cell products by disrupting the TCR alpha/beta chains through TALENs or CRISPR/Cas9 platform, is currently undergoing the evaluation of clinical trials (187). The inclusion of inducible caspase-9 safety switches to CARs could regulate the production of cytokines to prevent CRS (143). CRISPR/Cas9-mediated knockdown of negative immune checkpoints enables the CAR-T cells to resist the immunosuppressive TME. It is too early to appreciate the promising prospects of this novel immunotherapy approach in lung cancer treatment until more clinical trials to investigate these engineering strategies are conducted and evaluated.

AUTHOR CONTRIBUTIONS

B-TY and NW contributed significantly to fund support and the conception of the review. B-FX, J-TZ and Y-GZ contributed to wrote the manuscript. X-RC contributed to make preparations and revise the manuscript. Z-ML helped proposed some constructive suggestions. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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A Systematic Review on PD-1 Blockade and PD-1 Gene-Editing of CAR-T Cells for Glioma Therapy: From Deciphering to Personalized Medicine

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Background: Programmed cell death protein 1 (PD-1) can attenuate chimeric antigen receptor-T (CAR-T) cell-mediated anti-tumoral immune responses. In this regard, co-administration of anti-PD-1 with CAR-T cells and PD-1 gene-editing of CAR-T cells have been suggested to disrupt this inhibitory axis. Herein, we aim to investigate the advantages and disadvantages of these two approaches and propose a novel strategy to ameliorate the prognosis of glioma patients.

Methods: Scopus, Embase, and Web of Science were systematically searched to obtain relevant peer-reviewed studies published before March 7, 2021. Then, the current study was conducted based on the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statements. The random-effect model was applied to evaluate the effect size of administrated agents on the survival of animal models bearing gliomas using RevMan version 5.4. The Cochran Q test and I^2 were performed to assess the possible between-study heterogeneity. Egger's and Begg and Mazumdar's tests were performed to objectively assess potential asymmetry and publication bias using CMA version 2.

Results: Anti-PD-1 can substantially increase the survival of animal models on second-generation CAR-T cells. Also, PD-1 knockdown can remarkably prolong the survival of animal models on third-generation CAR-T cells. Regardless of the CAR-T generations, PD-1 gene-edited CAR-T cells can considerably enhance the survival of animal-bearing gliomas compared to the conventional CAR-T cells.

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Conclusions: The single-cell sequencing of tumoral cells and cells residing in the tumor microenvironment can provide valuable insights into the patient-derived neoantigens and the expression profile of inhibitory immune checkpoint molecules in tumor bulk. Thus, single-cell sequencing-guided fourth-generation CAR-T cells can cover patient-derived neoantigens expressed in various subpopulations of tumoral cells and inhibit related inhibitory immune checkpoint molecules. The proposed approach can improve anti-tumoral immune responses, decrease the risk of immune-related adverse events, reduce the risk of glioma relapse, and address the vast inter-and intra-heterogeneity of gliomas.

Keywords: glioma, CAR-T cells, engineered cell therapy, inhibitory immune checkpoint, single-cell sequencing, tumor microenvironment, neoantigen, personalized medicine

1 INTRODUCTION

High-grade gliomas are among the most common primary brain tumors; however, the current therapies have not led to meaningful outcomes for affected patients. Tumor invasion, heterogeneity, and immune escape are considered the daunting challenges for treating these highly aggressive tumors. Therefore, there is a pressing need to develop a safe and effective therapy for patients with high-grade gliomas (1).

Immunotherapy has offered a new treatment approach for some cancers; however, the overall low response rates of immunotherapy for some solid cancers have limited their widespread clinical translations. As "living drugs", CAR-T cells are engineered cells that can specifically target defined antigens expressed by tumor cells. The ectodomain of CAR-T cells consists of a single-chain variable fragment (scFv) that recognizes tumor-associated antigens and leads to the activation of its endodomain, CD3ζ. The endodomain of the first CAR-T cell generations does not contain other co-stimulatory factors besides CD3ζ. However, the second generation of CAR-T cells has other endodomain co-stimulatory components, i.e., CD28, CD137, or CD134. While the third-generation CAR-T cells were developed by adding two co-stimulatory factors to the CD3ζ, the fourth-generation ones were genetically edited to express desired factors following stimulation (2). Despite the food and drug administration (FDA) approval for CD19-targeting CAR-T cells in patients with B-cell malignancies, CAR-T cell therapy for other solid cancers has not been as promising (3).

Although the addition of co-stimulatory factors to the first generation of CAR-T cells has shown promising results in stimulating anti-tumoral immune responses, the immunosuppressive tumor microenvironment is now recognized as a critical culprit for the low response rates of CAR-T cells in solid cancers (4, 5). Indeed, the expression of inhibitory immune checkpoints, e.g., PD-1, on the CAR-T cells has been associated with a remarkable decrease in their ability to target tumoral cells (6). A recent clinical trial has shown that anti-EGFRVIII-CAR-T cell infusion can substantially promote immunosuppressive tumor microenvironment *via* upregulating inhibitory immune checkpoint molecules (7). In this regard, two approaches have been proposed to suppress PD-1 expression, i.e., PD-1-targeting monoclonal antibodies co-administration with CAR-T and PD-1 gene editing of CAR-T cells (8–11).

Here, we review the current evidence on the efficacy and safety of the combined therapy of PD-1-targeting monoclonal antibodies co-administration with CAR-T cells and PD-1 gene editing of CAR-T cells. Besides, we review the current trend in CAR-T cells therapy of high-grade gliomas in clinical trials and propose a novel strategy for immunotherapy of high-grade gliomas based on preclinical and clinical findings. Our proposed approach is based on the combination of fourth-generation CAR-T cell and single-cell sequencing technologies, which can address the shortcomings in terms of the safety and efficacy of CAR-T cells/immune checkpoint inhibitors for treating patients with high-grade gliomas.

2 MATERIAL AND METHODS

This study was conducted under the PRISMA statements (12).

2.1 The Search Strategy

Without imposing any restriction on the publication language and publication time, the Web of Science, Scopus, and Embase were systematically searched to obtain records published before March 7, 2021, with the following keywords: ("glioma" OR "glioblastoma" OR "glioblastoma multiforme") and ("programmed cell death 1" OR "PD-1" OR "PDCD1" OR "hSLE1" OR "CD279" OR "PD1" OR "SLEB2" OR "hPD-l" OR "programmed death 1 receptor" OR "hPD-1") and ("chimeric antigen receptor T-cell immunotherapy" OR "CAR-T" OR "CART" OR "CAR T" OR "CAR T cell" OR "chimeric antigen receptor T cell" OR "adoptive immunotherapy" OR "chimeric antigen receptor immunotherapy" OR "chimeric antigen receptor T"). We also used the Emtree terms to increase the sensitivity of our systematic search.

2.2 Eligibility Criteria

Studies with the following eligibility criteria were included in our study: (1) investigations that studied the effect of CAR-T cells on glioma, and (2) investigations with the objective of evaluating PD-1 suppression on the efficacy of CAR-T cells. Based on the following criteria, studies were excluded from the current systematic review: (1) studies that did not meet the

abovementioned inclusion criteria, (2) review papers, (3) meeting abstracts, (4) perspectives, (5) book chapters, (6) editorial articles, (7) commentaries, (8) opinion articles, and (9) duplicated papers.

2.3 Study Selection

Following the systematic search, the retrieved records were reviewed in two phases. In the first phase, the titles and abstracts of obtained papers were screened. In the second phase, the full text of the remaining papers and their supplementary data were reviewed for consideration to be included in the current study.

2.4 Data Extraction

The following data were extracted from the included studies: (1) the first author, (2) the year of publication, (3) the method of PD-1 suppressing, (4) their main findings, (5) the target of CAR-T cells, (6) the glioma cell line, (7) the schedule of anti-PD-1 administration in animal models, and (8) animal models. For the survival analysis, we extracted the hazard ratio (HR) and the 95% confidence interval (CI) for further analysis.

2.5 Evaluating the Quality of Included Studies

To enhance transparency and facilitate the translation of our results into the clinic, we used three quality assessment tools for evaluating the quality of clinical, *in vivo*, and *in vitro* studies. For our included clinical study, we used the "NIH quality assessment tool" (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools). For our included *in vivo* studies, we utilized the "SYRCLE's RoB" tool, adapted from the Cochrane RoB tool (13). For our included *in vitro* studies, we adapted the previously used quality assessment tool (14, 15).

2.6 Statistical Analysis

All meta-analyses were conducted using RevMan version 5.4. Because there might be unpublished investigations, the random-effect model was applied for the current meta-analysis. To objectively evaluate the effect of administrated agents on the survival of mice bearing glioma, the common effect sizes were calculated based on the obtained HRs from included studies. The standard chi-squared test and I^2 statistics were applied to evaluate potential heterogeneity between the included studies. The values over 75% for I^2 were considered considerable heterogeneity (16, 17). To assess the potential publication bias, funnel plots were provided using CMA version 2. Besides, Begg and Mazumdar's test was conducted to assess the potential publication bias objectively. Also, Egger's test was performed to evaluate potential publication bias statistically.

3 RESULTS

3.1 Selected Studies

Our systematic search retrieved 185 records: Embase (n= 98), Scopus (n= 61), and Web of Science (n= 26). After removing the duplicated studies, 122 studies were screened based on their title and abstracts. In the first phase, 102 records were excluded because they did not meet the abovementioned criteria. In the second phase, the full text of 20 studies and their supplementary data were reviewed for consideration to be included in the systematic review. After excluding twelve studies, we included eight studies in the current systematic review. The flowchart of literature identification is shown in **Figure 1**.

3.2 The Characteristics of Included Studies

The eight included studies were published in English between 2018 and 2021. One of the studies was from a phase I clinical trial, and the others were preclinical investigations. Four studies used monoclonal antibodies to block PD-1, and the other four studies inhibited PD-1 gene expression in CAR-T cells. The targets of CAR-T cells were epidermal growth factor receptor variant III)EGFRvIII(, interleukin 13 receptor alpha 2) IL13Rα2), human epidermal growth factor receptor 2 (HER2), and CD133; the most used tumor-antigen for CAR-T cell development was EGFRVIII. The cell lines were studied in the included studies were U87MG, U251, DKMG, U373, and D270. U251 was the most studied cell line in the included preclinical studies. **Table 1** demonstrates the summarized data extracted from the included studies.

3.3 Anti-PD-1 Can Substantially Increase the Survival of Glioma Animal Models on Second-Generation CAR-T Cells

Our results have demonstrated that the combined therapy of anti-PD-1 with second-generation CAR-T cells can significantly enhance the survival of animal-bearing gliomas compared to the monotherapy with second-generation CAR-T cells (HR = 0.17, 95% CI: 0.07 - 0.44, P = 0.0002). Besides, there has been no significant heterogeneity between the included studies (I^2 = 0%, P = 0.75) (**Figure 2**).

3.4 PD-1 Knockdown Can Remarkably Increase the Survival of Glioma Animal Models on CAR-T Cells

Our results have shown that regardless of the CAR-T generations, the PD-1 gene-edited CAR-T cells can significantly improve the survival of glioma animal models compared to the conventional CAR-T cells (HR = 0.34, 95% CI: 0.16 - 0.70, P = 0.004). Also, no significant heterogeneity between the included studies has been found (I^2 = 18%, P = 0.29) (**Figure 3**).

3.5 PD-1 Knockdown Can Considerably Increase the Survival of Glioma Animal Models on Third-Generation CAR-T Cells

Our results have shown that PD-1 gene-edited third-generation CAR-T cells can significantly improve the survival of glioma animal models compared to the conventional third-generation CAR-T cells (HR = 0.26, 95% CI: 0.10 - 0.73, P = 0.01). Besides, no significant heterogeneity between the included studies has been noted (I^2 = 34%, P = 0.22) (**Figure 4**).

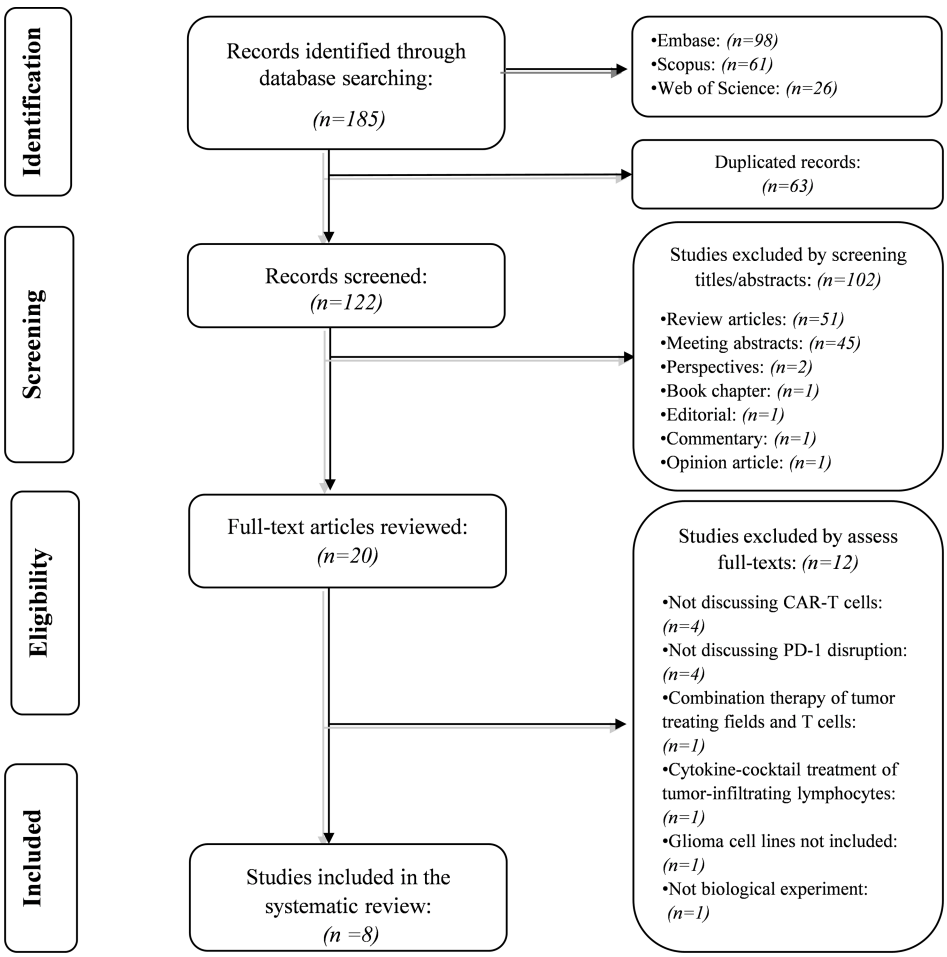

FIGURE 1 | The flow chart of the current study.

3.6 Evaluating Publication Bias

Begg and Mazumdar's and Egger's tests were performed to evaluate the asymmetry of funnel plots and potential publication bias. Our results have demonstrated that asymmetry is not present in the funnel plots, and there is no publication bias that can affect the obtained results (**Figure 5**).

3.7 Evaluating the Bias in the Included Studies

Table 2 evaluates the potential bias in the included clinical study based on the criteria of the NIH quality assessment tool. Overall, no considerable bias has been noted. **Table 3** assesses the potential bias in the included *in vitro* studies. Based on our results, the risk of potential bias is considered low. **Table 4** evaluates the potential bias among the *in vivo* investigations. The primary bias domains have been randomly selecting the animal models and their housing. Besides, only one *in vivo* study has evaluated the side effects of treatments, i.e., lymphoma development and graft versus host disease, in the mice (19).

4 DISCUSSION

The dismal prognosis of high-grade glioma patients with the current therapy requires developing new strategies to target cancer cells. Although CAR-T cells have demonstrated clinical benefit for patients with B-cell malignancies, this technology has not been that successful for patients with high-grade gliomas. The immunosuppressive tumor microenvironment and tumor heterogeneity are among the culprits of this failure. Because CAR-T administration has been associated with the upregulated expression of inhibitory immune checkpoint molecules in the CAR-T and tumoral cells, targeting inhibitory immune checkpoints, such as the programmed death-ligand 1 (PD-L1)/PD-1 axis, has shown promising results (9). In this regard, this systematic review and meta-analysis aimed to investigate the current approaches to target PD-1 expression in CAR-T cells, i.e., monoclonal antibody administration for targeting PD-1 and PD-1 gene-editing of CAR-T cells in high-grade glioma.

TABLE 1 | The characteristics of included studies.

No.	First author, publication year	PD-1 disruption approach	Target of CAR-T	CAR-T generation	Cell line	Anti-PD-1 schedule in animal models	Animal model
1	Song et al., 2020 (9)	PD-1 antibody	EGFRVIII	Second-generation	U87	14 to 21 days after tumor inoculation (once the majority of tumors exhibited an area greater than 100 mm2)	6- to 8-week-old female immunodeficient NPI mice
2	Nakazawa et al., 2020 (11)	CRISPR/Cas9-mediated gene therapy	EGFRVIII	Third-generation	U-251MG and DKMG	Not applicable	Not applicable
3	Portnow et al., 2020 (8)	PD-1 antibody	HER2 and IL13Rα2	Not mentioned	Not applicable	Not applicable	Not applicable
4	Zhu et al., 2020 (18)	PD-1 siRNA-mediated gene therapy	EGFRVIII	Third-generation	U373	Not applicable	BALB/c nude mice
5	Shen et al., 2019 (6)	PD-1 antibody	HER2	Third-generation	U251 and U87	Not applicable	Not applicable
6	Choi et al., 2019 (10)	CRISPR/Cas9-mediated gene therapy	EGFRVIII	Second-generation	U87 and U251	Not applicable	Immune compromised NSG mice
7	Hu et al., 2019 (19)	The nucleofection of plasmid DNA for CRISPR/Cas9-mediated gene therapy	CD133	Third-generation	U251	Not applicable	6- to 8-week-old female NPG mice
8	Yin et al., 2018 (20)	PD-1 antibody	IL13Rα2	Second-generation	U87, U251, and D270	From day 6 after tumor implantation	6- to-8-week-old female NSG mice

4.1 PD-1 Blockade and PD-1 Gene-Editing in CAR-T Cells for High-Grade Gliomas: What Does the Currently Available Evidence Say?

Our meta-analysis has indicated that co-administrating monoclonal antibodies for targeting PD-1 with second-generation CAR-T cells can significantly improve the survival of glioma-animal models compared to monotherapy with second-generation CAR-T cells (HR = 0.17, 95% CI: 0.07 - 0.44, P = 0.0002). It has been reported that administrating PD-1 inhibitors can remarkably increase the infiltration of immune cells into the tumor microenvironment and upregulate the expression of interleukin-2 (IL-2) and interferon-gamma (IFN-γ) (6, 9). Consistent with these, anti-PD-1 administration has been associated with a considerable decrease in the tumor size in mice bearing glioma (20). Of interest, a recent clinical trial has indicated that intravenous pembrolizumab, an anti-PD-1 monoclonal antibody, can result in a steady-state concentration of pembrolizumab in the cerebrospinal fluid (CSF) and suppress the PD-1 expression in CAR-T cells (6). Moreover, intravenous pembrolizumab can inhibit PD-1 expression in non-CAR T-cells, indicating its inhibitory role on other tumor-infiltrative immune cells, e.g., regulatory T cells (8). Consistent with the clinical study results, pembrolizumab can increase the persistency and anti-tumoral activity of CAR-T cells in patients with relapsed B-cell acute lymphoblastic leukemia (21). Since PD-1 is expressed by CAR-T cells and other immune cells in the tumor microenvironment, its blockade might be a promising strategy to increase the efficacy of CAR-T cells.

Our results have also demonstrated that regardless of the CAR-T generations, PD-1 gene-edited CAR-T cells can significantly improve the survival of glioma-animal models compared to the conventional CAR-T cells (HR = 0.34, 95% CI: 0.16 - 0.70, P = 0.004). Besides, PD-1 gene-editing of third-generation CAR-T cells can significantly improve the survival of glioma-animal models compared to the conventional third-generation CAR-T cells (HR = 0.26, 95% CI: 0.10 - 0.73, P = 0.01). Based on the limited currently available data, PD-1 gene-

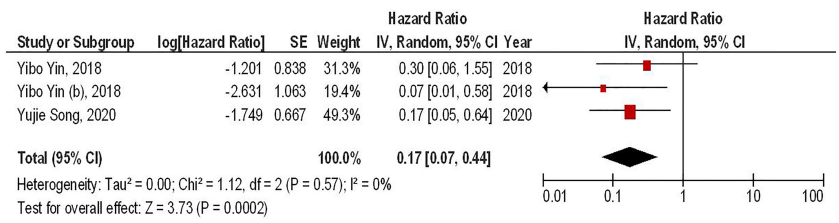

FIGURE 2 | The forest plot of studies evaluating the effect of anti-PD-1 administration on the survival of animal models treated with second-generation CAR-T cells.

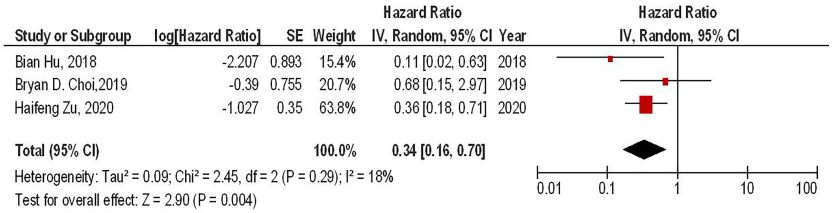

FIGURE 3 | The forest plot of studies evaluating the effect of PD-1 knockdown on the survival of animal models treated with CAR-T cells.

edited CAR-T cells do not lead to lymphoma development and graft versus host disease in mice bearing glioblastoma (17). Nevertheless, further investigations are needed to evaluate the safety of these approaches. Furthermore, PD-1 deletion has been associated with increased central memory T-cell-like properties, leading to elevated proliferation, increased persistence, and self-renewal features in glioblastoma (10). PD-1 deletion has also upregulated the expression of pro-inflammatory cytokines, i.e., IL-2, IFN-γ, and tumor necrosis factor-alpha (TNF-α), which are associated with increased anti-tumoral immune responses against glioblastoma (10, 18). Zhu et al. have shown that the increased anti-tumoral immune responses of PD-1 gene-edited CAR-T cells are more pronounced against glioblastoma that overexpress PD-L1 (18). Thus, tumoral PD-L1 expression might be a prognostic factor for this approach.

4.2 The Limitations of PD-1 Blockade and PD-1 Gene Editing

4.2.1 Anti-PD-1 in Treating Gliomas; One Piece of a Big Puzzle?

Although the currently available evidence has suggested that suppressing PD-1 can substantially increase the efficacy of CAR-T cells, the tumor-microenvironment is usually more complicated than its fate can be attributable to a single inhibitory immune checkpoint molecule. Indeed, other inhibitory immune checkpoints, e.g., cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoglobulin domain and mucin domain-3 (TIM-3), V-domain Ig suppressor of T cell activation (VISTA), lymphocyte activation gene 3 (LAG-3), PD-L1, and T cell immunoreceptor with Ig and ITIM domains (TIGIT), can also promote an immunosuppressive tumor microenvironment (4, 22). This has been reflected in the multiple clinical trials investigating the efficacy of immune checkpoint inhibitors in glioma patients. Pembrolizumab administration has resulted in no clinical/histologic improvements in patients with brain tumors (23). Nayak et al. have shown that the objective response rate of patients with recurrent glioblastoma to pembrolizumab is 0% (24). A recent phase 3 randomized clinical trial has also demonstrated that the response rate of glioblastoma patients to nivolumab, another anti-PD-1 monoclonal antibody, is poor, and the objective response rate of affected patients to this anti-PD-1 agent is 7.8% (25). Consistent with these, Omuro et al. have reported that the complete response rate of patients to nivolumab is 0%. Not only adding ipilimumab, an anti-CTLA-4 antibody, to the nivolumab regimen has not improved the complete response rate of patients with recurrent glioblastomas, but also the co-administration of ipilimumab and nivolumab has been associated with increased occurrence of treatment-induced adverse events (26). Indeed, administrating multiple inhibitory immune checkpoint inhibitors has been associated with an increased risk of autoimmunity development; because it paves the way for stimulating auto-reactive T cells. Matull et al. have reported that combined CTLA-4 and PD-1 inhibition can severely damage multiple organs following a single dosage of ipilimumab and nivolumab (27). Simonelli et al. have shown that nivolumab, as a PD-1 inhibitor, can severely damage the liver in a glioblastoma patient (28). Thummalapalli et al. have reported that suppressing PD-1 and indoleamine-pyrrole 2,3-dioxygenase (IDO) can lead to hemophagocytic lymphohistiocytosis, acute liver injury, cytopenia, and altered mental status in a patient with recurrent glioblastoma (29). A recent clinical trial has shown that 18.1% of patients with recurrent glioblastoma have manifested grade 3/4 treatment-related adverse events following nivolumab administration (25). In line with these, a recent systematic review has indicated that CTLA-4 inhibitors can promote immune-related adverse events and lead to organ-specific damage (30). Therefore, the safety issues

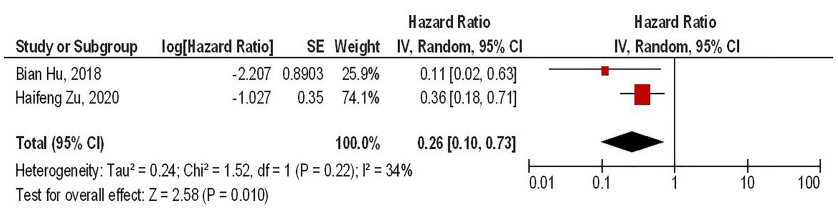

FIGURE 4 | The forest plot of studies evaluating the effect of PD-1 knockdown on the survival of animal models treated with third-generation CAR-T cells.

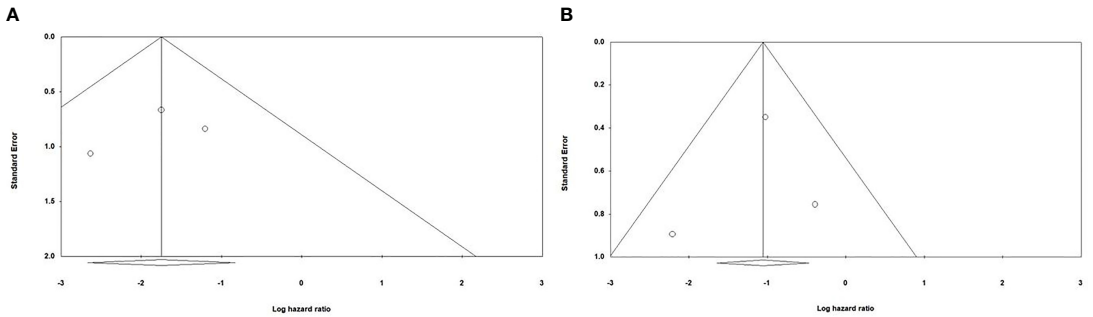

FIGURE 5 | Evaluating potential publication bias among the included studies **(A)** Evaluating publication bias among the studies investigating the effect of anti-PD-1 administration on the survival of animal models treated with second-generation CAR-T cells; Begg and Mazumdar' test one-tail P-value=0.30075 and two-tail P-value = 0.60151; Egger's test one-tail P-value=0.3272 two-tail P-value=0.65456 **(B)** Evaluating publication bias among the studies investigating the effect of PD-1 knockdown on the survival of animal models treated with CAR-T cells; Begg and Mazumdar' test one-tail P-value=0.30075 and two-tail P-value =0.60151; Egger's test one-tail P-value=0.40773 two-tail P-value=0.81545.

of the current method of immune checkpoint inhibitors administration might be a daunting challenge.

As discussed above, these unfavorable results to targeting one inhibitory immune checkpoint molecule might indicate that a network of the inhibitory immune checkpoint can regulate anti-tumoral immune responses, and targeting one axis can lead to the compensation of this network *via* other inhibitory immune checkpoint molecules. Indeed, the reason for the relatively favorable response rate of animal models or affected patients to immune checkpoint inhibitors can be stemmed from the fact that a specific inhibitory immune checkpoint molecule plays a predominant role in that network. Yin et al. have reported that anti-CTLA-4 administration has been associated with prolonged survival of glioma models treated with Hu08BBz compared to the administration of anti-TIM-3. However, the anti-PD-1 administration has been more effective in improving the survival of glioma models treated with 2173BBz, a second-generation CAR-T cell agonist EGFRVIII, compared to anti-CTLA-4 administration (20). In line with these, the response rates of affected patients to a specific immune checkpoint inhibitor vary substantially, and the overall response rate of glioblastoma patients is not favorable. A phase I clinical trial has demonstrated that the overall response rate of glioblastoma patients with positive tumoral PD-L1 to pembrolizumab has been 8% (31).

4.2.2 The Shortcomings of PD-1 Gene-Edited CAR-T Cells for Treating Gliomas

PD-1 gene-editing also harbors shortcomings. Single inhibitory immune checkpoint gene-editing of CAR-T cells cannot disrupt the inhibitory immune checkpoint axes between other cells residing in the tumor microenvironment. Besides, PD-1 disruption has slightly demonstrated off-target effects *via* targeting the T cell-related growth factor genes; thus, the proliferation of PD-1 gene-edited CAR-T cells can be slightly decreased compared to non-edited immune cells (11). In

TABLE 2 | Evaluating the potential risk of bias in the included clinical study.

Items	Yes	No	Other (CD, NR, NA)*
1. Was the study question or objective clearly stated?	*		
2. Were eligibility/selection criteria for the study population prespecified and clearly described?	*		
3. Were the participants in the study representative of those who would be eligible for the test/service/intervention in the general or clinical population of interest?	*		
4. Were all eligible participants that met the prespecified entry criteria enrolled?	*		
5. Was the sample size sufficiently large to provide confidence in the findings?		*	
6. Was the test/service/intervention clearly described and delivered consistently across the study population?	*		
7. Were the outcome measures prespecified, clearly defined, valid, reliable, and assessed consistently across all study participants?	*		
8. Were the people assessing the outcomes blinded to the participants' exposures/interventions?			*
9. Was the loss to follow-up after baseline 20% or less? Were those lost to follow-up accounted for in the analysis?			*
10. Did the statistical methods examine changes in outcome measures from before to after the intervention? Were statistical tests done that provided p values for the pre-to-post changes?	*		
11. Were outcome measures of interest taken multiple times before the intervention and multiple times after the intervention (i.e., did they use an interrupted time-series design)?	*		
12. If the intervention was conducted at a group level (e.g., a whole hospital, a community, etc.), did the statistical analysis take into account the use of individual-level data to determine effects at the group level?	*		

**CD, cannot determine; NA, not applicable; NR, not reported.*

TABLE 3 | Evaluating the potential risk of bias in the included *in vitro* investigations.

No.	First author, publication year	1. Was the studied cancer cell lines reported?	2. Was the duration of exposure to the CAR-T cells to tumoral cells reported?	3. Was the concentration of the studied CAR-T cells reported?	4. Was a standard culture media used for the study?	5. Were reliable tools used to assess the outcome?	6. Were the experiments conducted more than once?	7. Were more than one independent experiment performed?	The overall risk of bias
1	Nakazawa et al., 2020 (11)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
2	Shen et al., 2019 (6)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low

contrast, Song et al. have indicated that PD-1-targeting monoclonal antibody administration is not associated with decreased proliferation of CAR-T cells in glioblastoma (9). Therefore, special percussions are needed in developing gene-edited CAR-T cells *via* CRISPR/Cas9 technology to avoid the off-target effect. Also, siRNA-mediated PD-1 knockdown can be time-dependent; thus, PD-1-siRNA degradation can promote PD-1 upregulation on CAR-T cells. Therefore, further research is needed to address the gene-editing of PD-1 at the post-transcriptional level and deleting the PD-1 gene itself.

4.3 Glioblastoma Treatment in the Era of Single-Cell Sequencing and Fourth-Generation CAR-T Cells

4.3.1 How Can Single-Cell Sequencing Further Our Knowledge of the Very Dynamic Nature of the Tumor Microenvironment?

Single-cell sequencing technologies have revolutionized our knowledge of the cells that reside in the tumor microenvironment. Recently, Fu et al. have shown a substantial increase in the level of tumor-infiltrating TIM-3$^+$CD8$^+$ and PD-1$^+$CD8$^+$ T-cells in anaplastic astrocytoma tissues compared to corresponding cells in the peripheral blood mononuclear cells (PBMCs) from affected patients. The same trend has been true for tumor-infiltrative CD4$^+$ T-cells. These phenotypically exhausted T-cells, along with the increased level of Treg infiltration, can participate in the immunosuppressive tumor microenvironment development (32). Consistent with this, Davidson et al. have demonstrated that PD-1, LAG-3, and TIM-3 are substantially upregulated in tumor-infiltrating CD3$^+$ T-cells compared to corresponding cells of the PBMCs of glioma and normal individuals. Nevertheless, the tumor-infiltrating lymphocytes upregulate the expression of the genes involved in T-cell activation, i.e., CD38 and HLA-DR, and the genes pertained to T-memory phenotype, i.e., CD45RA, CD27, and CD127, compared to PBMCs of glioma patients (33). Therefore immune cells express both stimulatory and inhibitory molecules, and the traditional categorizing of immune cells based on one inhibitory immune checkpoint might not reveal the role of those immune cells. Besides, inhibitory immune checkpoint molecules, e.g., PD-1, can be transiently expressed following immune cells activation (34). In line with this, Clarke et al. have demonstrated that despite TIM-3 and PD-1 expression, tissue-resistant memory T-cells have demonstrated remarkable proliferation and upregulation of pro-inflammatory genes in lung cancer (35). In breast cancer patients, Bassez et al. have indicated that T-cells with PD-1, HAVCR2, LAG-3, and CD39 phenotype can substantially expand despite the expression of exhaustion-related markers. This phenomenon might be attributable to the fact that these cells also express the cytotoxic-related markers, antigen-presenting markers, and immune cell homing signals as well (36). In melanoma, Deng et al. have shown that cytotoxic subpopulation of CD8$^+$ T-cells, associated with improved prognosis, also demonstrate relatively increased expression of inhibitory immune checkpoint molecules, i.e., CTLA4, LAG3, PD-1, HAVCR2, and TIGIT (37). Thus, the expression of several inhibitory immune checkpoints does not always reflect attenuated anti-tumoral immune response, and a network of genes is involved in the fate of anti-tumoral immune responses.

The data obtained from the single-cell sequencing can help us decipher the unfavorable and variable response rate of affected patients to immune checkpoint inhibitors as well. Durante et al. have demonstrated that cytotoxic T lymphocytes (CTLs) do not overexpress PD-1/CTLA-4 molecules rather LAG-3 and provide evidence for the low response rate of uveal melanoma to the current version of immune checkpoint inhibitors (38). Darmanis et al. have demonstrated that only a minority of glioblastoma patients express the ligands for PD-1 and CTLA-4 on tumoral cells, which might be the reason for the overall unfavorable response rate of glioblastoma patients to anti-PD-1 and anti-CTLA-4 agents (39). Consistent with these, Yin et al. have shown that the administration of anti-CTLA-4 with Hu08BBz, a second-generation CAR-T cell against IL-13Rα2, is more effective in improving the survival of mice bearing glioma compared to the administration of anti-PD-1 (20). Indeed the different inhibitory immune checkpoint profiles of the tumor microenvironment, which can be different from case to case, might be the underlying reason for these disparities. A recent clinical trial has shown that the increased level of CD68$^+$ macrophages, which have strong associations with VISTA and B7-H3 expression, can be the underlying reason for the low response rate of glioblastoma patients to pembrolizumab (40). Therefore, the dynamic intercellular cross-talk in the tumor microenvironment can be implicated in the low response rate of glioma patients to immune checkpoint inhibitors.

Moreover, single-cell sequencing can identify specific tumor biomarkers for determining the response rate of affected patients to immune checkpoint inhibitors. It has been reported that the

TCF7 expression in CTLs can be a valuable prognostic factor for determining the response rates of melanoma patients to anti-PD-1 therapy (41). Furthermore, single-cell sequencing can help us identify novel inhibitory immune checkpoints. Li et al. have reported that sialic acid-binding Ig-like lectin-5, sialic acid-binding Ig-like lectin-7, sialic acid-binding Ig-like lectin-9, and sialic acid-binding Ig-like lectin-16 can be considered novel inhibitory immune checkpoint molecules that are functionally similar to TIM-3 and PD-L1. Besides, their combined inhibition might improve the prognosis of glioma patients (42). Collectively, single-cell sequencing can further our understanding of the tumor microenvironment.

4.3.2 Tumoral Antigen for CAR-T Cells and Single-Cell Sequencing

Identifying tumor-specific antigens for developing CAR-T cells might be one of the daunting challenges because of temporal, intra-, and inter-tumoral heterogeneity in the tumor bulk. This justifies the identification of multiple (neo-) antigens for each affected patient. Nejo et al. have classified tumoral antigen into four groups, i.e., virus-derived antigen, patient-specific neoantigen, shared neoantigen, and non-mutant shared antigen. EGFRVIII is an example of the shared neoantigens for glioma, and IL13Rα2 and HER2 are examples of the non-mutant shared antigens that our study has shown that CAR-T cells have been designed against them (43). Since discussing all aspects of these categories is out of the scope of the current study, we discuss the advantages and disadvantages of non-mutant shared antigens, shared neoantigens, and patient-specific neoantigen and highlight how single-cell sequencing data can improve the efficacy of CAR-T therapies.

4.3.2.1 Non-Mutant Shared Antigens: Time to Re-Think About Their Safety?

One of the advantages of this approach is that these antigens can be considered as "off-shelf." However, their relatively low specificity is the main disadvantage of this approach. The expression levels of non-mutant shared antigens are substantially higher in tumoral cells compared to normal cells. Besides, non-mutant shared antigens can be overexpressed in other malignancies as well as glioblastoma. For instance, HER2 can be overexpressed in pancreatic cancer, lung adenocarcinoma, and breast cancer (44–46). However, due to the vast temporal, intra-, and inter-tumoral heterogeneity in tumor bulk and application of immunohistochemistry (IHC) rather than investigating tumor bulk at single-cell levels, it is difficult to prescribe one non-mutant shared antigen for patients with a specific malignancy. Besides, non-mutant shared antigens can be expressed in normal cells at physiological levels, and the related CAR-T cells can severely damage normal tissues. For instance, Morgan et al. have reported a metastatic colorectal cancer patient treated with anti-HER2-CAR-T cells and developed cytokine release syndrome and respiratory distress after transfusion of CAR-T cells. This phenomenon might be stemmed from the fact that HER2 can be expressed in lung epithelial as well (47). Also, it has been shown that IL-13Rα2-targeting CAR-T cells can develop anti-tumoral immune responses against aortic and pulmonary artery smooth in glioma animal models (20). Therefore, developing CAR-T cells against non-mutant shared antigens can increase the risk of adverse events in affected patients.

4.3.2.2 Shared Neoantigens: Does Tumor Evolution Lead to Its Evasion?

Shared neoantigens can also be considered "off-shelf," and their high specificity is another advantage. However, tumor cells mutate, which leads to their evasion from the cytotoxic machinery of highly specific CAR-T cells. A recent clinical trial has shown that the expression level of EGFRVIII is substantially decreased following anti-EGFRVIII CAR-T cells infusion; however, the anti-EGFRVIII CAR-T cells have not entirely eradicated glioma cells (7). Zhu et al. have also demonstrated that although PD-1 gene-edited anti-EGFRVIII CAR-T cells can decrease glioma growth in affected mice, these CAR-T cells also can not entirely eradicate tumoral cells (18). Krenciute et al. have shown that developed genetically engineered CAR-T cells to express IL-15. Although these genetically modified CAR-T cells have demonstrated increased persistence and anti-tumoral effects in glioma-animal models, their efficacy has been limited over time. Because treating glioma cells with CAR-T cells that only target one tumoral (neo-) antigen can lead to (neo-) antigen loss in tumoral cells (48). Consistent with these, Bielamowicz et al. have reported that treating glioma with CAR-T cells with three different molecular targets can exhibit higher cytotoxicity. Besides, animal models treated with CAR-T cells with three different molecular targets have experienced more prolonged survival than those treated with one molecular target (49). Collectively, tumoral cells exhibit vast heterogeneity, and administrating multiple CAR-T cells that target multiple neoantigens can yield optimal results.

4.3.2.3 Patient-Specific Neoantigens and Single-Cell Sequencing in the Era of Personalized Medicine

Patient-specific neoantigens are the results of the genetic alteration of each patient. The main advantage of these neoantigens is that the immune system does not exhibit considerable tolerance against them, and normal cells do not physiologically express them. Nevertheless, identifying these neoantigens might be a daunting challenge. Besides, the relatively low mutation rate of glioblastoma distinguishes it from other cancers, leading to low tumoral neo-antigen development (50). In this regard, single-cell sequencing of tumor bulk can help identify (potential) patient-specific neoantigens. Single-cell sequencing technologies can provide valuable insights into the expression profile of tumoral cells and categorize tumoral cells based on their neoantigens (15, 51). Therefore, this categorization can allow us to develop personalized CAR-T cells with different molecular targets for each patient. In this approach, the vast intra- and inter-heterogeneity of glioma cells can be addressed, and the subsequent tumor recurrence can be prevented.

Nevertheless, single-cell sequencing-guided CAR-T cell generation harbors some limitations as well. One of the main disadvantages of this approach is that it is not "off-shelf," and its rapid availability requires further consideration and

implantation of high-tech centers. Besides, the conventional single-cell sequencing method is based on RNA sequencing; however, the mRNA expression level is not always well-correlated with its protein expression level. In this regard, applying RNA expression and protein sequencing (REAP-seq) and antibody sequencing can address this issue. Also, despite its promising future in eradicating glioma cells, the proposed strategy might be expensive, and assessing its cost-effectiveness requires further investigations (52). Lastly, the excessive immunosuppressive tumor microenvironment of glioma can substantially suppress the stimulation of CAR-T cells-mediated anti-tumoral immune responses even though the CAR-T cells are specifically designed for patient-derived antigens. For this issue, we propose single-cell sequencing-guided fourth-generation CAR-T cell development (see below).

4.3.3 The Combination of Single-Cell Sequencing and Fourth-Generation of CAR-T Cells: A New Perspective for Treating Glioblastoma?

Compared to systemic administration of multiple immune checkpoint inhibitors to reverse the immunosuppressive tumor microenvironment, the application of fourth-generation CAR-T cells can be promising in terms of decreasing the risk of immune-related adverse events development. In this approach, the stimulation of CAR-T cells can lead to the expression and release of desired factors in the microenvironment. This generation has shown encouraging results in expressing intended factors following the stimulation. Lanitis et al. have demonstrated that fourth-generation CAR-T cells can transform the immunosuppressive tumor microenvironment into a pro-inflammatory one, confer enhanced anti-tumoral immune responses, upregulation of B-cell lymphoma 2 (Bcl-2) in CAR-T cells, and activate natural killer cells *via* IL-15 expression (53). Daun et al. have shown that administration of fourth-generation CAR-T cells expressing IL-7 and CCL19 can remarkably increase the migration and cytotoxicity of CAR-T cells against multiple myeloma and substantially reduce urine protein-light levels in affected patients (54). Mei et al. have developed fourth-generation MUC-1-targeting CAR-T cells that release IL-22. They have shown that IL-22 release can considerably increase MUC-1 expression in head and neck squamous cell carcinoma cells; however, this effect mostly halted after 72 hours. Nevertheless, they have shown that these fourth-generation CAR-T cells can substantially decrease tumor volume and increase the infiltration of $CD3^+$ T-cells in animal models (55). A recent clinical trial has demonstrated that administration of CD19-targeting fourth-generation CAR-T cells to relapsed/refractory B cell non-Hodgkin lymphoma patients with the life-expediency of fewer than two months can lead to the median overall survival of 23.8 months. The overall response rate of the affected patients to these CAR-T cells has been 67%; however, the incidence of cytokine release syndrome development has been 14% (56).

Overall, one of the main advantages of fourth-generation CAR-T cells over others is their stimulatory effect on the "bystander" cells in the tumor microenvironment, which liberates them from exhaustion. The same concept can be applied for expressing inhibitory immune checkpoint inhibitors (**Figure 6**). Zhou et al. have engineered a fourth-generation CAR-T, EGFR BB-z/E30-CAR-T, that can express and release PD-1-targeting antibodies following its stimulation. This fourth-generation CAR-T cell has demonstrated higher

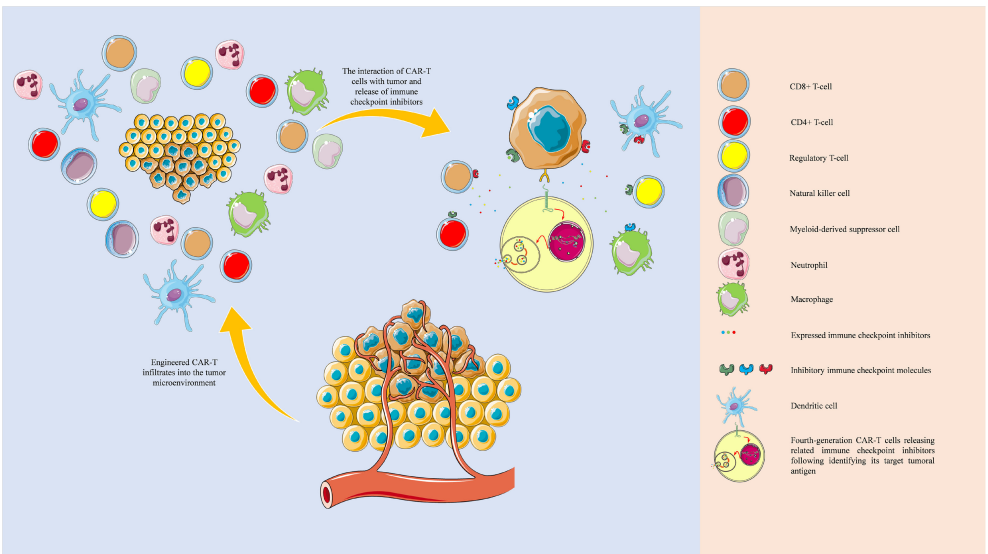

FIGURE 6 | Tumor microenvironment and single-cell sequencing-guided fourth-generation CAR-T cells. The development of fourth-generation CAR-T cells based on the single-cell sequencing-identified patient-derived neoantigens and the single-cell sequencing-guided inhibitory immune checkpoint molecules profiling can potentially eradicate tumoral sub-populations and effectively attenuate inhibitory immune checkpoint network present in the tumor microenvironment. The objects of this figure were obtained from https://smart.servier.com/.

TABLE 4 | Evaluating the potential risk of bias in the included *in vivo* investigations.

No.	First author and publication year	Sequence generation	Baseline characteristics	Allocation concealment	Random housing	Blinding (performance bias)	Random outcome assessment	Blinding (detection bias)	Incomplete outcome data	Selective outcome reporting	Other sources of bias
1	Song et al., 2020 (9)	***	***	***	**	**	***	**	***	**	Not noted
2	Zhu et al., 2020 (18)	***	***	***	**	**	***	**	***	**	Not noted
3	Choi et al., 2019 (10)	***	***	***	**	**	***	**	***	**	Not noted
4	Hu et al., 2019 (19)	***	***	***	**	**	***	**	***	***	Not noted
5	Yin et al., 2018 (20)	***	***	***	**	**	***	**	***	**	Not noted

****Not bias might be noted; **A slight bias might be noted; *Obvious bias might be noted.*

anti-tumoral immune responses and tumor infiltration rates than its corresponding second-generation one (57). Krenciute et al. have shown that IL13Rα2-targeting CAR-T cells, which express IL-15, can substantially demonstrate higher anti-tumoral immune responses (48). Nevertheless, genetically modified CAR-T cells are also prone to failure; because treating glioma cells with CAR-T cells that only target one tumoral antigen can result in antigen loss in tumoral cells. Therefore, there is a need to develop multiple types of CAR-T cells that their molecular targets are specifically expressed in all sub-populations of tumoral cells, i.e., patient-specific neoantigens. With the obtained data from the single-cell sequencing of cells in the tumor microenvironment, we can design multiple types of CAR-T cells that can cover tumoral neoantigens expressed in various tumor cell sub-populations and express the related immune checkpoint inhibitors following stimulation (**Figure 6**). In this approach, the pertained immune checkpoint inhibitors are released in the tumor microenvironment, which does not increase the risk of autoimmunity development in other organs. For this purpose, an atlas of neoantigens and inhibitory immune checkpoint molecules of the tumor microenvironment might be needed to link the certain phenotype of the patient's tumor with the personalized CAR-T cells. Therefore further studies are needed to build such an atlas, and the application of machine learning and artificial intelligence can facilitate this process. Besides, it remains to be determined how effective the single-cell sequencing-guided fourth-generation CAR-T cells approach can be; because it has been indicated that glioblastoma can induce systemic immunosuppression and T cell dysfunction (58–60). Therefore, further studies are needed before the translation of this approach into clinical practice.

4.4 The Current Trend of Clinical Trials of CAR-T Cells for Treating Patients With High-Grade Glioma

Based on our discussion, the combination of fourth-generation CAR-T cells with the data of single-cell sequencing of tumoral cells and cells residing in the tumor microenvironment can substantially improve anti-tumoral immune responses. Regarding the application of CAR-T cells in treating high-grade gliomas, the current trend in the clinical trials is summarized in **Table 5**. Although the combination of

TABLE 5 | The current trend in treating the high-grade glioma patients with CAR-T-based therapy.

No.	Intervention	Cancer type	Clinical trial phase	(estimated) study start date	The status	Clinicaltrials.gov Identifier
1	B7-H3 CAR-T + Temozolomide	Recurrent/refectory glioblastoma	Phase I	1-Jun-20	Recruiting	NCT04385173
2	NKG2D CAR-T	Recurrent glioblastoma	Not applicable	1-Sep-21	Not yet recruiting	NCT04717999
3	B7-H3 CAR-T + Temozolomide	Recurrent/refectory glioblastoma	Phase I/II	1-May-22	Recruiting	NCT04077866
4	GD2 CAR-T + Fludarabine + Cyclophosphamide	Glioma of spinal cord/glioma of brainstem	Phase I	4-Jun-20	Recruiting	NCT04196413
5	CD147-CAR-T	Recurrent CD147 positive glioblastoma	Early phase I	30-May-19	Recruiting	NCT04045847
6	IL13Rα2-CAR-T + Nivolumab + Ipilimumab	Recurrent/refectory glioblastoma	Phase I	26-Sep-19	Recruiting	NCT04003649
7	CAR-T + Radiation + TCR-T + GM-CSF	High-grade glioma	Phase I	1-Apr-18	Recruiting	NCT03392545
8	CAR-T	Recurrent malignant glioma	Phase I	2-Mar-18	Recruiting	NCT03423992
9	IL13Rα2-CAR-T Cell	Leptomeningeal metastases of glioblastoma	Phase I	15-Feb-21	Recruiting	NCT04661384
10	B7-H3 CAR-T	Diffuse glioma	Phase I	11-Dec-19	Recruiting	NCT04185038
11	Fludarabine + Cyclophosphamide + C7R-GD2.CAR-T	High-grade glioma	Phase I	3-Feb-20	Recruiting	NCT04099797

radiation/cytotoxic agents can considerably promote the immunogenicity of glioma cells *via* activating damage-associated molecular pattern (DAMP) signalings and promoting local inflammation, chemo-/radioresistance might attenuate the efficacy of this strategy. Therefore, further investigations for nurturing this combination therapy might be needed (61, 62). NCT04003649 clinical trial is the phase I clinical trial that investigates the combination of IL13Rα2-CAR T cells with nivolumab and ipilimumab in patients with recurrent/refectory glioblastoma. Based on the current evidence discussed in this study, potential immune resistance, tumor relapse, and low-response rates might be challenging.

The current study has some strengths. First, we have used a systematic and unbiased approach to identify and summarize the currently available evidence on the significance of co-administration of anti-PD-1 with CAR-T cells and PD-1 gene-editing of CAR-T cells for glioma therapy. Second, we included both preclinical and clinical studies, carefully evaluated their potential bias based on pertained checklists, and attempted to sort out the inconsistencies between these two. Third, we objectively evaluated the efficacy of these two approaches in improving the survival of animal models *via* applying multiple tests for assessing between-study heterogeneity and publication bias. Fourth, there has not been remarkable between-studies heterogeneity that poses questions about the significance of the interventions. Fifth, we proposed a new strategy to ameliorate the response rate of CAR-T cells based on the detailed discussion on the recent preclinical and clinical findings regarding tumor microenvironment interactions and tumor antigens. However, the current study also suffers from several limitations. First, we only included papers published in English. Second, the protocol of the current study was not publicly available.

5 CONCLUSION

The co-administration of anti-PD-1 with CAR-T cells and PD-1 gene-editing of CAR-T cells can substantially prolong the survival of glioma-animal models, and anti-PD-1 can effectively accumulate in the CSF of patients with high-grade gliomas. However, clinical trials have failed to report favorable response rates of anti-PD-1 for glioblastoma patients, which might be due to the regulated inhibitory immune checkpoint network in the tumor microenvironment. Indeed, the fate of the tumor microenvironment is usually more complex than its direction can be determined by a single inhibitory immune checkpoint molecule. Currently available limited evidence has demonstrated that the gene-edited CAR-T cells might not be associated with severe side effects in animal models. To further increase the response rates of immune checkpoint inhibitors/CAR-T therapy, the combination of data obtained from single-cell sequencing of cells residing in the tumor microenvironment with fourth-generation CAR-T cells is suggested. The data from single-cell sequencing of tumoral cells can provide valuable insights into the patient-derived neoantigens that are specifically expressed in tumoral cells and cover subpopulations of tumoral cells. Also, the data from single-cell sequencing of cells residing in the tumor microenvironment can demonstrate the expression profile of inhibitory immune checkpoint molecules and their intensity in the tumor microenvironment, which can be used for engineering fourth-generation CAR-T cells to express the related immune checkpoint inhibitors following their stimulation. The proposed approach increases the chance of glioblastoma cells eradication. Also, because the immune checkpoint inhibitors are released in the tumor microenvironment, the risk of immune-related adverse events, seen following systemic administration of heavy dosage of multiple immune checkpoint inhibitors, might be decreased. Collectively, the combination of fourth-generation CAR-T cells with the data from single-cell sequencing technologies can open a new chapter in treating high-grade gliomas in the era of personalized medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

All authors have substantially contributed to the manuscript. MA: developing the research question, conducting the systematic search, conducting data extraction, performing the meta-analysis, conceptualization, and writing the manuscript. FB: helping with analysis. VK and NH: extracting data. AD, RF, and OB: providing the figures and tables. RB: reviewing the manuscript. BB and NS: reviewing the manuscript and supervising the project. All authors contributed to the article and approved the submitted version.

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REVIEW
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Advances in CAR-T Cell Genetic Engineering Strategies to Overcome Hurdles in Solid Tumors Treatment

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During this last decade, adoptive transfer of T lymphocytes genetically modified to express chimeric antigen receptors (CARs) emerged as a valuable therapeutic strategy in hematological cancers. However, this immunotherapy has demonstrated limited efficacy in solid tumors. The main obstacle encountered by CAR-T cells in solid malignancies is the immunosuppressive tumor microenvironment (TME). The TME impedes tumor trafficking and penetration of T lymphocytes and installs an immunosuppressive milieu by producing suppressive soluble factors and by overexpressing negative immune checkpoints. In order to overcome these hurdles, new CAR-T cells engineering strategies were designed, to potentiate tumor recognition and infiltration and anti-cancer activity in the hostile TME. In this review, we provide an overview of the major mechanisms used by tumor cells to evade immune defenses and we critically expose the most optimistic engineering strategies to make CAR-T cell therapy a solid option for solid tumors.

Keywords: CAR-T cell immunotherapy, Tumor microenvironment, Solid tumor, Tumor Homing, Chemokines, Angiogenesis, Tumor stroma, Immune checkpoints

1 INTRODUCTION

Chimeric antigen receptor (CAR)-T cells are genetically engineered T lymphocytes with an extracellular antibody-like domain (consisting of a single chain variable fragment or scFv), a transmembrane domain and an intracellular signaling domain. Four main generations of CAR-T cells have been designed to date. The main driver of genetically engineered enhancements across all these generations is the improvement of the anticancer potential of this innovative immunotherapy. First-generation CAR-T cells are engineered with a single activating intracellular domain, CD3ζ, (known as signal 1), without any additional costimulatory domains. As these CAR-T cells cannot produce enough interleukin (IL)-2 –vital for proliferation and growth- exogenous administration of IL-2 (IL-2 immunotherapy) is necessary to enhance CAR-T cells persistence *in vivo* and, thus, anticancer activity. Second- and third-generation CAR-T cells are genetically engineered with one or more intracellular costimulatory domains (known as signal 2), which increases CAR-T cell

efficacy and persistence (1). In the case of fourth-generation CAR-T cells, also known as T cell redirected for antigen-unrestricted cytokine-initiated killing (TRUCKs), an additional cassette coding for a transgenic protein (such as a cytokine) is expressed. This protein is released by the genetically modified lymphocytes and modulates their anti-cancer response (2).

Adoptive transfer of CAR-T cells has shown immense success in treating B cell malignancies. In the contrary, the response rates of CAR-T cell immunotherapy among solid cancer patients are less favorable. Major obstacles in solid tumor immunotherapy with CAR-T cells are, first, difficulties in tumor targeting and second an insufficient trafficking and fitness of genetically modified T cells, especially in the hostile tumor microenvironment (TME). Because of the lack of tumor-specific antigens (TSA) or the heterogeneous expression of tumor associated antigens (TAA) with overlapping expression between healthy tissues and tumor cells, one of the roadblocks to effective CAR-T immunotherapy is specific tumor targeting. Hurdles in solid tumor targeting make it a challenge to develop safe immunotherapies devoid of on-target/off-tumor toxicities. Moreover, TAAs can be lost in case of tumor antigen escape (as the case of proliferating tumor subclones), with CAR-T cell immunotherapy becoming ineffective. Other drawbacks, some inherent to CAR-T cells, are represented by limited tumor trafficking and tumor infiltration, as well as an insufficient expansion and persistence of genetically modified T cells in the homeostatic cytokine-deprived TME. All these challenges have been addressed by various preclinical models recently and efforts to improve engineering are still ongoing. In this review, we expose the major obstacles that CAR-T cells face in solid tumors, especially the decrease of T lymphocytes infiltration to the tumor site, the immunosuppressive milieu and the inhibition of CAR-T cell activity by the negative immune checkpoints, and we propose, by reviewing the literature, an extensive list of solutions to each of the mentioned obstacles.

2 CHALLENGES AND ENGINEERING STRATEGIES TO OVERCOME CAR-T CELLS' LIMITATIONS IN SOLID TUMORS

2.1 Enhancing CAR-T Cells Tumor Trafficking and Penetration

Solid tumors are organ-like, disorganized structures composed of proliferating tumor cells surrounded by supporting stromal cells and by nourishing blood vessels of the tumor neovasculature and associated to a cellular immune infiltrate composed of both innate and adaptive immune cells. Tumor growth can be controlled by both the innate and adaptive components of the immune system. Therefore, the infiltrating cell populations in solid tumors are comprised of both innate immune cells: neutrophils, macrophages, dendritic cells (DCs), mast cells, natural killer cells (NK cells), and myeloid derived suppressor cells (MDSCs) and of adaptive immune cells: T and B lymphocytes, and regulatory T cells (Tregs). All these immune cells are associated with non-tumor-stromal cells composing the TME: endothelial cells, fibroblasts, pericytes, and mesenchymal cells. All these cells, as well as their secreted factors and molecules compose the TME, an immunosuppressive, hostile milieu for tumor-infiltrating T cells (TILs) and a physical barrier for T cell migration and tumor infiltration.

Among all aforementioned cells, the key player of the anti-tumor response are TILs, their capacity to infiltrate the tumor bed being related to tumor outgrowth and extension (3–5). It is well acknowledged that TILs are a trademark of ongoing tumor immunosurveillance as they have shown both therapeutic and prognostic significance in animals and in humans. Indeed, higher density of TILs in patients' TME correlates with improved clinical outcomes (6), whereas fail to respond to immunotherapy is associated with a low post-treatment infiltration of T cells (7).

Therefore, the key for successful therapeutic strategies is the switch from a poorly infiltrated "cold" TME or from an "immune-excluded" TME [i.e limited presence of T cells at the periphery of tumor nests without intra-tumoral infiltration (8)] to a "hot" TME, with a rich, active, immune cell infiltrate in the tumor core, especially including functional TILs (9).

Despite initial expectations in solid tumor treatment with CAR-T cell therapies, one major roadblock in treating solid tumors turned out to be the limited access of cellular therapies to the tumor bed, as T cells must face additional barriers before inducing their antitumor activity (10). Indeed, great response to systemically infused CAR-T cells in hematological cancers is due, at least in part, to the easy access of CAR-T cells to malignant cells residing in hematologic organs readily accessible to the blood flow (bone marrow, lymph nodes, spleen) (10).

2.1.1 T cell Trafficking and Homing to Tumor Sites

T cell trafficking to both lymphoid organs and peripheral tissues is tightly regulated by chemotactic cues and controlled by chemokine/chemokine receptors axis and adhesion molecules interactions. T cell migration from the bloodstream and homing into peripheral tissues is a regulated, three-step process starting with 1) an initial transitory attachment and selectin-mediated rolling on the endothelium, followed by 2) chemokine-receptor mediated activation of integrins and finally by 3) integrin-dependent transmigration and extravasation (11, 12). Homing and retention of naïve T cells to lymph nodes is regulated by the expression of CD62L and of the CC chemokine receptor 7 (CCR7, which binds lymph-nodes chemokines CCL19 and CCL21), accompanied by the activation of LFA-1 (13). After T cell priming by antigen presentation, central memory T cells (TCM) lose the expression of both CCR7 and CD62L to acquire an effector memory (CD45RO+) phenotype (TEM), thereby losing their ability to access lymph nodes through the high endothelial venules (HEV).

Therefore, TEMs recirculate in the bloodstream to migrate to peripheral tissues and their migration back to the lymphoid organs is inhibited. Instead, activated T cells gain expression of a cohort of homing molecules that enable them to migrate to diseased/inflamed tissues (14). The T cell effector population presenting with homing capacity to tumor sites expresses homing molecules including ligands for E-selectin (CD62E)

and P-selectin (CD62P) expressed on activated endothelial cells as well as chemokine receptors for inflammatory chemokines, such as CXCR3 which binds inflammatory chemokines CXCL9 and CXCL10 (14) and CCR5 which binds respectively to CCL3/CCL3L1/CCL4/CCL5/CCL8/CCL11/CCL13/CCL16 ligands produced by tumor tissues (15, 16). Moreover, the activation of chemokine receptors enables adhesion to the endothelium by inducing the expression of two integrins: β2-integrin leukocyte function-associated antigen-1 (LFA-1), and very late antigen-4 (VLA-4, also known as α4β1), which bind respectively to ICAM-1 and VCAM-1 receptors expressed on the endothelium (17). Upon activation, integrins express binding sites that interact with cell adhesion molecules on the blood vessel walls, leading to T cell firm adhesion and transmigration into the tumor site (18) (**Figure 1**).

Peripheral tissues are the homing site for specialized memory T cell subsets identified and characterized extensively in the context of infectious diseases, called tissue resident memory T cells or TRM, and whose presence in solid cancer is associated with better outcomes. TRMs are localized in non-lymphoid peripheral tissues, do not recirculate and have a unique surface phenotype characterized by the lack of expression of receptors/transcription factors enabling egress from the tissues and lymph node homing (CCR7, CD62L, S1pr1 and Klf2). TRMs express the activation marker CD69, and the integrins CD103 (αEβ7) and CD49a (α1β1), which bind to E-cadherin and type IV collagen on epithelial and endothelial cells, respectively. They also upregulate the LFA1 integrin (αL(CD11a) β2), which binds to the ICAM-1 adhesion molecule on endothelial cells (19). Moreover, CD8+ TILs with a TRM phenotype expressing the adenosine producing ectonucleotidase CD39 and the CD103 integrin are a unique, specific tumor-reactive population found exclusively in the TME, both in primary and metastatic tumors, and whose frequencies are associated with overall survival (OS) in some cancer patients (20). Furthermore, it has recently been shown that a high density of CD8+CD103+CD49a+CD69+ TRM TILs correlates with an improved response to anti-programmed death 1 (PD-1) immune checkpoint blockade (19). Immune checkpoint inhibitors (i.e. ICI) represent a new Nobel-Prize worth immunotherapy with immense success in some incurable cancers, which target s inhibitory costimulatory molecules on the surface of T cells (like PD-1 or CTLA-4).

Barriers limiting access of CAR-T cells to the tumor bed are both physical (represented by surrounding blood vessels and the

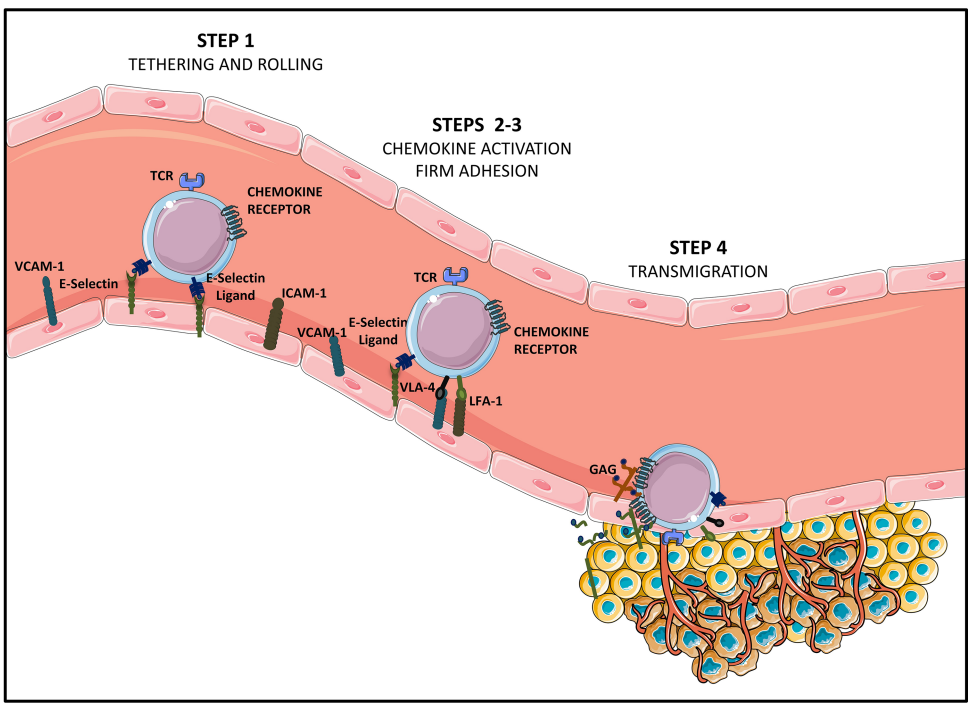

FIGURE 1 | Steps of T cell homing to tumor tissues [Adapted from Sackstein et al. (11)]. Tumor infiltrating CD8+ effector T lymphocytes (Teffs) presenting a specific tumor antigen circulate in the blood stream. They express homing molecules allowing for their oriented migration towards the tumor (like CXCR3 and CCR5-chemokine receptors), as well as ligands allowing binding to endothelial cells (E-selectin ligands and VLA-4 and LFA-1 integrins at suboptimal levels). Circulating Teffs tether and roll on the endothelium (STEP 1) *via* engagement of E-Selectin ligands with endothelial E-Selectin, which slows down Teffs, and allows firm adhesion to the endothelium (STEP 2). In this second step of Teffs entry into tumoral tissues, chemokines produced by cancer cells or by stromal cells from the TME (CXCL9, CXCL10, CCL5…) bind chemokine receptors. This binding of chemokine receptors to their ligands elicits activation of VLA-4 and LFA-1, allowing for VLA-4/VCAM-1 and LFA-1/ICAM-1 firm adhesion (STEP 3). Firmly adherent Teffs undergo transendothelial migration (STEP 4), to infiltrate the TME and establish cell-to-cell contact with tumor cells, *via* TCR-based recognition of cancer antigens presented on HLA molecules.

tumor stroma) and functional (represented by immunosuppressive molecules and soluble factors in the TME). Briefly, infused CAR-T cells need, in order to exert their cytotoxic effect, to: 1) traffic through the blood stream and migrate to the tumor tissue, in a chemokine directed manner, 2) cross the limiting blood vessels during the transmigration step 3) infiltrate the tumor and migrate to the vicinity of tumor cells by degrading TME components and 4) generate stable cell to cell contacts with tumor cells. Finally, success of adoptive cell therapy (ACT) is warranted by an increased persistence of infused CAR-T cells, which is dictated by their capacity to proliferate and survive in the hostile (acidic, hypoxic and nutrient and cytokine derived) tumor environment. Moreover, tumors have developed "escape mechanisms" in order to divert the immune-patrol process (21).

Therefore, CAR-T cell trafficking to and infiltration of the tumor is the first roadblock that needs to be overcome. Defective CAR-T cell infiltration is caused by: (i) chemokine/chemokine receptors mismatch or downregulated tumor-derived chemokines (22), (ii) an aberrant vasculature with downregulated or deficient adhesion molecules (23) and (iii) a remodeled tumor stroma, mainly composed of extracellular matrix (ECM) and cancer associated fibroblasts (CAFs) (24, 25).

2.1.2 Overcoming the Mismatch or the Dysregulation of Chemokine Receptor/Ligand Axes

Recent studies have shown that endothelial cells lining the tumor vasculature are able to prevent the trafficking, the adhesion and to eventually hijack anti-tumor activity of T cells (26). Some tumors block T cell homing by reducing the expression of adhesion molecules such as ICAM-1, VCAM-1, and CD34 on the tumor endothelium (14). For instance, the overexpression of endothelin B receptors (ETBR) on the tumor vasculature in ovarian cancer represses T cell trafficking by preventing ICAM-1 clustering on endothelial cells, which has a central role in T cell arrest and migration (27). Furthermore, as CXCR3 and CCR5 are often used by activated T cell to infiltrate tumors that should express their respective ligands (28), an insufficient expression of CXCR3 and CCR5 ligands by some tumors leads to a decrease in T cell recruitment (29, 30).

Since efficient trafficking is the first critical step for CAR-T cells to mediate their anti-tumor activity, several strategies targeting chemokine-chemokine receptor signaling are currently being explored in solid tumors. Some of them have already been tested in preclinical and clinical studies. To this end, CAR-T cells were genetically modified to co-express either chemokine receptors, among which we can cite: CCR2b, CXCR1/CXCR2, CCR4, CX3CL1, CSF-1R and CCR8 or to produce various chemokines: CCL19, CCL21 or CXCL11 (**Figure 2**). In more recent studies, co-expression of tissue homing molecules, as CD103 or CD39 was used to direct CAR-T cells to the tumor sites more efficiently

The chemokine ligand CCL2 or monocyte chemoattractant protein 1 (MCP-1) mediates the trafficking of immune cells into the TME in many types of malignancies, such as melanoma, colorectal, breast, prostate and pancreatic cancer (32). Therefore, co-expression of the CCL2 chemokine receptor, CCR2b, in CAR-T cells improves their anti-tumor activity, by enhancing their ability to traffic to the tumor bed. Craddock et al. demonstrated an improved homing (>10-fold) of GD2-specific CAR-T cells co-expressing CCR2b to CCL2-secreting neuroblastoma, as compared to CCR2-negative CAR-T cells (33). Likewise, co-expression of CCR2b was also associated with an increased migration (12.5-fold) of mesothelin (MSLN)-targeted CAR-T cells toward malignant pleural mesothelioma, in a study conducted by Moon et al. (34).

Furthermore, IL-8/CXCL8 was shown to be a pro-inflammatory chemokine that plays an important role in a variety of human cancers, including melanoma (35), prostate (36), colon (37), breast (38) and ovarian (39) cancers, by mediating tumorigenesis and angiogenesis. Some researchers took advantage of tumor-produced IL-8 in order to guide the IL-8 receptor (CXCR1 or CXCR2)-expressing CAR-T cells to infiltrate solid tumors (glioblastoma, hepatocellular carcinoma (HCC), ovarian and pancreatic cancer), and stimulate an antitumor immune response. Results showed a significantly enhanced tumor trafficking and persistence of genetically modified T cells, which triggered tumor regression, durable immunologic memory and better toxicity profile in mice (40–42). A clinical trial (NCT01740557) was initiated to evaluate the efficacy of T cells transduced with CXCR2 and with nerve growth factor receptor (NGFR), associated with Recombinant Human IL-2 (Aldesleukin) infusion in melanoma (**Table 10**). Exogenous supplementation of the Il2 vital support cytokine is widely used in the clinical setting (See *Targeting Fibroblast Activation Protein (FAP)* and *Tregs*), and is resumed in the table dedicated to CAR-T cells clinical trials (**Table 10**).

Moreover, it has been noted that two CCR4 ligands -CCL17 and CCL22-, are overexpressed in lymphoid malignancies such as Hodgkin's lymphoma (HL) (43), and in many other types of human cancers (44) including ovarian (45), breast (46), esophageal (47) and gastric (48) cancers. The aberrant overexpression of those ligands at the tumor site plays a central role in recruiting CCR4+ Th2 and regulatory T cells (Tregs) to such malignancies, resulting in an immunosuppressive TME (43). Since CCR4- effector T cells are barely present at the tumor site, the forced co-expression of surface CCR4 in CAR-T cells appears to be a promising therapeutic strategy in the treatment of certain types of lymphomas. Taking advantage of a mouse model of HL, Di Stasi et al. demonstrated that CAR-T cells engineered to co-express the chemokine receptor CCR4 together with the effector antigen receptor CD30 (CAR-CD30 T cells), had improved migration towards the tumor and enhanced anti-lymphoma activity as compared to CD30 CAR-T cells lacking CCR4 expression (49). A clinical trial (NCT03602157) was initiated to ascertain the effectiveness of CAR-T cells co-expressing CD30 and CCR4 in relapsed/refractory CD30+ HL and cutaneous T cell lymphoma (CTCL) (**Table 10**).

More recently, mesothelin specific CAR-T cells (MSLN-CAR) transduced to express either CCR2b or CCR4 chemokine receptors of Mcp-1 were engineered by Wang et al. and tested *in vitro* and *in vivo* in a non-small-cell lung carcinoma (NSCLC) model. MSLN-CCR2b-CAR-T cells

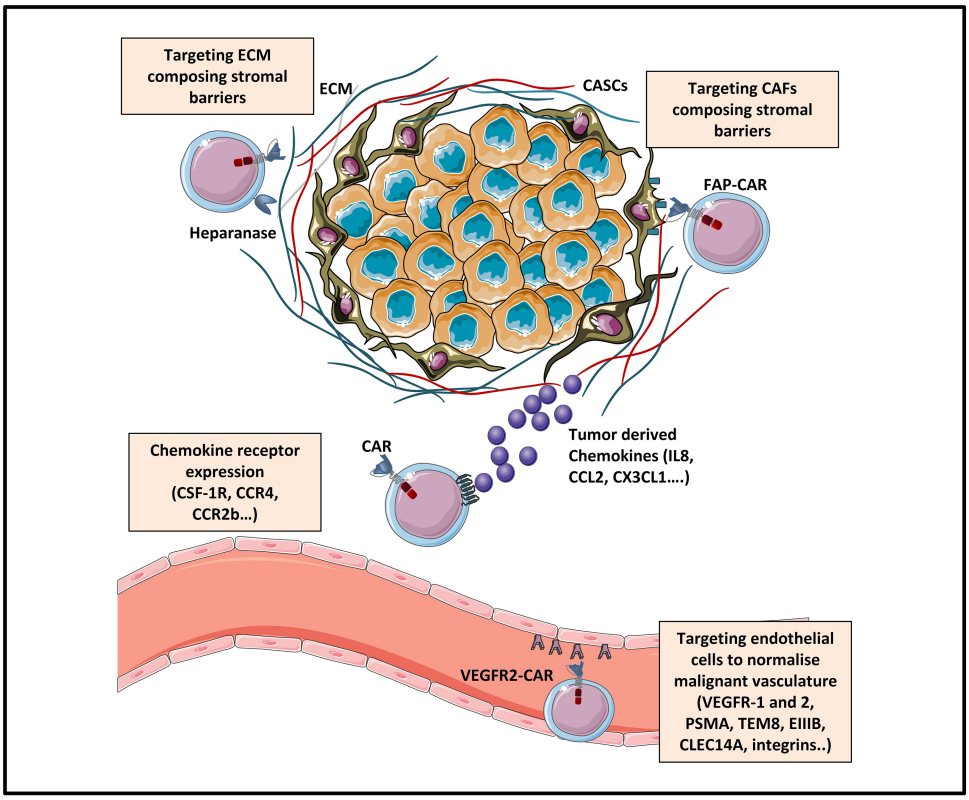

FIGURE 2 | Strategies enhancing tumor trafficking and penetration [Adapted from Rafiq et al. (31)]. The trafficking of CAR-T cells towards tumor sites can be enhanced by engineering CAR-T cells expressing chemokine receptors (as for example CSF-1R, CCR4 or CCR2b) specific for tumor-derived chemokine ligands (IL8, CCL2, CXCL1…). Tumor penetration of CAR-T cells can be enhanced by various strategies: (1) normalizing the malignant vasculature by targeting tumor blood vessels *via* CAR targeting of endothelial/tumoral antigens (like VEGFR, EIIIB, TEM8, integrins.), and (2) targeting physical barriers in the tumor microenvironment (TME) like the extracellular matrix (ECM) or the cancer associated fibroblasts (CAFs).

displayed superior anti-tumor function due to enhanced migration and infiltration into tumor tissues as well as no obvious toxicity (no organ damage). The MSLN-CCR4-CAR-T cells showed enhanced migration and potent cytotoxic function and cytokine production *in vitro* but were not further tested *in vivo* (50).

As previously mentioned, CXCR3 is highly expressed on effector T cells and plays a key role in their trafficking (51). Therefore, tumors expressing chemokines such as interferon-γ (IFN-γ)-inducible CXCR3 ligands would attract effector lymphocytes. CXCR3 binds three ligands: CXCL9 (monokine induced by IFN-γ), CXCL10 (interferon-induced protein-10) and CXCL11 (interferon-inducible T cell alpha chemoattractant) (52). Moon et al. used CAR-T cells as vehicles to deliver CXCL11 to the cancer site in order to increase its expression within the tumor and therefore recruit effector TILs. Unfortunately, this approach was not able to improve T cell tumor infiltration, despite of the local increase in CXCL11 (53). Given the success of oncolytic vaccinia viruses (VVs) expressing CXCL11 in increasing the numbers of effector T lymphocytes in specific murine tumors (54, 55), the same team combined the use of a VV engineered to produce CXCL11 with MSLN CAR-T cells administration. Results showed increased efficacy in CAR-T cells trafficking and tumor progression control of this combined strategy, as compared to VV.CXCL11 alone (53).

On another front, data showed that the unique member of the CX3-chemokine subfamily, termed fractalkine or CX3CL1, can be exploited to help overcome the poor homing of CAR-T cells to tumor sites. The CX3CL1-CX3CR1 axis is involved in chemotaxis and adhesion of leukocytes and in the recruitment of immune cell subpopulation such as NK cells, Th1 lymphocytes and macrophages (56). CX3CL1 is expressed in breast (57), pancreatic (58), gastric (59) and colon (60, 61) cancers. Siddiqui et al. demonstrated that CAR-T cells engineered to express CX3CR1 have increased infiltration towards CX3CL1-producing tumors in mice as well as decreased tumor growth (62).

In a proof-of-concept *in vitro* model, Lo et al. induced forced expression of the macrophage colony-stimulating factor 1 receptor (CSF-1R) to render CAR-T cells sensitive to CSF1, a monocyte recruiting chemokine enriched in various tumor tissues. Forced expression of CSF-1R exploits the T cell signaling machinery to enhance CAR-T cells Il-2 driven proliferation and costimulate production of IFN-γ, without reducing cytotoxicity and without inducing transdifferentiation to the monocytic/macrophagic lineage. CSF-1 forced expression

is a cytokine engineering strategy which could improve both CAR-T cell effector function (i.e. persistence/proliferation and cytokine production) and CAR-T chemotaxis to the tumor site (63).

More recently, Cadilha et al. employed a combined CAR-T cells engineering strategy enabling enhanced recruitment by CCR8 expression together with shielding from immunosuppression by the expression of a dominant-negative TGF-β receptor 2 (TGF-β DNR). The team exploited the CCR8-CCL1 recruitment axis, by which various tumors with poor prognosis attract Tregs, to empower effector CAR-T cells with enhanced chemotaxis. The team validated this strategy in a murine model of pancreatic cancer and in human xenograft tumor models. Furthermore, this strategy exploits activated T cell derived CCL1 to potentialize a positive feedback loop in CCR8+ cells recruitment to the tumor site (64).

Two other teams designed fourth generation CAR-T cells producing/co-expressing both IL-7 and CCL19 or CCL21 (65, 66). These combinatorial strategies associating co-expression of chemokine receptors/ligands with production of homeostatic cytokines could enhance both migration of CAR-T cells to the tumor site and proliferation/persistence of CAR-T cells in the hostile TME. Adachi et al. engineered CAR-T cells specific to fluorescein isothiocyanate (FITC) co-expressing IL-7 and CCL19 (7 × 19 CAR-T cells), two factors produced by T-zone fibroblastic reticular cells and essential for the maintenance of T cell zones in lymphoid organs. Treated mice achieved complete remission of pre-established tumors and 7 × 19 CAR-T cells showed superior anti-tumor activity than conventional CAR-T cells, as well as an improved ability of both migration and proliferation in the TME. Response to 7 × 19 CAR-T cells was dependent on the recipient's immune system (i.e activation and recruitment of dendritic cells and of tumor-reactive recipient T cells). Moreover, recipient conventional T cells also generated tumor –antigen-specific memory, probably due to epitope spreading. The authors raised security concerns about this engineering strategy, as gain of function (GOF) mutations of the IL-7 receptor (CD127) are frequent in pediatric T cell acute lymphoblastic leukemia (T-ALL) and as CCR7 could play a role in tumor metastasis. This engineering strategy could, therefore, benefit from the integration of a suicide gene system in order to prevent an eventual leukemic change of 7 × 19 CAR-T cells before clinical application (65). The same team validated the use of anti-mesothelin IL-7/CCL19-producing human CAR-T cells in a preclinical model of orthotopic pre-established malignant mesothelioma, as well as in patient derived xenograft (PTX) models of mesothelin-positive pancreatic cancer. As in the previous study, IL-7/CCL19-producing human CAR-T cells exerted a significant inhibition of tumor growth and prolonged survival of treated mice. Tumors showed increased infiltration with T recipient no-CAR-T cells as well as downregulation of exhaustion markers PD-1 and T cell immunoreceptor with Ig and ITIM domains (TIGIT) on T cells (67). Similar results were obtained with 7 × 19 CAR-T cells *in vivo* in the context of hepatocellular carcinoma (HCC) and pancreatic carcinoma (68).

There are two clinical trials on CAR-T cells co-expressing IL-7 and CCL19. Results from a first six-case cohort preliminary phase I clinical study (NCT03198546) in advanced HCC/PC/ovarian carcinoma (OC) patients with glypican-3 (GPC3) or MSLN expression have been published recently and show encouraging results: two complete responses (CR), two partial responses (PR) and 2 steady diseases (SD). There were no grade 2–4 adverse events or major complications (68). Another ongoing clinical trial (NCT03932565) evaluates intratumoral injection of Nectin4/FAP-targeted fourth-generation CAR-T cells (expressing IL-7 and CCL19, or IL12) for the treatment of Nectin4-positive advanced malignant solid tumors (NSCLC, breast, ovarian, bladder or pancreatic cancer). This represents an engineering strategy designed to enhance migration (CCL19), proliferation/maintenance (IL-7/IL-12) of CAR-T cells and to simultaneously target the stromal CAFs (anti-FAP). Three other clinical trials are evaluating the efficacy of this type of immunotherapy in the context of B cell lymphoma/multiple myeloma: NCT04833504 evaluating CD19-CAR-T expressing IL-7 and CCL19 in the context of relapsed/refractory B cell lymphoma, NCT04381741 evaluating CD19 CAR-T expressing IL-7 and CCL19 combined with PD-1 mAb for relapsed or refractory diffuse large B cell Lymphoma (DLBCL) and NCT03778346 evaluating fourth generation CAR-T cells simultaneously expressing IL-7 and CCL19 and directed against single or compound targets (Integrin β7, BCMA, CS1, CD38 and/or CD138) in the context of refractory/recurrent multiple myeloma (R/R MM). Results for the first two patients treated with BCMA-7 × 19 CAR-T cells (NCT03778346) in the context of R/R MM show encouraging results: an objective response within 1 month after BCMA-7 × 19 CAR-T cell infusion with one patient reaching CR and one a very good partial response (VGPR) and responses lasted more than 12-months (**Table 10**). There was no clinically significant toxicity. It is worth noticing that this CAR-T cell therapy was associated with a high proportion of stem cell memory (TSCM) among produced CAR-T cells, possibly due to IL-7 production (69). Indeed, several clinical studies have shown that the modifications to induce differentiation toward a TCM/TSCM profile improve CAR-T cell responses in subjects (70–72).

Another similar approach was to engineer Claudin18.2 (CLDN18.2)-specific CAR-T cells to co-express IL-7 together with the chemokine receptor CCR7 ligand CCL21 (7 × 21 CAR-T cells). CLDN18.2-specific second-generation CAR-T cells coexpressing IL-7 and CCL21 were tested *in vitro* and *in vivo* in three tumor models (breast, pancreatic and hepatocellular carcinoma) and revealed superior therapeutic effects to either conventional CAR-T cells or 7 × 19 CAR-T cells, without preconditioned lymphodepletion. As for 7 × 19 CAR-T cells, 7 × 21 CAR-T cells showed significantly improved survival and tumor infiltration. Treated mice showed increased infiltration of DCs as well as an inhibition of the tumor angiogenesis (presumed effect of CCL21) (66). No clinical trials evaluating the efficacy of CCL21 expressing CAR-T cells have been designed to date. However, various clinical trials use CCL21 gene modified dendritic cells (DCs-adenovirus CCL21) as anticancer vaccination strategies in

lung cancer or melanoma (NCT00601094, NCT01433172, NCT01574222, NCT03546361 and NCT00798629).

Genetically engineered expression of TRM-type markers CD103 or CD39 on CAR-T cells has recently been evaluated as a strategy to overcome insufficient trafficking and infiltration of solid tumors (HCC) or hematologic cancers (human Raji lymphoma). In a HCC model, hepatitis B virus (HBV) surface protein-specific CAR-T cells (HBVsCAR-T cells) were genetically manipulated to express CD39 and showed increased cytotoxicity in an *in vitro* model of HCC organoids and T lymphocytes coculture and in a PDX mouse model. To prevent an exhausted phenotype of CD39+ CAR-T cells, the team used a combinatorial strategy of CD39 expression on CAR-T cells, together with knockdown of inhibitory immune-checkpoints (triple knockdown of PD-1, T cell immunoglobulin domain and mucin domain-3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3) with shRNAs). CD39+ CAR-T cells showed enhanced cytokine production and antitumor effect. According to the authors, CD39 can serve as a biomarker to identify both personalized tumor-reactive CD8+ T cells as well as active CAR-T cells. Besides phenotypic identification, CD39 expression is also necessary for the cytotoxic effect of CD8 CARs and positively regulates antitumor activity (73). The TRM marker CD103 is a tissue homing molecule important for effector T cell trafficking as well as a promising prognosis biomarker for assessment of tumor-reactive TILS in various types of cancer, such as lung cancer, ovarian cancer and cervical cancers. CD103 is an integrin protein (αE) that binds integrin β7 to form the heterodimeric integrin complex αEβ7. Sun et al. used an E-Cadherin positive human lymphoma preclinical model (human Raji leukemia/lymphoma cells injected in NSG mice) to test therapeutic effects of CD103 expression on CD19-specific human CAR T cells. The gene encoding for the αE integrin was incorporated in the CD19-specific CAR structure to generate CD103-CD19-BBz-CAR T cells. These CAR-T cells showed more immature phenotypes (expressing high levels of CD62L and CD45RA), as compared to conventional CD19-BBz-CAR T cells, an increased production of IL-2 and greater expansion in culture, as well as improved anti-tumor efficacy (increased persistence, infiltration and eradication of lymphoma distant metastasis) upon adoptive transfer in immunodeficient mice (74).

The aforementioned preclinical studies on chemokine receptors expressing CAR-T cells are summarized in the table below (**Table 1**).

2.1.3 Handling Neovasculature Aberrancies

Tumor angiogenesis is a hallmark of cancer growth and progression (75). The generation of a tumor-associated neovasculature enables the growing tumor mass to obtain nutriments and oxygen. Moreover, the tumor uses these new vessels as a principal route to enter the circulation and to metastasize and proliferate to distant areas (76). Tumor neovasculature is a disorganized labyrinth of vessels at risk of vascular collapse. It lacks a hierarchical vessel division, which gives rise to abnormal blood flow and permeability, diffusion-limited nutrient delivery, oxygen deprivation, and an increased interstitial fluid pressure in the tumor (77). Tumor-induced angiogenesis is induced by the imbalanced production of proangiogenic factors by the tumor cells, including vascular endothelial growth factor-A (VEGF), platelet-derived endothelial growth factor (PDGF), transforming growth factor (TGF)-α, angiopoietin (Ang), basic fibroblast growth factor (bFGF), fibroblast growth factor (FGF), and placental growth factor (PGF) (78, 79). These soluble factors bind to and activate diverse tyrosine kinase (TK) receptors, such as VEGFR1, VEGFR2, PDGFRA, and endothelial growth factor receptor (EGFR), promoting angiogenesis, among other biological events (80).

As stated previously, in order to reach the tumor site, T cells encounter a physical barrier, represented by this abnormal vasculature, which operates though as a first obstacle for lymphocyte recruitment into the tumor. Therefore, vascular targeting, using anti-angiogenic molecules, has been proposed as a novel strategy to block tumor growth. This approach aims at correcting the structural and functional abnormalities of the tumor vasculature, in order to improve T cell infiltration and immunotherapy efficacy (81). The first Food and Drug Administration (FDA) approval of an anti-angiogenic monoclonal antibody (mAb) (Bevacizumab) dates back to more than a decade ago (82). In more recent studies, substantial efforts were deployed to develop CAR-T cells with a chimeric receptor comprising a scFv antibody against specific angiogenic growth factors/receptors or adhesion molecules abnormally expressed on the tumor vasculature (83). To this end, Kershaw et al. were the first to suggest an indirect strategy to target stromal tumors by the usage of CAR-T cells targeting the vascular stroma instead of the cancer cell itself (84).

In order to inhibit tumor angiogenesis, Chinnasamy et al. genetically modified murine and human T cells to express a CAR targeted against VEGFR-2 (85). VEGFR-2 is overexpressed in tumor vasculature and is known to be critical for both physiological and pathological/tumor angiogenesis, as well as for VEGF-mediated tumor progression (86). VEGFR-2 is overexpressed in many types of solid tumors, including breast cancer, cervical cancer, NSCLC, hepatocellular carcinoma, and renal carcinoma (87). Chinnasamy et al. demonstrated that the antitumor effect of VEGFR-2 targeting CAR- T cells was not mediated through their direct cytotoxicity on the tumor cells but rather through their ability to eliminate VEGFR-2-expressing cells in the tumor vasculature. A single dose of VEGFR-2 CAR-T cells was effective in increasing tumor infiltration, and inhibiting the growth of 5 vascularized syngeneic tumors of various histological origins (85). The same group showed, in another study, that the coadministration of anti-VEGFR-2 CAR-T cells along with tumor-specific TCR transduced T cells (premelanosome (Pmel) TCR, tyrosinase-related-protein-1 (TRP-1) TCR, and tyrosinase-related-protein-2 (TRP2) TCR traduced T cells) resulted in a synergic anti-tumor effect and an extended tumor-free survival (TFS) of mice with metastatic melanoma tumors. These results emphasize the advantageous effects of dual targeting adoptive therapy including an anti-angiogenic strategy (88). Recently, Englisch et al. suggested

TABLE 1 | Summary of preclinical studies on chemokine receptors/ligands or homing molecules expressing CAR-T cells.

Expressed chemokine receptor/ligand	CAR	Type of cancer	Reference
CCR2b	GD2	Neuroblastoma	(33)
	MSLN	Malignant pleural mesothelioma	(34, 50)
		Non-small-cell lung carcinoma (NSCLC)	
CXCR1 or CXCR2	CD70	Glioblastoma, ovarian or pancreatic cancer	(40)
	αvβ6	Ovarian or pancreatic cancer	(41)
	GPC3	Hepatocellular carcinoma	(42)
CCR4	CD30	HL	(49)
	MSLN	Non-small-cell lung carcinoma (NSCLC)	(50)
CCR8	MSLN	Pancreatic cancerPancreatic ductal adenocarcinoma (PDAC)	(64)
CXCL11	MSLN	Lung cancer	(53)
CX3CR1	CX3CR1	Colorectal cancer	(62)
CSF-1R	P28z	Prostate carcinoma	(63)
CCL19	FITC	MastocytomaLung carcinomaPancreatic adenocarcinoma	(65)
	MSLN	Malignant mesotheliomaPancreatic cancer	(67)
	GPC3	HCC	(68)
	MSLN	Pancreatic carcinoma	
CCL21	CLDN18.2	Breast cancerPancreatic carcinomaHCC	(66)
CD39	HBVs	HCC	(73)
CD103	CD19	Leukemia/Lymphoma	(74)

that VEGFR-2 expressed on tumor vasculature could be a potential CAR target in Ewing sarcoma (EwS) (89), especially that this type of cancer is characterized by a limited TSA expression on cancer cells (90). Contact with their target triggered a powerful antigen-specific degranulation response, increased proliferation and cytokine secretion of VEGFR-2 CAR-T cells. Data showed that VEGFR-2 CAR-T cells with short-length or medium-length hinge domains effectively destroyed VEGFR-2-expressing tumor-associated endothelial cells (89). Similarly, in a study from Taheri et al. nanobody-based anti-VEGFR2 CAR showed effective activation, degranulation and lysis of VEGFR2+ cell lines in an *in vitro* model (91). Unfortunately, adoptive transfer of VEGFR-2 CAR-T cells in a clinical setting was devoid of great success in a phase 1 clinical trial NCT01218867 on patients with metastatic cancer. The trial was terminated due to lack of objective responses: out of 24 infused patients, only one reached a PR and another one had a stable disease (SD) after CAR-T cell injection. There were no CR (**Table 10**).

While VEGFR-2 plays a critical role both in physiological and pathological angiogenesis, VEGFR-1, another member of the VEGFR family, is strictly involved in pathological angiogenesis (92). Even though both are abnormally expressed at high levels on tumor vasculature, their signaling characteristics are different (93). However, VEGFR-1 is not restricted to endothelial cells as expression has also been proven on monocyte/macrophages, and on various types of tumor cells (92). VEGFR-1 has been shown to be a key regulator of macrophage' function and of cancer metastasis, among others, which makes it an interesting target in the development of novel approaches for cancer ACT (94). Wang et al. demonstrated that VEGFR-1 CAR-T cells can be a promising solution to break the resistance to traditional anti-angiogenic therapies, with higher efficacy than strategies blocking separately cancer growth or angiogenesis. This study also showed that co-administration of IL-5 producing CAR-T cells enhanced the anti-metastasis activity mediated by VEGFR-1 CAR-T cells (95).

Prostate-specific membrane antigen (PSMA) is another transmembrane protein highly expressed on the tumor-associated endothelium of a great variety of solid tumors - including bladder, oral, hepatocellular, gastric, colorectal, breast, ovarian, renal, and pancreatic ductal carcinoma as well as NSCLC and melanoma - (96, 97). Although not expressed by the normal endothelium, like it is the case of VEGFR, PSMA is still expressed at low levels in normal tissues as the brain, liver, kidney, intestine, colon and the prostate (98). Moreover, PSMA has a crucial role in tumor neovascularization. Santoro et al. directed a proof-of-concept study showing that PSMA CAR-T cells can recognize primary tumor PSMA-expressing endothelial cells and disrupt the tumor vasculature both *in vitro* and *in vivo*. Contrary to traditional anti-angiogenic agents, anti-PSMA CAR-T cells showed long-term *in vivo* persistence. However, in order to improve the safety profile of PSMA CAR-T cells, toxicity control mechanisms like the use of split-signaling CAR-T cells should be needed (97). PSMA has especially been targeted in prostate cancer patients, with various ongoing clinical trials (NCT01140373 (99, 100), NCT01929239, NCT00664196; and NCT03089203) (*see Tregs* and **Table 10**) (101).

Tumor endothelial marker 8 (TEM8), also known as anthrax receptor 1 (ANTRX1), is another cell membrane glycoprotein consistently overexpressed in the tumor vasculature and in many types of cancer, including breast (102), gastric (103),, skin (104), colon (105), and lung (106) cancers. Blocking or knocking out TEM8 inhibited pathological angiogenesis in several preclinical cancer models (104, 107). Moreover, anti-TEM8 CAR-T cells can serve as a potential targeted therapy for triple-negative breast cancers (TNBC). Results from Byrd et al. showed that TEM8-targeted CAR-T cells were able to concomitantly destroy TNBC tumor cells, breast cancer stem-like cells (BCSC) as well as tumor endothelial cells, and to cause regression of lung metastatic TNBC cell line-derived xenograft tumors (108). Unfortunately, a study published by Petrovic et al. raised concerns over possible on-target/off-tumor toxicities of TEM8-specific CAR-T cells (109).

It has been shown that fibronectin (FN) splice variants EIIIA and EIIIB are overexpressed in the vasculature of many types of tumors, including breast, lung and prostate cancers and high-grade glioma, whereas absent in normal tissues (110–112). These properties make EIIIA and EIIIB ideal targets for CAR-T cell therapy. Genetically engineered CAR-T cells targeting EIIIB were able to inhibit the growth of solid cancers in immunocompetent mice by compromising the blood supply of the tumor (113). Based on three tumor models, Wagner et al. reported similar results using immunodeficient mice treated with anti-EIIIB CAR-T cells (114).

Recently, C-type lectin domain family 14 member A (CLEC14A) has been identified as part of a molecular gene signature for tumor angiogenesis based on a meta-analysis on breast cancer, head and neck squamous cell carcinoma (HNSCC), and clear-cell renal cell carcinoma (ccRCC) (115). This protein is mainly overexpressed in the three aforementioned cancers (116, 117). CLEC14A could be a promising target for antiangiogenic therapy. A single injection of CLEC14A-specific CAR-T cells was sufficient for a significant suppression of tumor growth in 3 distinct tumor models. Use of anti-CLEC14A CAR-T cells could be combined with CAR-T cells targeting another tumor endothelial marker, in order to increase tumor vessel targeting capacities (118).

The integrin αvβ3 emerges as another potential target for cancer immunotherapy. Integrin αvβ3 is expressed on different types of cancer, including glioblastoma (119), melanoma (120), pancreatic (121), breast (122) and prostate cancers (123). Even though expressed on activated endothelial cells and newly formed vessels, it is not detectable in resting endothelial cells and normal tissues, making it a valid target for the treatment of many solid tumors (124). Wallstabe et al. generated αvβ3 targeted CAR-T cells and investigated antitumor effects of such approach in preclinical models *in vitro* and *in vivo*. They concluded that this strategy was able to inhibit tumor growth, but without achieving tumor eradication. Presence of haematomas in the tumor tissues proved that engineered T cells damaged tumor vessels, due to αvβ3-expression on tumor endothelium. Results also showed that adoptive therapy with αvβ3 CAR-T cells was more effective than immunotherapy with anti-αvβ3 mAbs (125).

Another integrin, the integrin αvβ6, whose expression on endothelial cells is restricted to development and remodeling processes (like wound healing, chronic inflammation and cancer), is upregulated in various cancers (126) and associated with more invasive tumor phenotypes, characterized by high tumor invasion and shorten survival in colon and cervix cancers or in NSCLC (127). This integrin emerged as an interesting target for immunotherapy with CAR-T cells in cholangiocarcinoma (CCA), a lethal bile duct cancer with poor responses to classic therapy. Targeting of CCA with anti- αvβ6 fourth generation CAR-T cells showed anti-tumor function against αvβ6 expressing CCA tumor spheroids, *in vitro* (128). In a previous study from Whilding et al., anti-αvβ6 CAR-T cells showed *in vivo* efficacy in other solid tumors expressing intermediate to high levels of this integrin (ovarian, breast, and pancreatic tumor xenografts in SCID beige mice). For selective expansion, CAR-T cells were engineered to co-express the IL-4-responsive fusion gene (4αβ, obtained by fusing the human IL-4 receptor α ectodomain to the shared human IL-2/IL-15 receptor β transmembrane and endodomain regions). Moreover, despite expression of this integrin in non-tumor endothelium, toxicities related to anti-αvβ6 CAR-T infusion were mild and reversible and only associated to systemic infusion of supra-therapeutic doses (129). There is no ongoing clinical trial with anti-αvβ6 CAR-T cells in cancer patients, but anti-αvβ6 cancer targeting, either by monoclonal antibodies or by peptides has already been tested in *in vitro* or preclinical animal models of breast (130) and pancreatic cancers (131, 132).

The aforementioned preclinical studies on proangiogenic factors/receptors-targeting CAR-T cells are summarized in the table below (**Table 2**).

2.1.4 Targeting the Tumor Stroma

Besides strategies aiming at targeting tumor blood vessels, engineering modifications targeting stromal cells may also be promising strategies for CAR-based immunotherapy. Targeting non-cancer cell components of the tumor stroma could help to enhance the anti-cancer effect of this immunotherapy for many reasons. First, stromal cells are less prone to immune-escape from the CAR-T cells attack as they show higher genetic stability than tumor cells, and are less likely to lose antigen expression *via* immunoediting (133). Second, since tumor stroma can be found in almost all human adenocarcinomas, CAR-T cells targeting the extracellular matrix (ECM) and/or the nonmalignant cancer-associated stromal cells (CASCs) could generate "broad-spectrum" CAR-T cells (134). Finally, tumor stroma plays a major role in tumor survival, growth, invasion, and angiogenesis, by producing growth factors, and chemotactic factors that attract immunosuppressive cells, and by expressing inhibitory surface checkpoint proteins (135). However, as extracellular matrix components are vital components of connective tissues, this targeting strategy needs identification and usage of specific tumor-ECM targets, in order to avoid on-target/off-tumor toxicities. To this regard, some studies focused on targeting ECM components by using CAR-T cells expressing ECM degrading enzymes while others chose as an attractive stromal candidate the fibroblast activation protein (FAP) expressed in CASCs (**Figure 2**).

a. ECM components modifying enzymes

Another strategy aiming at facilitating cellular penetration into solid tumors is genetic manipulation of CAR-T cells to secrete ECM-modifying enzymes. Indeed, the ECM is a complex structural component of the TME and the main physical barrier that hinders T cell-cancer cell contacts. The ECM is synthesized by malignant cells and cancer associated fibroblasts (CAFs) and can constitute up to 60/% of the tumor mass (136). Different ECM molecules, such as fibrillar collagen, hyaluronan (HA), proteoglycans (chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate), elastin, fibronectin and laminins are highly expressed in many solid cancers (136). Therefore, in order

TABLE 2 | Summary of preclinical studies on proangiogenic factors/receptors-targeting CAR-T cells.

Target	Type of cancer	Reference
VEGFR-2	Solid tumors	(85)
	Metastatic melanoma	(88)
	EwS	(89)
	Experimental cancer	(91)
VEGFR-1	Lung cancer	(95)
PSMA	Ovarian cancer	(97)
TEM8	TNBC	(108)
EIIIB	Solid tumors	(113)
	Lung cancerSarcomaHigh-grade glioma	(114)
CLEC14A	Lung carcinomaPancreatic cancer	(118)
αvβ3 integrin	Metastatic melanoma	(125)
αvβ6 integrin	Cholangiocarcinoma(CCA)	(128)
	Ovarian, breast and pancreatic cancer	(129)

to access the tumor sites and mediate their anti-tumor functions, T cells must be able to degrade the main components of the ECM. Lymphocytes secrete specific enzymes to disrupt the ECM, including: (i) heparinase (HPSE), an endoglucuronidase that cleaves heparan sulfate side chains of heparan sulfate proteoglycans (137), (ii) hyaluronidase, an endoglycosidase that cleaves glycosidic bonds of hyaluronic acid (138), and (iii) matrix metalloproteinases (MMPs), endopeptidase proteases that cleave the majority of ECM and non-ECM components (**Figure 2**). Caruana et al. noted that *in vitro*-engineered and cultured T cells lose heparinase expression following *TP53* binding to the HPSE gene promoter, which may restrict CAR-T cell infiltration in stroma-rich solid tumors. To this regard, the authors engineered CAR-T cells to express heparinase and demonstrated that it's expression led to improved cell migration in neuroblastoma xenograft models (139). Xiong et al. studied, *in vitro* and *in vivo*, the ability of GPC3 (Glypican 3 protein)-targeted CAR-T cells co-expressing IL-7 and the PH20 hyaluronidase to infiltrate hepatocellular carcinoma (HCC) xenograft models. Their results showed that the co-expression of the two aforementioned genes improved CAR-T cells trafficking, which may significantly enhance their efficacy in solid tumors (140).

Similarly, Zhao et al. reported the construction of MSLN (mesothelin)-targeted CAR-T cells with the overexpression of a secreted form of the human hyaluronidase (sPH20-IgG2) and found that this enzyme can promote the antitumor activity of these CAR-T cells *in vitro* and *in vivo* in gastric cancer cell xenografts, by promoting their infiltration (141). Use of a pegylated form of the human recombinant hyaluronidase (PEGPH20) has already been tested in the clinical setting in two randomized trials, as an adjuvant to chemotherapy in metastatic pancreatic adenocarcinoma (142, 143). Results of one of the trials (NCT01959139) could claim certain caution as adjuvant PEGPH20 therapy resulted in a diminished OS as well as an increased toxicity (gastrointestinal and thromboembolic events) (142). The other clinical trial (NCT01839487) did not confirm the reduction in survival (143) (**Table 10**). Even so, both studies confirmed an increased thromboembolic risk of the PEGPH20 therapy and imposed the adjunction of an anticoagulant prophylaxis with low molecular weight heparin in the study from Hingorani et al. (143). In a more recent phase III trial adjunction of PEGPH20 to chemotherapy had no benefits in terms of OS or progression free survival (PFS) in the case of metastatic pancreatic carcinoma (144).

Not least, another strategy to enhance CAR-T cells migration through the collagen barriers of the ECM could be CAR-T cell production of another ECM-modifying enzyme, the MMP8 metalloproteinases (also known as collagenase-2), as suggested by Mardomi and Abediankenari (145). However, transgenic production of MMPs has not been applied yet to CAR-T cells engineering. This type of engineering strategy, can also seam tempting for genetically engineered Macrophages (CAR-Macrophages) (146).

The aforementioned preclinical studies on CAR-T cells expressing ECM degrading enzymes are summarized in the table below (**Table 3**).

b. Targeting Fibroblast Activation Protein (FAP)

Growing evidence proves that many cell types within the TME play a key role in oncogenesis. Among them, CAFs, a major component of the tumor stroma, represent a reactive tumor-associated fibroblast population that secretes various active factors promoting tumor development, progression, metastasis, and therapeutic resistance (147) (**Figure 3**). CAFs express various molecules that can be targeted by immunotherapies. Among them, FAP has recently emerged as the most promising target (149). FAP is a cell surface serine protease that is highly expressed on the CASCs of various human cancer types (150), such as lung (151), prostate (152), pancreatic (153), colorectal (154), and ovarian cancer (155). In contrast, the expression of this proteolytic enzyme on normal quiescent adult stromal cells and benign tumors is reported to be low to undetectable. Moreover, several studies have shown that tumors expressing FAP are associated with poor prognosis (150), enhanced tumorigenesis (150) and an increased neo-angiogenesis (156). Therefore, different strategies have been used to target FAP using antibodies (157, 158), vaccines (159, 160), immunoconjugates (161, 162), peptide-drug complexes (163–166), FAP gene knock-down by siRNA delivery (167), and CAR-T cells (168).

A large number of preclinical studies using FAP-targeted CAR mouse T cells have been reported to date (**Table 4**). Tran et al. genetically modified T cells to express a scFv from the FAP-specific monoclonal antibody (MAb) FAP5, reactive both to human and mouse FAP. They report effective cytotoxic effect of FAP-reactive CAR T cells *in vitro*. However, adoptive transfer of FAP5-CAR-T cells into mice bearing a variety of subcutaneous tumors mediated limited antitumor effects and induced significant cachexia and lethal bone toxicities in two

TABLE 3 | Summary of preclinical studies on CAR-T expressing ECM degrading enzymes.

ECM degrading enzyme	CAR	Type of cancer	Reference
HPSE	GD2	Neuroblastoma	(139)
PH20	GPC3	HCC	(140)
	MSLN	Gastric cancer	(141)

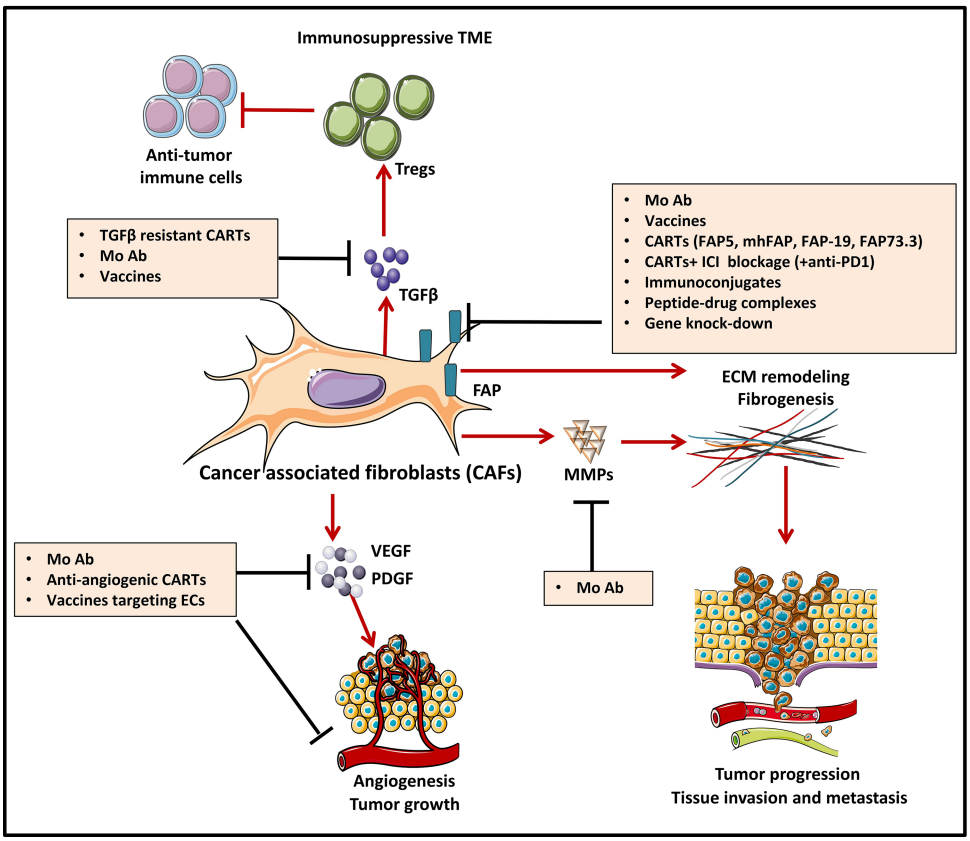

FIGURE 3 | Strategies to counteract protumorigenic effects of CAFs [Adapted from Kakarla et al. (148)]. Cancer associated fibroblasts (CAF)-directed anti-cancer therapies are one of the weapons of tumor targeting which is directed against the stromal compartment. Strategies depicted in this figure aim at inhibiting cancer associated fibroblasts (CAFs) functions and are based on targeting crucial signals and effectors of CAFs such as cytokines (TGFβ) and growth factor pathways (VEGF, PDGF…). For instance, CAF-derived extracellular matrix proteins (MMPs) and associated signaling can be targeted with monoclonal antibodies (MAb), to induce stromal depletion and increase immune T cell infiltration. Blocking some targets like TGFβ, can act both upstream and downstream, by blocking CAF formation and attenuating downstream signaling in CAFs that are already established. FAP targeting aims at blocking CAFs ability to exert tumor promoting effects in the TME. Targeting FAP can be done by using either MAb/antibody-drug conjugates, immunoconjugates or peptide-drug complexes, FAP-specific CAR-T cells or strategies of gene-knock out. Some other strategies, not depicted in this figure aim at CAFs direct depletion or CAFs normalization towards an inactive phenotype.

TABLE 4 | Summary of preclinical studies on FAP-targeted CAR-T cells.

CAR	Intracellular signaling domains	T cell origin	Type of cancer	Reference
FAP-5	CD28, 4-1BB and CD3ζ	Mouse	MelanomaColorectal cancerPancreatic cancerBreast cancer	(169)
mhFAP	CD28 and CD3ζ	Human	NSCLC	(170)
FAP-F19	ΔCD28 and CD3ζ	Human	Mesothelioma	(171)
FAP-73.3	CD8α, 4-1BB and CD3ζ	Mouse	MesotheliomaLung cancer	(172)
FAP-73.3	KIR2DS2 and DAP12	Human	Mesothelioma	(173)
FAP-F19 (+ Anti-PD-1)	ΔCD28 and CD3ζ	Human	Mesothelioma	(174)

mouse strains, due to low-level expression of FAP in multipotent bone marrow stromal cells (BMSCs) (169). Moreover, low level expression of FAP has been documented in other healthy tissues like: adipose tissue, skin, muscle and pancreas (150). Other on-target/off tumor toxicities after FAP+ stromal cell depletion with CAR-T cells, reported by Roberts et al., were bone marrow hypoplasia, anemia, pancreatic toxicity and loss of muscle mass (175). In an established lung cancer model, Kakarla et al. generated a CAR specific for both murine and human FAP (mhFAP) using the scFv from MO36 (previously generated by phage display from an immunized FAP/knock-out mouse) (176). They noted that mhFAP CAR-T cells were able to significantly reduce FAP+ stromal cells and tumor growth, with no toxicity or negative effects on wound healing. This study shed the light on the advantage of co-targeting CAFs and cancer cells since the authors

demonstrated that combining mhFAP CAR-T cells with EphA2 CAR-T cells increased overall antitumor activity (170). Schuberth et al. developed FAP CAR-T cells using F19 CAR that only recognizes the human version of FAP. They removed the binding site of lck from the CD28 intracellular signaling domain in order to impede IL-2 secretion upon FAP CAR-T cell engagement with its target, and thus reduce Tregs persistence. The authors found that the redirected T cells successfully lysed FAP+ mesothelioma cells in an antigen-specific manner *in vitro* and *in vivo*. However, the authors could not evaluated the on-target/off-tumor toxicity of their CAR-T cells since F19-FAP antibody targets only the human version of FAP, with no cross-reactivity with the mouse version (171).

Wang et al. developed FAP-73.3 CAR mouse T cells against mouse FAP and demonstrated that depletion of FAP+ cells reduced tumor growth in an immune-dependent manner, as the antitumor effect was only seen in fully immunocompetent mice. Moreover, no clinical toxicities have been observed in mice following the administration of FAP-73.3 CAR mouse T cells *in vivo*. In order to enhance the antitumor activity, the authors successfully increased the efficacy of their FAP CAR-T cells either by reinfusing a second dose one week later or by combining the redirected T cells with an HPV-E7 vaccine (Ad.E7) (172). The same group designed an alternative chimeric immunoreceptor by fusing the FAP CAR to the transmembrane and cytoplasmic domains of KIR2DS2, a stimulatory killer immunoglobulin-like receptor (KIR), instead of the conventional cytoplasmic domain of CD28 used previously. The aim of this study was to evaluate whether KIR-based CAR-T cells expressing FAP-KIR2DS2 and DAP12 (an immunoreceptor tyrosine-based activation motif (ITAM)-bearing transmembrane adaptor associated with NK-activating receptors) can exhibit a more powerful antitumor response as compared to CD3ζ-based CAR-T cells. Therefore, they generated murine FAP-KIRS2/DAP12-modified T cells using the same scFv from the FAP-73.3 hybridoma. Results showed an enhanced antitumor effect with a complete inhibition of tumor growth, as compared to the significant but minimal slowing of tumor growth with CD3ζ-based CAR-T cells. However, despite the lack of toxicity of the CD3ζ-based FAP-specific CAR-T cells, FAP-KIRS2/DAP12 CAR-T cells showed similar toxicity to the one reported by Roberts et al. in the aforementioned study, suggesting that higher efficacy of FAP targeting is also associated with higher risk of on-target/off-tumor toxicity (173). This issue prompted Gulati et al. to investigate which intracellular signaling domains should be combined with FAP CAR for malignant pleural mesothelioma treatment. When comparing CAR-T cells expressing the CD28/CD3ζ, ΔCD28/CD3ζ and 4-1BB/CD3ζ CAR, the authors noted that 4-1BB/CD3ζ CAR-T cells persisted the most (until day 44) in the peripheral blood of humanized mice, and that the deletion of lck in ΔCD28/CD3ζ CAR enhanced antigen-specific proliferation. Despite higher persistence of 4-1BB/CD3ζ CAR-T cells, statistically significant tumor control *in vivo* was only obtained when combining FAP-ΔCD28/CD3ζ CAR-T cells with the immune checkpoint PD-1 inhibitor antibodies (174).

To date, two clinical trials using FAP CAR-T cells have already been conducted. The first one is a phase I clinical trial (NCT01722149) using CD3ζ/CD28-based FAP-specific CAR-T cells in three patients with malignant pleural mesothelioma (**Table 10**). A single dose of 1x 10^6 CAR-T cells was administered through a pleural catheter. This therapy was well tolerated without any significant toxicity. In addition, one of the three patients received an anti-PD-1 checkpoint inhibitor antibody 8 months after FAP CAR-T cell administration; no clinical toxicity has been reported and 2 out of 3 patients were still alive after a follow-up of 18 months (177, 178).

The second one, cited earlier, is a phase I clinical trial (NCT03932565) using fourth-generation CAR-T cells coproducing IL-7 and CCL19/IL-2 in patients with Nectin4-positive advanced malignant solid tumors such as NSCLC, breast, bladder, pancreatic and ovarian cancer. An approach of intravenous infusion combined with intratumoral injection of Nectin4/FAP-targeted CAR-T cells will be undertaken. The clinical trial is ongoing and still recruiting (**Table 10**).

2.2 Counteracting the Immunosuppressive TME

The solid tumor microenvironment is composed, as stated previously, by stromal cells (including CAFs), surrounded by the tumor vasculature and by an immune infiltrate of immunosuppressive cells, among which myeloid cells (myeloid-derived suppressor cells or MDSCs), tumor-associated macrophages (TAMs) and Tregs (**Figure 4**). As previously shown (see *Targeting the Tumor Stroma*), stromal cells strongly impact the TME as well as the interactions between the immune system and the tumor. Various cell types of hematopoietic origin contribute to the generation of an immunosuppressive TME. This immunosuppressive TME is maintained both by contact mechanisms as cancer cells and stromal cells express a broad range of inhibitory immune-checkpoint ligands (for PD-1, TIGIT, LAG-3 and TIM-3) and by suppressive soluble factors produced by immune cells or by CAFs (cytokines like TGF-β or IL-10). Moreover, other soluble factors with known effects on angiogenesis and produced by this pro-tumorigenic cells, like VEGFA and prostaglandin E2 (PGE2), also induce immunosuppression by inhibiting cytotoxic T lymphocytes (CTLs) and NK cells and by inducing accumulation and proliferation of Tregs (179–181). Therefore, targeting immunosuppressive cells in the TME could improve the efficacy of immunotherapies by increasing tumor recognition by the immune system. In this section, we will discuss the mechanisms by which pro-tumorigenic immune cells from the TME hijack T cell function as well as the different molecular strategies deployed to enhance the efficacy of genetically modified T cells to surmount these roadblocks.

2.2.1 TAMs

Macrophages, one of the main effector cells of the immune system, play a key role in both innate and adaptive immune responses. They constitute the first line of defense against foreign pathogens and help trigger an adaptive antigen-specific response.

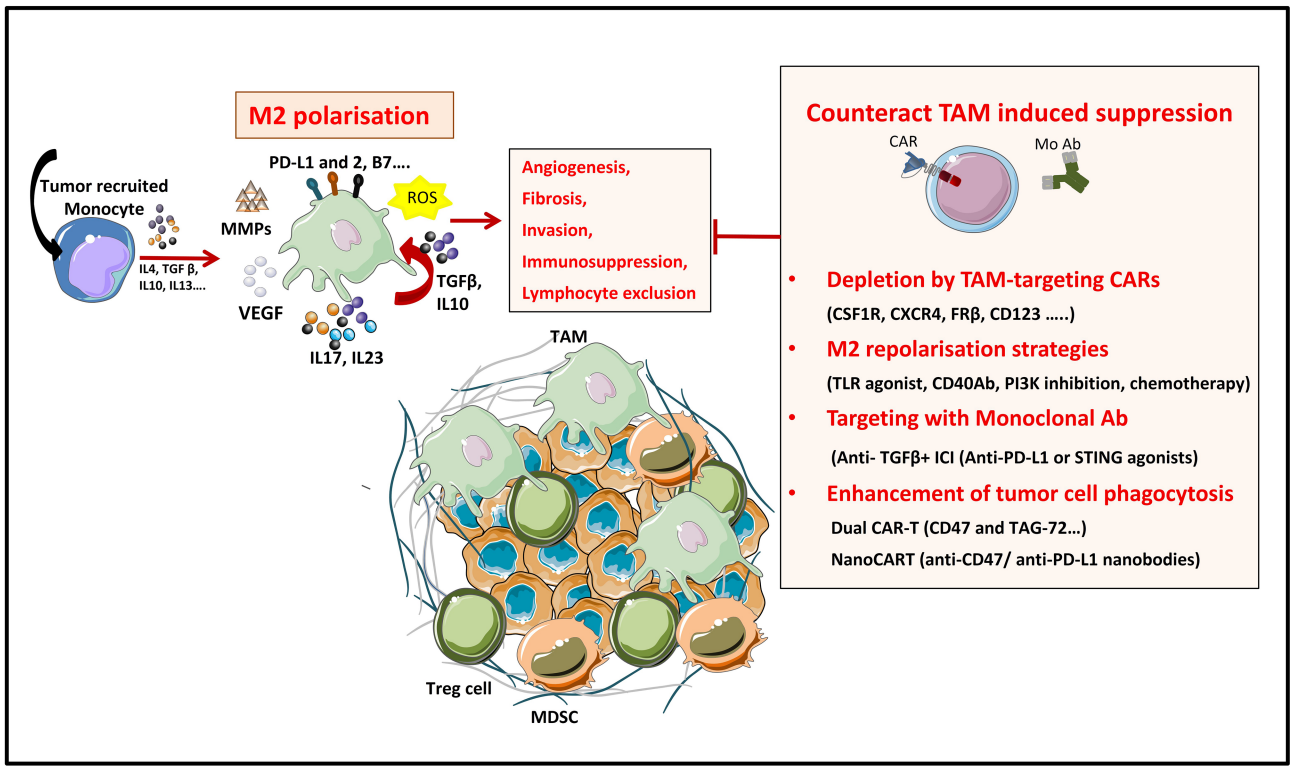

FIGURE 4 | Strategies to overcome TAM's induced suppression in the TME. TAMs are a tumor promoting immune populations derived under a specific cytokine milieu either from blood circulating monocytes or from tumor resident macrophages. TAMs exert their tumor promoting and immunosuppressive role by means of cell-to cell contact (inhibitory check point ligands), by secreting soluble factors (like cytokines IL10, IL17, L23), by producing ECM-modifying enzymes (MMPs) or by producing reactive species of oxygen (ROS). All these factors promote tumor progression. TAMs directed therapies in the TME aim either at (1) specifically depleting the TAM population, at (2) reprogramming M2 towards proinflammatory M1 phenotypes, at (3) targeting TAM-secreted factors or 4) at enhancing TAM's phagocytic functions in the TME.

Macrophages are potent immune effector cells with extensive plasticity and heterogeneity. Some types of macrophages play a crucial role in maintaining tissue homeostasis, by promoting wound healing, whereas others promote inflammation (182). Moreover, impaired macrophage function may lead to the development of many pathologies such as cancer (183). Macrophages are polarized into two contrasting groups: classically activated macrophages or M1 macrophages (pro-inflammatory and usually anti-tumor) and alternatively activated macrophages or M2 macrophages (anti-inflammatory and pro-tumor). This polarization is induced by exposure to soluble factors or pathogen derived molecules in the tissues. M1 macrophage polarization is driven by GMCSF, IFN-γ, TNF-α, lipopolysaccharide (LPS), or other pathogen-associated molecular patterns (PAMPs). M1 macrophages are proinflammatory and play an important role in anti-tumor immunity by: (i) orienting cellular immunity towards à TH1 type response by secreting TNFα, IL-1β, and IL-12, (ii) recruiting Th1 lymphocytes to sites of inflammation through secretion of CXCL9 and CXCL10 chemokines and (iii) presenting processed antigens and expressing costimulatory molecules which enhance T cell responses (184). M2 polarization on the other hand, occurs in the presence of cytokines like MCSF, IL-4, IL-10, IL-13, or TGF-β. Despite their role in tissue homeostasis (stimulating Th2 responses to eliminate parasites, immune regulation, wound healing and tissue repair), M2 macrophages can also promote tumor progression.

Tumors secrete and produce a variety of soluble and mechanical factors to recruit both circulating monocytes and tissue resident macrophages to the TME and convert them to TAMs. TAMs are a specialized population of M2-like macrophages, located in the TME, that share some phenotypic characteristics with M1 and M2 macrophages but have a particular transcriptional profile which is distinct from both types. TAMs enhance tumor progression and metastasis by promoting genetic instability and by enhancing angiogenesis, fibrosis, invasion, immunosuppression and lymphocyte exclusion (185, 186).

On the one hand, TAMs produce inflammatory cytokines like IL-17 and IL-23, which increase genetic instability and on the other hand they can impede tumor immunosurveillance, and thus T cell-mediated antitumor immunity, by secreting immunosuppressive cytokines like TGF-β and IL-10, by expressing immune checkpoint ligands such as PD-L1, PD-L2, B7-H4, or VISTA (4, 187) or by producing reactive oxygen

species (ROS). Furthermore, immunosuppressive cytokines produced by TAMs have a role in Treg recruitment. Nonetheless, other factors produced by TAMs are VEGF and MMP enzymes, which promote tumor angiogenesis and metastasis by inducing TME remodeling, increased blood vessel formation, and tumor cell migration (184). All these characteristics make TAMs targeting a promising strategy for cancer treatment (188).

Up to date, various therapeutic strategies targeting TAMs have already been tested in preclinical studies and clinical trials (**Figure 4** and **Table 10**) (189). Macrophage-focused immunotherapeutic strategies aimed either to deplete or to repolarize TAMs. Therefore, the first approach was to reduce or deplete TAMs by eliminating existent TAMs or by inhibiting further TAM recruitment, by targeting: (i) colony-stimulating factor 1 (CSF1)/CSF1 receptor (CSF1R) signaling pathway (190), (ii) chemokines/chemokines receptors axis such as CCL2/CCR2, CCL5/CCR5 (191, 192), (iii) IL-8/CXCR2 (193) or (iv) CXCL12/CXCR4 axis (194).

Second approach is to repolarize TAMs toward an M1-like phenotype, by inhibiting the PI3Kγ signaling pathway (195), by triggering inflammatory activating toll-like receptor (TLR: TLR3, TLR4, TLR7/8 and TLR9) signaling pathway with TLR agonists (196), or by using agonistic CD40 antibodies (197). Third approach of TAM reprogramming is to promote antigen presentation and phagocytosis of TAMs by blocking anti-phagocytic surface proteins called "don't eat me" signals, like SIRPα or Siglec-10, with antibodies blocking CD47 or CD24 expressed on cancer cells (198, 199) (**Figure 4**).

Nonetheless, another molecule of interest for targeting TAMs is TGF-β, an anti-inflammatory cytokine typically expressed by macrophages during injury resolution. Macrophages are both a source and a target for TGF-β, causing a positive feedback loop for TAMs and maintaining the immunosuppressive TME by promoting the secretion of additional TGF-β. TAM targeting by TGF-β blockade has already been employed, either in association with STING agonists or with anti-PDL1 blockade and showed tumor regression in preclinical models (200–202). For example, STING agonists DMXAA and cGAMP promote CAR-T cell persistence in the TME of immunocompetent mice in a breast cancer preclinical model. Association of STING agonists with CAR-T cell immunotherapy reprograms macrophagic and myeloid immunosuppressive populations in the TME. This is proven by an increased expression of CXCL9 and CXCL10 by myeloid cells within the TME, with increased recruitment of CXCR3+ TH1 to the tumor as well as by the enhanced expression of genes associated with M1-like macrophages and a marked loss of genes associated with M2-like macrophages and MDSC-like cells (201).

Additional potential molecular targets are discussed by Li et al. in a recent review (189). An increased research aimed at identifying TAM-associated or even TAM-specific targets and some have been used to redirect CAR-T cells against TAMs. Lynn et al. identified, in 2015, folate receptor beta (FRβ), a glycophosphatidylinositol-anchored receptor, as a potential target, as it is highly expressed in monocyte-derived TAMs from primary ovarian cancer. They, thereby, developed mouse FRβ-specific CAR-T cells to target the immunosuppressive M2-like subset of TAMs, while sparing M1-like subpopulations. The preliminary data showed that adoptive transfer of FRβ CAR-T cells into ID8 tumor-bearing mice depleted FRβ+ TAMs and delayed tumor development (203). Similarly, in the study from Rodriguez-Garcia et al., infusion of FRβ-specific CAR-T cells resulted in depletion of FRβ+ TAMs and controlled tumor progression in ovarian cancer, melanoma and colon adenocarcinoma (204). Ruella et al. found that CD123, the α chain of the receptor for IL-3, is expressed within Hodgkin lymphoma (HL) tumor masses both on cancer cells and on the M2-like TAMs. They demonstrated that CD123-specific CAR-T cells target both malignant cells and the surrounding immunosuppressive TME, and lead to the eradication of HL tumor xenografts. Moreover, anti-CD123 CAR-T immunotherapy induced long-term remission and the generation of an antitumor memory response. However, the use of immunodeficient mouse models in this studies does not enable for an accurate evaluation of the role of all endogenous immune system components (205).

New studies have underlined the importance of multiple antigen targeting as a means to both enhance the effectiveness of CAR-T cell therapy and to reduce off-target reactivity (206). To this end, Shu et al. generated CAR-T cells with two tandem CARs targeting CD47 and TAG-72 (Tumor-Associated Glycoprotein 72) (207). CD47 is a cell surface antigen highly expressed in ovarian tumors that functions equally as a macrophage "don't eat me" signal enabling malignant cells to escape cell phagocytosis and thus detection by the immune system, by interacting with macrophage' surface signal-regulatory protein-α (SIRPα) (208). TAG-72 is a pancarcinoma antigen and a tumor marker highly expressed in ovarian cancer (209). Blocking both CD47 and TAG-72 with CAR-T cells was associated with increased levels of macrophage-inflammatory protein (MIP)-1α and MIP-1β chemotactic factors in breast cancers, indicating functionality of the CD47 receptor in this model. The dual targeting strategy demonstrated enhanced ability of CAR-T cells to destroy tumor cells expressing low antigen levels, in favor of an increased binding avidity of the tandem CARs to the tumor cell. Another study, conducted by Xie et al., indicated that NanoCAR-T cells engineered to secrete anti-CD47 nanobodies (variable domain of heavy chain-only antibodies or V_HH) were able to inhibit tumor growth, while avoiding toxicity encountered with systemic anti-CD47 therapy. This strategy of TAM reprogramming showed superior antitumor activity compared with standard CAR-T cells (210). The team also engineered anti-PD-L1 or anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) nanobodies secreting NanoCAR-T cells, which showed increased persistence. Moreover, this strategy to modify the intra-tumoral immune landscape by nanobody/V_HH secretion can offer antitumor agents for multiple targets, has the advantage of being applied to immunocompetent animals and could limit systemic toxicity by means of local delivery at the tumor site (210).

Another approach to increase CAR-T cell infiltration and counteract the immunosuppressive TME is to induce tumor remodeling with adjuvant therapies (like chemotherapy or immune-checkpoint blockade). Srivastava et al. demonstrate that adding oxaliplatin to the lymphodepletion regimen given before ROR1 CAR-T cell infusion activates lung tumor macrophages to produce T cell-recruiting chemokines (reprogramming of TAMs to M1-macrophage). This results in improved CAR-T cell infiltration, tumor remodeling, and response to anti-PD-L1 checkpoint blockade, providing a strategy to improve CAR-T cell efficacy in the clinic. Moreover, a positive control loop has been noted in this model: CAR-T cells remodel the tumor microenvironment to amplify recruitment of endogenous T cells (211).

The aforementioned preclinical studies on CAR-T cells engineered to overcome the immunosuppressive effect of TAMs are summarized in the table below (**Table 5**).

2.2.2 Tregs

For years, regulatory T cells have been known to participate in the immunosuppressive environment of tumors. Due to their suppressive functions, Tregs are able to inhibit the effector functions of tumor-specific cells and reduce the effectiveness of active immunotherapy strategies based on the adoptive transfer of cytotoxic effectors. Therefore, several approaches have been developed to reduce the negative impact of Tregs in CAR-T cell therapies (**Figure 5**), and evaluated further on in various *in vitro* and pre-clinical studies (**Table 6**). Strategies to overcome immunosuppressive impact of the Treg population can be resumed as follows: (i) depletion strategies aiming to reduce cellular density of Tregs in the tumor, (ii) expression of interleukin receptors, hybrid interleukin receptors or switch receptors (iii) optimization of costimulatory domains of CAR-T cell, (iv) transgenic production of various cytokines by TRUCKs and, not least, (v) shielding of CARs from the suppressive effect of TGF-β by gene editing (**Figure 5**).

For instance, the modification of CAR-T cells to produce IL-12 resulted in improved anti-tumor immune response by different mechanisms and in particular by decreasing CAR-T cells sensitivity to inhibition by regulatory T cells (214) but also by reduction of Tregs densities in the TME (215, 216). In a similar way, it was shown that CAR-T cells producing IL-18 promote antitumor immune responses (218, 219) by modifying the tumor environment notably by increasing the density of M1 macrophages and NK cells and by decreasing Treg infiltration, CD103+ suppressive DCs and M2 macrophages frequency (217, 219). It was also demonstrated that these two cytokines improve the antitumor response by increasing *in vivo* the survival and the proliferation of CAR-T cells that produce them (215, 216, 218). Promoting the proliferation of CAR-T cells *in vivo* is an important issue and initial strategies were based on injection of IL-2, a stimulator of T cells proliferation. Unfortunately, this adjuvant treatment has the major inconvenient of inducing the proliferation of Tregs in cancer patients. In order to overcome this side effect, some approaches have sought to increase CAR-T cells dependency on proliferative cytokines different from IL-2, such as IL-7. In various murine solid cancer models, the use of CAR-T cells expressing a constitutively active IL-7 receptor (IL7R) promotes *in vitro* activation, proliferation and cytotoxicity of CAR-T cells and increases survival of animals by eliciting a protective immune response (220–222). Moreover, unlike the case of IL-2 utilization, IL-7 conjoint injection upon CAR-T infusion does not result in increased proliferation and immunosuppressive function of Tregs, which have low expression of the IL-7R (220). On the other hand, endogenous production of IL-2 by activated T cells has a similar effect as exogenous IL-2 administration, and participates in the generation of Tregs in the TME. Therefore, in order to abrogate production of IL-2 by CAR-T cells, optimization of costimulatory domains of CARs, like the genetic modifications of the intra-cytoplasmic part of the CD28 molecule were designed (171, 174, 228). Preliminary results showed that the elimination of CD28-mediated IL-2 induction impairs CAR engraftment *in vivo*. However, when impairment of the IL-2 autocrine signaling is compensated for by another costimulatory molecule such as 4-1BB, the CARs accumulate in the bloodstream, suppress tumor growth and resist Tregs-induced immunosuppression (228). As IL-2 release and autocrine IL-2 receptor signaling seemed crucial in counteracting TGF-β repression, but CAR-T cell-released IL-2 negatively impacts the anti-tumor activity through sustaining survival and function of Treg cells, another elegant strategy to improve resistance to TGF-β is the engineering of a hybrid IL7/IL2 receptor to provide IL2 signaling upon IL7 binding. Therefore, Golumba-Nagy et al. designed TRUCKs releasing IL-7 and co-expressing hybrid IL-7Rα/IL-2Rβ receptor, which showed improved survival over a prolonged period and improved activity against TGF-β+ tumors (227).

Indeed, Tregs represent one of the major sources of TGF-β, an immunosuppressive cytokine which impacts the efficiency of immune effectors in the TME. Therefore, a different strategy to resist to TGF-β-induced immunosuppression is to inhibit or delete its receptor on the surface of CAR-T cells. Accordingly, the absence of a functional TGF-β receptor in CAR-T cells promotes proliferation as well as cytokine secretion, resistance to exhaustion and long-term *in vivo* persistence. Engineering methods for TGF-β receptor deletion/inhibition are: (i) the expression of a dominant negative (DN) receptor, and (ii) genetic disruption by gene-editing techniques like the CRISPR/Cas9 technology. To this regard, CAR-T cells expressing a dominant negative (DN) TGF-β receptor gene are more

TABLE 5 | Summary of preclinical studies on CAR-T cells engineered to overcome the immunosuppressive effect of TAMs.

Targeted antigen	Type of cancer	Reference
mFRβ	Ovarian cancer	(203)
	Ovarian cancerMelanomaColon adenocarcinoma	(204)
CD123	Hodgkin lymphoma	(205)
CD47 & TAG-72	Ovarian cancer	(207)
CD47	MelanomaColon adenocarcinoma	(210)

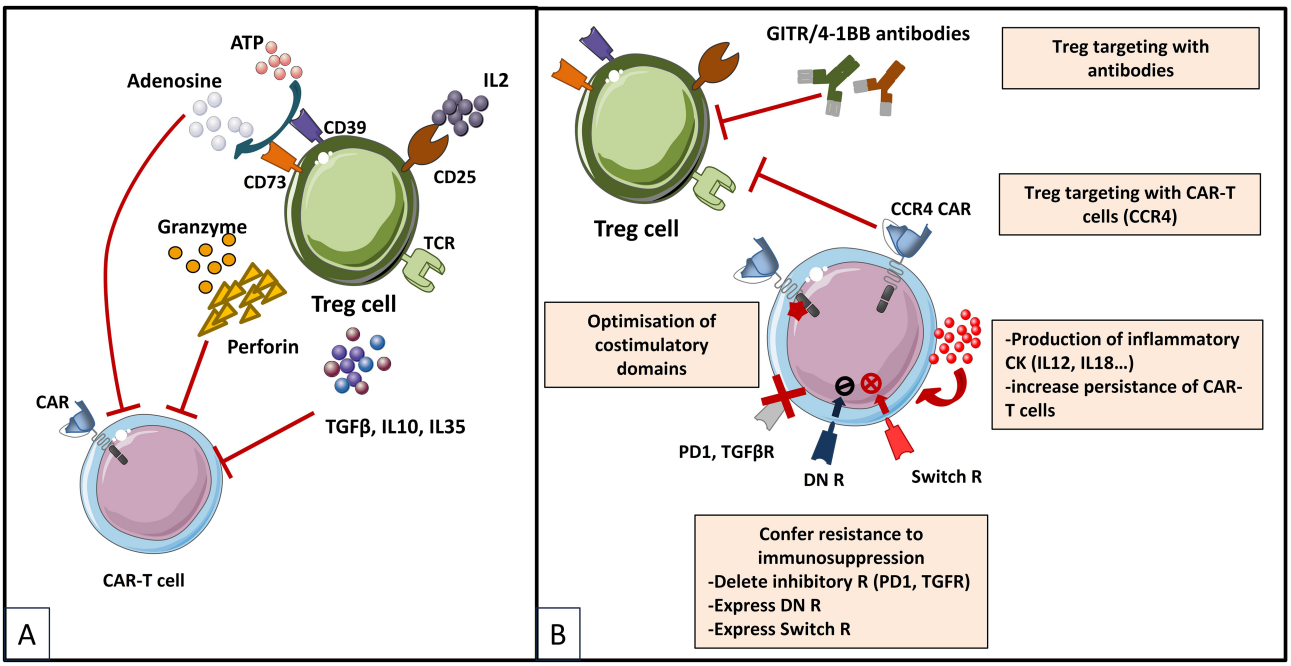

FIGURE 5 | Immunosuppressive mechanisms exerted by Tregs in the TME **(A)** and engineering strategies to surmount Treg-induced immunosuppression **(B)** [Adapted from Togashi et al. (212), and Rodriguez-Garcia et al. (213)]. **(A)** depicts mechanisms for regulatory T (Treg) cells immunosuppressive effects on CAR-T cells based on their physiologic roles. Tregs are immunosuppressive cells highly dependent on IL-2. They bind to and deplete IL-2 from their surroundings, thus reducing availability to effector T (Teff) cells by constitutively expressing the high affinity IL-2 receptor (IL2R) subunit-α (CD25). Treg cells also produce immunosuppressive cytokines (IL-10, IL-35 and TGFβ), which can downregulate the activity of both Teffs and antigen presenting cells (APCs) and they exert direct cytotoxic effects by secreting granzymes and perforin. Moreover, Treg cells release large amounts of ATP, which is converted to adenosine (by CD39 and CD73) that can provide immunosuppressive signals to Teff cells and APCs. Other indirect mechanisms not depicted in the figure by which Tregs exert immunosuppressive effects are mediated by APC, as for instance Tregs expression of cytotoxic T lymphocyte antigen 4 (CTLA-4), which binds to CD80/CD86 on APCs, thereby transmitting suppressive signals to these cells and reducing their capacity to activate Teff cells. **(B)** shows therapeutic strategies to overcome the immunosuppressive TME sustained by Tregs. Some strategies are based on elimination of Tregs by CAR-T cells or combinations of CAR-T cells with monoclonal antibodies (mAbs) or drugs. CAR-T cells have been designed to target antigens expressed by Tregs for direct depletion. Other strategies are based on immunomodulation of the TME in order to increase CAR-T cells performance: 1) expression of proinflammatory cytokines by CAR-T cells and 2) optimization of costimulatory signaling domains in order to reduce IL-2 secretion and impair Treg expansion and tumor infiltration. Last type of strategies are meant to confer an intrinsic resistance to immunosuppression to CAR-T cells, either by endowing them with 1) dominant-negative receptors (DN R) meant to disrupt signaling, or 2) a chimeric switch receptor (CSR or Switch R) to convert negative signaling into a positive one, or by abrogating the expression of inhibitory receptors (like PD1 of TGFβ receptors) using genome-editing tools (knock out).

efficient at inducing protective responses (224, 225) and elimination of endogenous TGF-β receptor II (TGFBR2) in CAR-T cells using CRISPR/Cas9 technology reduces the induction of Treg cells and prevents CAR T cell depletion (226). These strategies aiming to use CAR-T cells modified to resist the immunosuppression induced by the TME derived TGF-β are currently undergoing clinical trials in hematological cancers and solid tumors such as prostate cancer (NCT03089203, NCT04227275.). The first clinical trial (NCT03089203) showed encouraging preliminary results (101) (**Table 10**).

In contrast to these strategies, which aim to reduce the negative impact of TGF-β on the anti-tumor response of CAR-T cells by inhibiting the expression of its receptor, other approaches are currently evaluating the therapeutic benefit of CAR-T cells modified to express a chimeric TGF-β receptor (switch receptor) whose activation by the cytokine would promote their functions (229). The aforementioned preclinical studies on CAR-T cells engineered to overcome the immunosuppressive effect of Tregs are summarized in the table below (**Table 6**).

2.2.3 MDSC

MDSCs are a heterogeneous group of immature myeloid cells at various stages of differentiation and which differ from differentiated mature myeloid cells, such as neutrophils, macrophages, and dendritic cells (Dcs). As their name implies, MDSCs are a major group of immunosuppressive cells abundant in different types of cancers (230–233). Recent reports have suggested that MDSCs exert their immunosuppressive activity both on the innate and the adaptive immune system, both by cell-to-cell contact and by the secretion of soluble factors. MDSCs can also facilitate cancer progression by regulating cell mobility or even angiogenesis (234). MDSCs induce

TABLE 6 | Summary of preclinical studies on CAR-T cells to overcome the immunosuppressive effect of Tregs.

Targeted antigen	Expressed gene	Type of cancer	Reference
	A. Interleukin expression (TRUCK)		
CD19	IL-12	Thymoma tumors	(214)
MUC16	IL-12	Ovarian cancer	(215)
GPC3	IL-12	HCC	(216)
CEA	IL-18	Pancreatic carcinoma	(217)
CD19	IL-18	Melanoma	(218)
MUC16	IL-18	Ovarian cancer	(219)
	B. Interleukin receptor expression		
GD2	IL-7Rα	Neuroblastoma	(220, 221)
AXL	IL-7Rα	TNBC	(222)
	C. TGF-β targeting or inhibition		
DNRII/sRII	–	Melanoma	(223)
PSMA	TGF-βRII	Prostate cancer	(224)
TGF-β	–	Melanoma	(225)
MSLN	*TGFBR2*-KO	MesotheliomaOvarian cancer	(226)
	D. TGF-β resistance		
CCR8-DNR	CCR8	Pancreatic cancer	(64)
CEA	ΔCD28, IL-7 and IL-7Ra/IL-2Rb	Colon carcinoma	(227)
	E. Deletion of the LCK binding domain in CD28		
FAP-F19	ΔCD28	Mesothelioma	(171, 174)
EGFRvIII	ΔCD28-4-1BB	Melanoma	(228)

CEA, Carcinoembryonic antigen.

immunosuppression of T cell immune responses by various mechanisms: (i) degradation of amino acids essential for activation and proliferation (such as arginine, cysteine, or tryptophan) by production of arginase 1 (Arg1) and indoleamine 2,3-dioxygenase 1 (IDO1) enzymes (235), (ii) Treg induction *via* IL-10 and TGF- β secretion (236), (iii) suppression of T cell proliferation by MDSC-derived nitric oxide (NO) inhibition of the Jak/STAT5 pathway (237), (iv) impairment of T cell migration into tumor sites by the cleavage of the ectodomain of L-selectin by a disintegrin and metalloproteinase 17 (ADAM17) expressed by MDSCs, and reduction of E-selectin expression on endothelial cells caused by MDSC-derived NO (238), and (v) release of MDSC-derived reactive oxygen species (ROS) implicated in MDSC-mediated T cell suppression (239). In addition, accumulating research showed that MDSCs have potent mechanisms to promote cancer growth (via downregulation of IFN-γ and expression of MMP9) and metastasis (via TNFα, TGFβ, CXCL2 and S100A8/9) by establishing an immunotolerant environment (240). A meta-analysis, published by Zhang et al., concluded that the presence of MDSCs was correlated with poor prognosis in patients with solid cancer (241). Recent reports have also indicated that MDSCs may play a role in resistance to immunotherapy and CAR-T cell therapy (242).

Several strategies have been deployed to increase CAR-T cells resistance to the immunosuppressive effects of MDSCs (**Table 7**). Among these various strategies, some are based on combinatorial therapies. Briefly, strategies aiming at counteracting MDSC are based on: (1) preventing differentiation and recruitment of MDSCs to the tumor bed, (2) depleting tumor infiltrating MDSCs or (3) mitigating MDSCs immunosuppressive effects (**Figure 6**). For instance, blocking of MDSC differentiation could be obtained by using a multitargeted TK inhibitor (TKI), Sunitinib, which inhibits STAT3 signaling and induces apoptosis of murine MDSCs. Li et al. demonstrated that coadministration of carbonic anhydrase IX (CAIX)-CAR-T cells with Sunitinib significantly enhanced therapeutic efficacy against a mouse model of human metastatic renal cancer and resulted in prolonged survival of mice, as well as reduction in the number of MDSCs at the cancer site (246). Sunitinib has already shown positive results in a clinical trial (NCT03277924), in combination with anti-PD1 blocking in advanced sarcoma (251) (**Table 10**).

Blocking recruitment of MDSC to the tumor bed has been obtained for example by impeding the chemotaxis axis SDF1α/CXCR4 by Sun et al. They proved that olaparib, a Poly(ADP-ribose) polymerase inhibitor (PARPi), could enhance the anti-tumor immune response of EGFRvIII-specific CAR-T cells as well as the recruitment of CD8+ T cells in mouse breast cancer models. Moreover, mice treated with a combination of olaparib and EGFRvIII-specific CAR-T cells showed decreased expression of SDF1α (CXCL12), one of the main MDSC chemoattractant, as well as decreased MDSC recruitment. In parallel, a decrease of the CXCL12 receptor, CXCR4, expression has been noted in MDSCs treated with olaparib, proving that Olaparib might reduce MDSC recruitment by interfering with the SDF1α/CXCR4 axis (245). Other strategies to mitigate MDSCs recruitment to the tumor bed could by the blocking of MDSCs chemokine receptors, as it is the case of CCR2. The CCR2 blocking technique has already been employed but, it can, unfortunately also impede TILs recruitment to the tumor bed (252, 253). Even so, combination of CCR2 blockade with anti-PD1 therapy enhanced anti-tumor responses in many preclinical cancer models (melanoma, breast cancer) (254). Moreover, CCR2 antagonism decreased MDSC counts in pancreatic cancer patients (NCT02345408) (255) (**Table 10**).

Other strategies already deployed to deplete intra-tumoral MDSC were antagonism or inhibition of specific receptors, like

TABLE 7 | Summary of preclinical studies on CAR-T cells strategies counteracting the immunosuppressive effect of MDSCs.

Targeted antigen	Expressed genes	Co-administered agent	Type of cancer	Reference
GD2	–	ATRA	Osteosarcoma	(243)
GD2MSLNEGFRvIII	–	GO	Solid tumors	(244)
EGFRvIII	–	Olaparib	Breast cancer	(245)
CAIX	–	Sunitinib	Metastatic renal caner	(246)
EGFRvIII	–	Poly I:C	Breast cancerColon cancer	(247)
GD2	–	NKG2D.ζ CAR-NK	Neuroblastoma	(248)
MUC1	TR2.4-1BB	–	Breast cancer	(249)
HER2	TR2.4-1BB	–	Breast cancer	

Gr1, CD40 (with mAbs) or CD33 (256, 257). CD33 was identified as a common surface marker of MDSCs and Fultang et al. provided evidence that Gemtuzumab ozogamicin (GO), an antibody-drug conjugate consisting of a humanized mAb targeting CD33 linked to an intracellular toxin named calichamicin, can eliminate MDSCs, leading to CAR-T cell reactivation against multiple cancers. In this study, coadministration of GO restored GD2, MSLN and EGFRvIII CAR-T cell proliferation, leading to increased tumor cell death (244). Moreover, depletion or expansion reduction of MDSC in combination with CAR-T cell therapy has also been employed by using GM-CSF or PD-L1 neutralization as it has been recently shown the immunosuppressive capacities of MDSCs are modulated by GM-CSF through the PD1-PD-L1 axis (258). In a different approach, Parihar et al. demonstrated the effectiveness of specific CAR-NK cells in suppressing MDSCs (248). It has been reported that MDSCs overexpress NKG2D ligands, which are able to activate the NKG2D cytotoxicity receptor on NK cells (259). The immunosuppressive TME restricts, however, NK cell activation. To overcome this obstacle and enhance MDSC depletion *in vivo*, they generated a CAR that fuses the NKG2D receptor to CD3ζ, the NKG2D.ζ CAR-NK cells. Coadministration of these NKG2D.ζ CAR-NK cells increased the anti-tumor activity of GD2 CAR-T cells in a xenograft model of neuroblastoma (248).

In a 2021 ASCO annual meeting's abstract, Nalawade et al. demonstrated that MUC1-specific CAR-T cells engineered with a novel chimeric co-stimulatory receptor, TR2.4-1BB, comprising a ScFv derived from a TNF-related apoptosis-inducing ligand receptor 2 (TR2) mAb with a 4-1BB endodomain., induced MDSC apoptosis. As MDSCs express TR2, this strategy could warrant selective MDSC depletion. MUC1.TR2.4-1BB CAR-T cells showed increased cytotoxic activity against breast cancer tumors and inhibited tumor growth more effectively than either MUC1 CAR-T cells or TR2.4-1BB T cells. Similar results have been observed with HER2.TR2.4-1BB CAR-T cells in a HER2+ breast cancer model (249).

Furthermore, Di et al. proved that administration of the toll-like receptor 3 (TLR3) ligand polyinosinic-polycytidylic acid (poly I:C) can increase EGFRvIII-specific CAR-T cell efficacy in immune competent mice bearing colon and breast cancers by enhancing specific lysis of cancer cells and cytokine release upon antigen stimulation. Poly I:C also impeded the suppressive effect of MDSCs on T cell proliferation (247)

And finally, Long et al. identified all-trans-retinoic acid (ATRA) as an effective agent in decreasing the suppressive effect of MDSCs. Co-treatment with ATRA and GD2 CAR-T cells led to an increased antitumor activity compared to ATRA or CAR-T cells treatment alone. These positive effects of ATRA treatment can be explained by an augmented expression of glutathione synthase in MDSCs resulting in higher glutathione synthesis and neutralization of ROS (which contribute to T cell depletion and impede MDSC differentiation) (243).

2.2.4 Enhancing Persistence/Fitness of Genetically Modified T Cells by Interleukin Production

In order to increase persistence and/or maintenance in the immunosuppressive TME, CAR-T cells can be genetically engineered to produce vital cytokines. This engineering strategy is meant to complete the third signal of the immunological synapse (i.e. cytokine stimulation), which is lacking or insufficient in the TME. Various cytokines have already been transgenically expressed in CAR-T cells, the most common being: IL-7, IL-12, IL-15, IL-18, IL-21 and IL-23 (**Table 8**). Some of them hace been addressed in the Treg section (see section 2.2.2 on Tregs). For an extensive review on gene-edited interleukin CAR-T cells published recently, see Zhang et al. (268).

Therefore, another strategy to overcome the immunosuppressive TME, is the use of fourth generation CAR-T cells genetically redirected for antigen-unrestricted cytokine-initiated killing (TRUCKs), modified to secrete immune stimulatory cytokines (2). Yeku et al. reported the efficacy of IL-12 secreting TRUCKs directed against mucin-16 (MUC16), known as 4H1128ζ-IL-12 T cells, in an aggressive disseminated mouse ovarian cancer model. Indeed, these CAR-T cells induced the eradication of TAMs *via* Fas/FasL pathway, secreted more inflammatory cytokines (such as IFN-γ) and exhibited increased cytotoxicity *in vitro* and *in vivo*. Moreover, cytokine stimulation of 4H1128ζ-IL-12 T cells was associated with increased resistance of CAR-Ts in the TME. The armored CAR-T cells also showed a decreased expression of eomesodermin (Eomes), forkhead box P3 (FOXP3), CTLA-4, LAG-3, TIM-3 and programmed death-ligand 1 (PD-L1), all of which play a major role in the establishment of an immunosuppressive environment (260). Similarly, Chmielewski et al. found that treatment with IL-18 secreting TRUCKs directed against carcinoembryonic antigen (CEA), enhanced CAR-T cell function and survival of mice with advanced pancreatic and lung cancers, and induced an acute Th1 inflammatory response. Data showed an increase in the number of

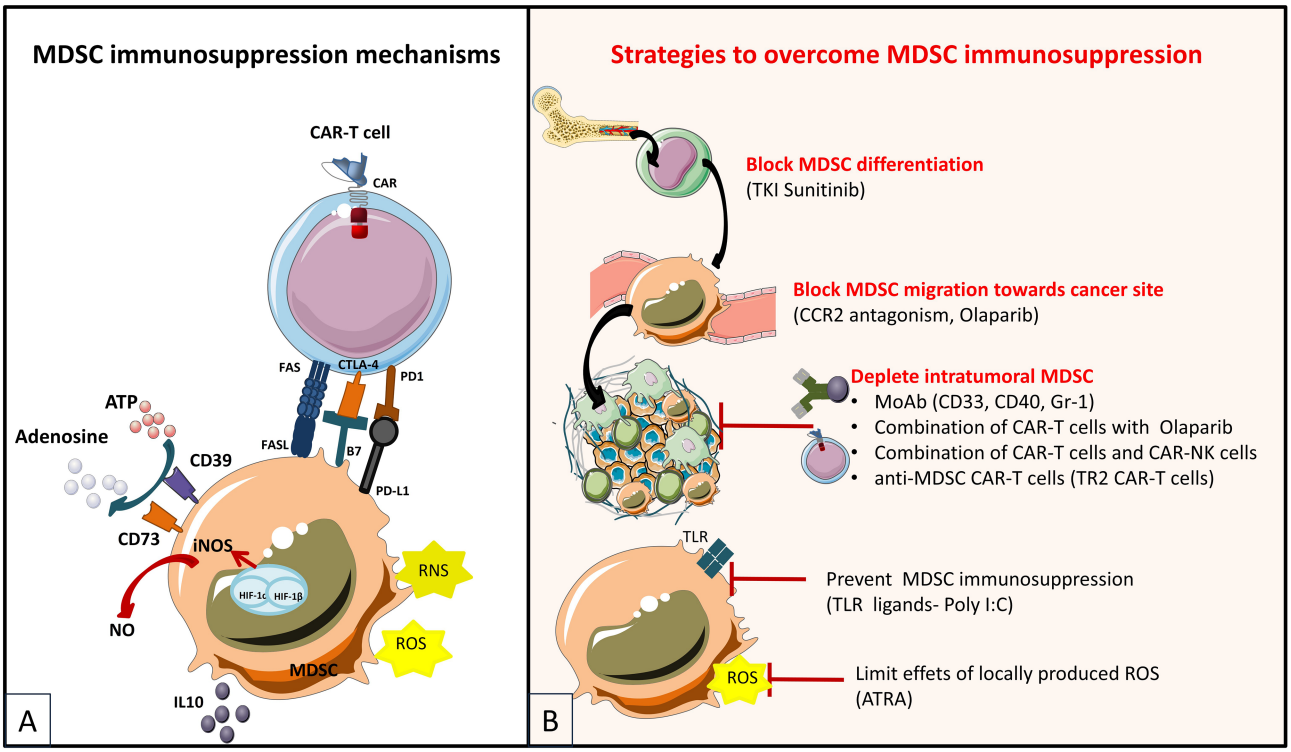

FIGURE 6 | Immunosuppressive mechanisms exerted by MDSCs in the tumor microenvironment **(A)** and engineering strategies to surmount MDSC-induced immunosuppression **(B)** [Adapted from Krishnamoorthy et al. (250)]. **(A)** MDSC exert immunosuppressive effects in the TME (tumor microenvironment) by secretion of IL10 (which activates other immunosuppressive cells such as Tregs). Moreover, MSDCs can induce upregulation of checkpoint molecules (CTLA4, PD1) on T-cells, further inducing T-cell anergy, or can upregulate Fas which induces T-cell apoptosis by contact Fas/Fas-L mechanism. As an effect of hypoxia in the TME, MDSC can contribute to adenosine production by upregulation of CD73 and CD39. MDSCs also produce reactive oxygen (ROS) and nitrogen (RNS) species that can decrease T-cell proliferation and alter antigen recognition capabilities. **(B)** Strategies for targeting MDSCs in cancer are for example the prevention of MDSC differentiation from hematopoietic stem cells by the usage of Sunitinib, a tyrosine kinase inhibitor (TKI) that inhibits crucial factors for MDSC differentiation (VEGF and STAT3 activity). Second type of strategy is to prevent MDCS migrating to the tumor by targeting chemokine/chemokine receptor axes (CCR2/CCL2). Third, MDSCs depletion from the tumor can be achieved by using immunotherapy (depleting antibodies targeting CD33/gemtuzumab ozogamicin (GO), CD40 or Gr1) or chemotherapy. And least, mitigating the immunosuppressive effects of MDSCs at the tumor site can be realized by reducing the local effects of ROS (with ATRA or all-trans-retinoic acid) or by using TLR stimulation with specific ligands (TLR3 ligand polyinosinic-polycytidylic acid Poly I:C)

NK cells and M1-like macrophages, and a decrease in the number of M2-like macrophages, Tregs, and inhibitory DCs, allowing for an enhanced antitumor activity (217). The interleukin IL-15 emerged as an immunomodulatory cytokine with anti-tumor effects thanks to its roles in inducing expansion and activation of NK, natural killer T (NKT) cells, and long-lasting memory CD8+ T cells (CTLs). Indeed, IL-15 promotes memory CTL survival and effector function (cytotoxic activity and IFN γ release) and could prevent Tregs from influencing the effector functions of CD4 and CD8 T cells. Moreover, IL-15 can inhibit IL-2 activation induced cell death of effector lymphocytes (269). To this regard, many groups found that preconditioning with IL-7 and IL-15 resulted in better *in vitro* expansion of CAR-T cells as well as superior antitumor effects *in vivo* and even increased efficacy upon immune-checkpoint blockade (increased CAR T cell responses to anti-PD-1 adjuvant therapy) (262, 270–272).

Therefore, transgenic expression of IL-15 seemed like an appealing strategy to enhance CAR-T cell effector function, by enhancing proliferation and persistence of CAR-T cells in the TME and has been used in preclinical models of acute myeloid leukemia (AML), melanoma, glioblastoma or neuroblastoma (261–265, 273). In preclinical models of AML, CLL1-directed CAR-T cells with transgenic expression of IL-15 showed increased expansion, survival and antileukemic potency. Unfortunately, co-expression of IL-15 was associated with lethal cytokine release syndrome (CRS), a fatal adverse effect that could be prevented with anti-TNF-antibodies pretreatment and depletion of IL-15 secreting CARs by the inducible caspase-9 (iCas-9) suicide switch (261).

Another fourth generation CAR targeting VEGFR2 on the tumor vasculature and co-expressing murine IL-15 has been evaluated as a tool to overcome TME immunosuppression in immunocompetent, syngeneic melanoma-bearing mice. As in previous studies, expanded CAR-T cells transduced to co-express IL-15 cultivated with both IL-7 and IL-15 showed enhanced expansion as well as a TCM cell phenotype predominantly. *In vivo*, CAR-T cells up-regulated the antiapoptotic marker Bcl-2 and down-regulated the inhibitory receptor PD-1. These CARs

TABLE 8 | Summary of preclinical studies on CAR-T cells engineered to produce vital cytokines or to express transgenic cytokine receptors.

Targeted antigen	Expressed cytokines/Cytokine receptors	Type of cancer	Reference
MUC16	IL-12	Ovarian cancer	(260)
CEA	IL-18	Advanced pancreatic cancerAdvanced lung cancer	(217)
CLL1	IL-15	AML	(261)
VEGFR2	IL-15	Melanoma	(262)
IL-13Rα2	IL-15	Glioma	(263)
GD2	IL-15	Neuroblastoma	(264)
GPC3+	IL-15 & IL-21	HCC	(265)
GD2	p40 of IL-23R	Neuroblastoma	(266)
PSMA & IL-23	–	Prostate cancer	(267)
GD2	IL-7R	Neuroblastoma, Glioblastoma	(221)

showed enhanced effector functions, engraftment and tumor control, in part through reprograming of the TME in favor of protective endogenous immunity, including NK cell activation and reduced presence of M2 macrophages (262). IL-15 co-expression was also used in a model of IL-13Rα2-positive glioma (IL-13Rα2-CAR.IL-15 T cells) and results showed better *in vivo* persistence and greater antiglioma activity. Unfortunately, despite enhanced recognition of glioma cells, greater proliferative capacity and increased production of cytokines, the improved T cell persistence was associated with recurrence of gliomas with down-regulated IL-13Rα2 expression. Therefore, at least in GBM treatment, this engineering strategy should be coupled with multiple antigen targeting techniques (263). Expression of IL-15 as a reinforcement proliferative signal was also used in a neuroblastoma model. As in previous studies, GD2 specific CARs armored with IL-15 (GD2.CAR.15-Ts) showed reduced expression of the PD-1 receptor as well as superior antitumor activity. IL-15 forced expression resulted in enrichment in stem cell-like cells (TSCM-like). CAR-Ts were engineered to contain the inducible caspase 9 (iCas9) safety switch (264).

Another method of interleukin gene-editing of CAR-T cells is the co-expression of two simultaneous cytokines. Batra et al. engineered fourth generation CAR-T directed against GPC3+ composed of a 4-1BB costimulatory motif and co-expressing both IL-21 and IL-15 and found superior expansion and antitumor activity against HCC in a preclinical model. IL-21 and IL-15 armored GPC3+ CAR-T cells showed higher proliferation at least in part by maintaining the expression of T cell factor-1 (TCF-1), a transcription factor critical for T cell development and survival. Moreover, manufacturing outcome showed a higher percentage of TSCM and TCM populations. For effective management of toxicity risk in the clinical setting, the authors also proved that the iCas-9 "suicide switch" can effectively eliminate these CAR-T cells. Two clinical trials were open to explore anti-tumoral benefit: NCT02932956 and NCT02905188 (265) (**Table 10**).

Nonetheless, co-expression of cytokines by genetically engineered CAR-T cells as a mean of boosting anti-tumor activity has the inconvenience of constitutive cytokine signaling in T cells and activation of bystander cells which may cause toxicity. To prevent hyper-activation and excessive cytokine production of CAR-T cells, another team engineered CAR-Ts containing signaling TCR-responsive nanoparticles containing human IL-15 superagonist complex. These protein nanogels embedded in CARs were associated with enhanced selective expansion of CAR-T cells within tumors and improved therapeutic efficacy (273).

In order to circumvent interleukin forced expression, Xincong et al. engineered CAR-T cells expressing the p40 subunit of the IL-23 receptor (p40-Td cells). The p40 subunit of the IL-23R is the only subunit which is not up-regulated upon TCR stimulation. Over-expression of the p40 subunit induced selective proliferation of CAR-T cells *via* an IL-23 autocrine loop. Moreover, p40-Td cells showed improved antitumor capacity both *in vitro* and *in vivo* as well as attenuated side effects in comparison to CAR T cells expressing IL-18 or IL-15 (266).

The interleukin IL-23 has also been targeted with a different strategy, in the context of prostate cancer, by designing CAR-Ts targeting both PSMA and IL-23 (IL-23 mAb). The inflammatory cytokine IL-23 plays an active role in tumorigenesis, by upregulating certain MMPs (MMP9), by increasing angiogenesis and infiltration of M2 macrophages and neutrophils and by reducing CD8 T cell infiltration in the TME. Moreover, it has been proven that IL-23 secreted by MDSCs drives castration-resistant prostate cancer by activating the androgen receptor pathway (274). Therefore, Wang et al. designed three types of CAR-T cells in order to simultaneously target PSMA expressing cells and capture local soluble IL-23 produced by tumor cells or by MDSCs: either (i) dual or duo CAR-Ts expressing 2 CARs at the surface (IL-23mAb-T2A-PSMA), or (ii) a tandem CAR IL-23mAb/PSMA or (iii) PSMA-CARs secreting anti-Il-23 Ab. IL-23 and PSMA targeted duo-CAR-Ts (IL-23mAb-T2A-PSMA) were more efficient in prostate cancer eradication than PSMA CARs only and induced stronger T cell activation, and increased cytokine production when compared to single-molecule tandem CAR IL-23mAb/PSMA (267).

In a different approach, Shum et al. engineered CAR-T cells constitutively expressing the IL-7 receptor (C7R CAR-T cells), in order to deliver potent stimulation and increase CAR-T persistence and antitumor activity (221). An ongoing clinical trial evaluates efficacy of C7R-GD2.CAR T cells in the treatment of brain tumors (NCT04099797) (**Table 10**). Except armored CAR-T cells secreting IL7 and/or IL2 cited above (see *Overcoming the Mismatch or the Dysregulation of Chemokine Receptor/Ligand Axes*), armored CAR-T cells secreting other survival cytokines have also gone to the clinic (like IL-15 and/

or IL-21: NCT04715191, NCT05155189, NCT02932956 and NCT02905188, NCT03721068, NCT04377932, and NCT05103631) (**Table 10**).

2.2.5 Overcoming Inhibition by Negative Immune Checkpoints

The tumor-related immune response is regulated by various stimulating and inhibitory signals. Immune checkpoints (ICs) insure the maintenance of immune homeostasis, and thus self-tolerance, by regulating the time course and the intensity of the immune reaction. However, receptor-based signal cascades emerging from ICs play a negative regulatory role in T cells, by inducing immune tolerance and therefore tumor escape from immunosurveillance (275). The first main ICs identified as essential receptors for T cell and CAR-T cell inhibition and apoptosis are CTLA-4 and PD-1 (276). Other immunoreceptors extensively studied in cancer are LAG-3, TIGIT, T-cell immunoglobulin and mucin containing protein-3 (TIM3) and B and T lymphocyte attenuator (BTLA). Since, many different monoclonal Abs (mAbs) and bispecific antibodies (BsAbs) that prevent ligand- inhibitory IC receptor engagement have been used to block immune checkpoints. IC receptors use mono-tyrosine signaling motifs, such as ITIM and immunoreceptor tyrosine-based switch motifs (ITSM), to exert their inhibitory activity (276). IC inhibition either in monotherapy or as supplementary therapy turned out to be a very efficient weapon to fight cancer (277). As PD1/PD-L1 inhibition is the most studied axis, this chapter mainly focuses on PD-1/PD-L1 inhibition in CAR-T cell therapy (**Table 9**).

PD-1 is a member of the B7/CD28 family which exerts its role in modulating T cell activity by interacting with two ligands- PD-L1 and PD-L2. PD-1/PD-L1 binding impedes the synthesis of IFN-γ and IL-2, which decreases T cell proliferation (290). Furthermore, the overexpression of PD-L1 is correlated with poor prognosis in many cancers (291–294). In order to block the interaction between PD-1 and PD-L1, various mAbs, such as nivolumab, pembrolizumab, avelumab, lambrolizumab, and atezolizumab, have been developed (295). After encouraging results from different clinical trials, the FDA granted accelerated approval to nivolumab for the treatment of advanced melanoma in 2014 (296), and advanced squamous NSCLC in 2015 (297). Nivolumab has also shown positive results in many other cancers, such as R/R HL (298) and HCC (299). Furthermore, other anti-PD-1 mAb, pembrolizumab (humanized IgG4 kappa anti-PD-1 mAb), has been approved by the FDA for the treatment of many types of cancer, including unresectable or metastatic melanoma in 2014 (300), advanced NSCLC in 2015 (301), recurrent or metastatic HNSCC in 2016 (302), and locally advanced or metastatic urothelial carcinoma in 2017 (303).

Given acknowledged CAR-T cell dysfunction following engagement of IC receptors and especially spectacular results obtained with anti-PD1 and anti-PD-L1 ICI mAbs, different strategies were deployed in order to enhance CAR-T cell efficacy (**Figure 7**).

John et al. were the first to demonstrate that inhibiting an important immunosuppressive pathway such as PD-1 can significantly increase adoptive immunotherapy efficacy using genetically modified T cells (278). To this regard, PD-1 expression was shown to be significantly enhanced on CAR-T cells cocultured with PD-L1+ HER-2+ tumor cells, whereas PD-1 inhibition enhanced CAR-T cell proliferation and activity *in vitro* and *in vivo*. Moreover, the coadministration of anti-PD-1 mAbs together with HER-2 specific CAR-T cells enhanced HER-2+ tumor regression and mice survival in a transgenic animal model without any sign of autoimmunity. Interestingly, CAR-T cell and anti-PD-1 mAbs combined therapy significantly depleted MDSCs but not Tregs at the cancer site, as compared to untreated control mice. Similar results have been observed by Gargett et al. with GD2-specific CAR-T cells combined to pembrolizumab against neuroblastoma and melanoma cell lines (279), and Gulati et al. with FAP-specific CAR-T cells combined to anti-PD-1 mAbs against a malignant pleural mesothelioma cell line (174). Many ongoing clinical trials are evaluating the efficacy of the administration of CAR-T cells with anti-PD-1/PD-L1 blocking antibodies in patients with solid tumors (NCT04995003, NCT02414269, NCT01822652, NCT04003649, NCT03980288, and NCT03726515).

In order to avoid repeated anti-PD-1/PD-L1 mAbs administration and the toxicity associated with it, new approaches have been proposed to inhibit the PD-1/PD-L1 axis,

TABLE 9 | Summary of preclinical studies on CAR-T cells to overcome the inhibitory effect of ICs.

Targeted antigen	Expressed genes	Co-administered agent	Type of cancer	Reference
HER-2	–	Anti-PD-1	Breast cancer	(278)
GD2	–	Pembrolizumab	NeuroblastomaMelanoma	(279)
FAP	–	Anti-PD-1	Malignant pleural mesothelioma	(174)
MUC16	PD-1 scFv	–	Ovarian cancer	(280)
EGFR	PD-1 scFv	–	Gastric cancer	(281)
CAIX	PD-L1 scFv	–	RCC	(282)
MSLN	PD-1 DNR	–	Malignant pleural mesothelioma	(283, 284)
MSLN	PD1/CD28switch-receptor	–	Mesothelioma	(285)
PSCA		–	Prostate cancer	
CD133	PD-1 KO	–	Glioma	(286)
MSLN	PD-1 KO	–	TNBC	(287)
GPC3	PD-1 KO	–	HCC	(288)
EGFRvIII	PD-1 KO	–	GBM	(289)

TABLE 10 | Summary of clinical trials on CAR-T cells designed to improve tumor homing and penetration, CAR-T cell persistence and resistance to immunossupresssion in solid tumors.

Type of strategy	Targeted antigen	Additional engineering strategies	Type of cancer	Other therapy	Phase/NCTnumber	Number of patients	Preliminary outcomes	Reference
Homing	–	CXCR2 and NGFR Expression	Metastatic melanoma	Lymphodepletion (cyclophosphamide & fludarabine),High-dose Aldesleukin (rhIL2)	Phase I/II NCT01740557	Active, not recruiting3/36 enrolled	None posted	(304)
Homing, Maintenance & Stroma targeting	Nectin4 FAP	IL-7 and CCL19/IL-2 production	Nectin4+ advanced NSCLC, breast, bladder, OC or PC	Intravenous minimally invasive surgery	Phase I NCT03932565	Recruiting	None posted	–
Homing, Maintenance & Protection from IS	MSLN GPC3, and/or TGFβ	CCL19 expression, IL7 production and/or scFv against PD1/CTLA4/TIGIT +/-PD1 KO	Advanced HCC/ PC/OC	Lymphodepleting chemotherapy	Phase I NCT03198546	6 patients	2 CR, 2 PR and 2SD No high grade toxicities	(68)
Stroma targeting	FAP	–	Malignant pleural mesothelioma	Anti-PD1 (in 1/3)	Phase I NCT01722149	3 patients	2/3 alive at 18 months follow-up	(169)
Neovasculature targeting	VEGFR2	–	Metastatic melanoma or renal cancer	Non-myeloablative lymphodepletion (fludarabine & cyclophosphamide) + Aldesleukin (rhIL-2)	Phase I/II NCT01218867	24 patients	Lack of objective responses: 1/24 PR, 1/24 SD, 22/24 PD Grade 3/4 toxicity in 5/24	–
	PSMA	–	Prostate cancer	Non-myeloablativeconditioning, Low/moderate dose of IL-2	Phase I NCT00664196	Suspended	None posted	–
	PSMA	HSVtk gene for ganciclovir elimination	Metastatic CRPC	Cyclophosphamide	Phase I NCT01140373	Active, not recruiting7 patients	2/7 SD, 2/7 PD	(305)
	PSMA	–	Metastatic prostate cancer	Non-Myeloablative Conditioning (fludarabine & cyclophosphamide) Low dose of IL-2	Phase I/II NCT01929239	Suspended	2/5 PR	(306)
Antigen Escape Prevention or increased immune engagement/ Bispecific or Dual CARs	c-Met/PD-L1	–	Primary HCC	–	Early Phase I NCT03672305	Recruiting	None posted	–
	B7H3CD19	Suicide mechanisms: EGFRt or Her2tG	Pediatric Solid Tumor	+/- Cetuximab (anti- EGFRt) or Trastuzumab (anti-HER2tG)	Phase INCT04483778	Recruiting	None posted	–
	EGFRCD19	Suicide mechanisms: EGFRt or H2tGand express a EGFR-specific and a CD19-specific receptor	R/R Solid Tumors in Children and Young Adults	–	Phase I NCT03618381	Recruiting	None posted	–
	HER2PD-L1	–	Pleural or Peritoneal Metastasis of HER2+ Cancer	–	Early Phase INCT04684459	Active, not recruiting	None posted	–

(Continued)

TABLE 10 | Continued

Type of strategy	Targeted antigen	Additional engineering strategies	Type of cancer	Other therapy	Phase/NCTnumber	Number of patients	Preliminary outcomes	Reference
Potentiating activation/ Switchable CARs	PSCA	Inducible MyD88/CD40 co-activation switch	Metastatic prostate and pancreatic cancer	Fludarabine & cyclophosphamide Rimiducid (inducible MyD88/ CD40 co-activation switch)	Phase I/ IINCT02744287	RecruitingPreliminary results in 15 patients	8/15 SD, 3/15 PD, No CRS, 1/15 serious Grade 2 AE	(307)
Universal CARs	–	UniCAR02-T Cells and recombinant antibody derivative TMpPSMA	Prostate Cancer	Fludarabine & cyclophosphamide	Phase I NCT04633148	Recruiting	None posted	–
Protection from IS by PD-1 gene deletion/gene expression inhibition	MSLN	CRISPR-Cas9 MediatedPD-1 and TCR/CD3 Gene-knocked Out (PDCD1 and TRAC KO)	MSLN+ metastatic solid tumors	No prior lymphodepletion	Phase I NCT03545815	15 patients	2/15 SDNo dose-limiting toxicity or unexpected AEs	(308)
	MUC1	CRISPR-Cas9 PD-1 gene KO	Advanced NSCLC(IIIb to IV)	PD-1 mAb (PD-1 antibody treated group) or sham (control)	Phase I/ IINCT03525782	Recruiting(Preliminary results on 20 patients)	11/20 SD, 9/20 PD,no grade 3-5 AEs, no CRS	(309)
	–	CRISPR-Cas9 PD-1 gene KO	Advanced prostate cancer	–	Phase I/II NCT03525652	Unknown status	None posted	–
	–	CRISPR-Cas9 PD-1 gene KO	Metastatic NSCLC	Cyclophosphamide	Phase I NCT02793856	22 enrolled/12 treated	2/12 SD, no PR, 11 cancer progression related deaths,Grade 1/2 AEs	(310)
	–	CRISPR Cas9 PD-1 gene KO	Advanced ESCC	Hydrocortisone	Phase INCT03081715	21 enrolled/17 treated	6/17 SD, 11/17 PD (10 cancer progressionrelated deaths), no PR,no grade 3/4 AEs	(311)
	Cancer-specific TCR transgene (NY-ESO-1)	NY-ESO-1 TCR expressions and multiplex CRISPR-Cas9 editing (KO of endogenous TCR and PD-1)	MM, sarcoma (SS, MRCL)	Lymphodepleting chemotherapy (Cyclophosphamide & Fludarabine)	Phase INCT03399448	3/4 infused	2/3 SDNYCE T cells detectable in circulation for up to 9 months (1 MM)	(312)
	–	PD-1 gene KO	Metastatic Renal Cell Carcinoma	CyclophosphamideIL2	Phase I NCT02867332	Withdrawn (No funding)	None posted	–
	EBV-specific autologous CTLs	CRISPR-Cas9 PD-1 gene KO	EBV+ Stage IV cancers: Gastric and Nasopharyngeal Carcinoma, TCL, Adult HL and DLBCL	Fludarabine Cyclophosphamide IL-2	Phase I/ IINCT03044743	Recruiting	None posted	(313)
	–	CRISPR-Cas9 PD-1 gene KO	Invasive Bladder Cancer (Stage IV)	Cyclophosphamide IL-2	Phase I NCT02863913	Withdrawn (No funding)	None posted	–
	MSLN	CRISPR-Cas9 PD-1 gene KO	MSLN+ multiple solid tumors	Paclitaxel, Cyclophosphamide+/- Gemcitabine	Phase INCT03747965	9 treated (6 pancreatic, 2 ovarian and 1 colorectal cancer)	7 evaluable patients:2/ 7 PR, 4/7 SD,2/7 grade 1 CRS	(314)
	MUC-1	CRISPR-Cas9 PD-1 gene KO	Advanced esophageal	–	Phase I/ IINCT03706326	8 enrolled		(315)

(Continued)

TABLE 10 | Continued

Type of strategy	Targeted antigen	Additional engineering strategies	Type of cancer	Other therapy	Phase/NCTnumber	Number of patients	Preliminary outcomes	Reference
			cancer/ ESCCNSCLC (IIIb to IV)				5/8 SD, 3/8 PD,no grade 3-5 AEs, no CRS	
		PD-1 gene KO	Refractory Thyroid Cancer	–	Phase I ChiCTR1900022620**	Not yet recruiting	None posted	–
		PD-1 gene KO	Advanced, recurrent or metastatic solid tumors	–	Phase I ChiCTR-OIN-17012136**	Recruiting	None posted	–
		PD-1 gene KO	Lung cancer	–	ChiCTR1800016023**	Not yet recruiting	None posted	–
	MUC-1	PD-1 gene KO	MUC1 +advanced breast cancer	–	Phase 0 ChiCTR1900025088**	Recruiting	None posted	–
Protection from IS by immune-checkpoint blockade	MSLN	αPD1 antibodies secretion	MSLN + advanced malignancies	–	Phase I/II NCT03030001	Unknown status	–	–
	MSLN	αPD1 antibodies secretion	MSLN + solid malignancies	Apatinib (anti-angiogenic drug)	Phase I/II NCT03615313	Unknown status (50 to include)	1 advanced/refractory OC infused patient: 1PR and survival > 17 months	(316)
	MSLN	αPD1 nanobodies secretion	NSCLC Mesothelioma	Lymphodepletion (cyclophosphamide)	Early Phase I NCT04489862	Recruiting	None posted	–
	MSLN	αPD1 nanobodies secretion	Advanced CC and OC	Lymphodepletion (cyclophosphamide)	Early Phase I NCT04503980	Recruiting	None posted	–
	MSLN	αPD1 nanobodies secretion	CC	–	Phase I NCT05089266	Not yet recruiting	None posted	–
	–	αPD1 antibodies expression	EGFR+ solid tumors	–	Phase I/II NCT02873390	Unknown	–	–
	MUC-1	CTLA-4 and PD-1 antibodies expression	MUC1+ Advanced Solid Tumors	Cyclophosphamide	Phase I/II NCT03179007	Unknown	None posted	–
	EGFR	CTLA-4 and PD-1 antibodies expression	EGFR + advanced recurrent/ refractory solid tumors	Cyclophosphamide	Phase I/ IINCT03182816	Unknown	9 NSCLC:1/9PR (lasted> 13m),6/9 SD and 2/9 PD, no grade 4 AEs, Grade 1-3 AE	(317)
	MSLN	CTLA-4/PD-1 antibodies expression	Advanced solid tumors	Cyclophosphamide	Phase I/ IINCT03182803	Unknown	None posted	–
	HER2, MSLN PSCA, MUC-1, Lewis-Y, GPC3, AXL, EGFR, Claudin18.2/6, ROR1, GD1, or B7-H3	CRISPR-Cas9 PD1 KO and CTLA-4/PD-1 -scFv secretion	Solid tumors	–	Phase I NCT04842812	Recruiting	None posted	–
	PSCA, MUC1, TGFβ, HER2, MSLN, Lewis-Y,	CD4+: TGFβ-CAR and expression and secretion of IL7/CCL19 and/	Lung cancer	–	Phase INCT03198052	Recruiting	None posted	–

(Continued)

TABLE 10 | Continued

Type of strategy	Targeted antigen	Additional engineering strategies	Type of cancer	Other therapy	Phase/NCTnumber	Number of patients	Preliminary outcomes	Reference
	GPC3, AXL, EGFR, B7-H3, Claudin18.2	or SCFVs against PD1/CTLA4/ TIGITCD8+: PD1 KO						
Protection from IS	PD-L1	PD1/CD28 chimeric switch receptor truncated EGFR (tEGFR) for CSR ablation	Glioblastoma	Cyclophosphamide and Fludarabine)	Phase I NCT02937844	Unknown	None posted	–
	PSMA	Non-viral PD-1integration	CRPC	Cyclophosphamide and Fludarabine	Phase INCT04768608	Not yet recruiting	None posted	–
	MSLN	CD3z signaling domain with loss-of-function mutations within 2 of 3 ITAM motifs and PD-1 dominant-negative receptors (DNR)	Mesothelioma	Cyclophosphamide	NCT04577326	Recruiting	None posted	–
	PSMA	Dominant negative TGFβ receptor (TGFβDNR)	Advanced CRPC	+/- Cyclophosphamide	Phase INCT03089203	Active, not recruiting	4/10 reached PSA30 response, 5/10 CRSgrade ≥ 2, 1/ 10grade 5 AE	–
	PSMA	TGFβDNR	Metastatic CRPC	Fludarabine & CyclophosphamideAnakinra	Phase INCT04227275	Active, not recruiting	None posted	–
	CD22	anti-PD-L1 scFv	Cervical cancer, sarcoma, NSCLC	–	Phase 1NCT04556669	Recruiting	None posted	–
CAR-T cell maintenance	GD2	Constitutively active IL-7 Receptor	GD2+ brain cancers (HGG, DIPG, medulloblastoma)	Lymphodepleting chemotherapy (Cyclophosphamide & Fludarabine)	Phase INCT04099797	Recruiting	None posted	–
	GPC3	IL15 & IL21armoredInducible caspase 9 safety switch (iCas9)	Pediatric solid tumors*	Lymphodepleting chemotherapy (Cyclophosphamide &Fludarabine)	NCT04715191	Not yet recruiting	None posted	–
	GPC3	IL15 & IL21armored	HCC/ Hepatoblastoma	–	NCT04093648	Withdrawn/ incorporated into another study	None posted	–
	GPC3	IL15 armored	Pediatric solid tumors	–	Phase INCT04377932	Recruiting	–	–
	GPC3	IL15 armored	HCC	–	Phase INCT05103631	Not yet recruiting	–	–
	GPC3	ArmoredCAR-T cells	Advanced HCC	–	Phase INCT05155189	Not yet recruiting	None posted	–
	GPC3	Expression of IL-21 and/or IL-15	Pediatric Liver Tumors	Cyclophosphamide & Fludarabine	Phase I NCT02932956	Recruiting	None posted	(318)
	GPC3	Expression of IL-21 and/or IL-15	HCC	Cyclophosphamide & Fludarabine	Phase INCT02905188	Recruiting	None posted	–
	GD2	iCas9 safety switch,expression of IL-15	R/R neuroblastoma and osteosarcoma	Cyclophosphamide & Fludarabine	Phase INCT03721068	Recruiting	None posted	–

(Continued)

TABLE 10 | Continued

Type of strategy	Targeted antigen	Additional engineering strategies	Type of cancer	Other therapy	Phase/NCTnumber	Number of patients	Preliminary outcomes	Reference
Combinatorial strategies	GPC3	–	HBV-related metastatic HCC	Lymphodepletion+ Tyrosine kinase inhibitors (TKIs) sorafenib/regorafenib (in 67%) +/- PD-1/PD-L1 mAb	Phase I NCT03980288	6 patients	1 PR persisting at 18 months	(319)

*Registered trials listed in the table were either from www.clinicaltrials.gov or from www.Chictr.org.cn (Chinese Clinical Trial Registry, marked with **).*

AE, adverse effects; ALL, Acute Lymphoblastic Leukemia; BCMA, B-cell maturation antigen; CLL, Chronic Lymphocytic Leukemia; CC, colorectal cancer; CR, complete response; CRPC, Castration Resistant Prostate Cancer; CRS, Cytokine release syndrome; CTCL, Cutaneous T-Cell Lymphoma; RR DLBCL, relapsed or refractory diffuse large B cell Lymphoma; DIPG, diffuse intrinsic pontine glioma; FAP, Fibroblast activation protein; GPC3, glypican-3; HCC, Hepatocellular Carcinoma; HGG, high grade glioma; HL, Hodgkin Lymphoma; Hsvtk, herpes simplex virus-1 thymidine kinase; Interleukin, IL; IS, immunosuppression; KO, Knock-out; mAb, monoclonal antibody; MM, Multiple Myeloma; MRCL, Myxoid/ Round Cell Liposarcoma; MSLN, mesothelin; NGFR, Nerve Growth Factor Receptor; NHL, Non Hodgkin Lymphoma; NSCLC, non-small-cell lung carcinoma; OC, ovarian carcinoma; OS, overall survival; ESCC, esophageal squamous cell carcinoma; PC, pancreatic cancer; PD, progressive disease; PFS, progression-free survival; PR, partial response; PSCA, Prostate stem cell antigen; rhIL2, Recombinant Human IL-2; RR BCL, relapsed/refractory B cell lymphoma; RR MM, refractory/recurrent multiple myeloma; scFv, single chain variable region; SD, steady disease; SS, Synovial Sarcoma; VGPR, very good partial response.

by engineering CAR-T cells expressing PD-1-blocking scFv (280, 281) or PD-L1-blocking scFv (282), expressing PD-1 DNR (283, 284), or chimeric switch receptors (CSR, like PD1/CD28 CSR) (285) (**Figure 7**). Various ongoing clinical trials are evaluating anti-PD-1 antibodies secreting (NCT03030001, NCT03615313, NCT04489862, and NCT02873390), anti-PD-L1 scFv secreting (NCT04556669) or PD-1 nanobodies secreting CAR-T cells (NCT04489862, NCT04503980 and NCT05089266) (**Table 10**). Genetic engineering strategies aiming at counteracting immunosuppressive signaling by the design of PD1/CD28 CSR are also evaluated in the clinical setting (clinical trials NCT03932955, NCT04850560, NCT02937844). Other clinical trials evaluate efficiency of CAR-T cells designed to resist immunosuppression by various other mechanisms, employing mutant PD-1 proteins, PD-1 dominant negative receptors (DNR), cytoplasmic activated PD-1 or PD-1 downregulation (NCT03540303, NCT04768608, NCT04577326, NCT04163302, NCT04162119, and NCT04836507) (**Table 10**).

A different strategy to provide an enhanced version of genetically modified T cells with increased antitumor activity against solid tumors was to engineer PD-1 knockout (KO) CAR-T cells. The breakthrough of gene editing techniques has, thus, allowed to genetically disrupt PD-1 function in CAR-T-cells (320). As a strategy that increases lymphocyte fitness in the immunosuppressive TME, PD-1 KO engineering in CAR-T cells proved enhanced antitumor activity in preclinical models, both *in vitro* and *in vivo* (283, 321). PD-1 deletion was first applied to autologous T cells and its efficacy was variable, depending on gene editing techniques. To this regard, Beane et al. used the zinc finger nuclease-mediated (ZFN) gene editing technology to KO PD1 expression in TILs drawn from melanoma patients, with an average reduction of 76% in PD-1 surface-expression. In addition, PD-1 KO TILs showed enhanced *in vitro* activity and a significantly superior polyfunctional cytokine profile (IFNγ, TNFα, and GM-CSF) as compared to unmodified TILs in 66.67% (2 of 3) patients tested (322). Menger et al. knocked out the PD-1 gene in melanoma-reactive CTLs and in fibrosarcoma-reactive polyclonal T cells, using the TALEN technology and noticed that modified T cells had better persistence at the cancer site and were able to control the tumor progression more efficiently than non-modified T cells (323). Other groups have also succeeded in inactivating the PD-1 gene in TILs by using the CRISPR-Cas9 technology (321, 324, 325).

Considering immune-related adverse events related to anti-PD-1 mAbs administration and the success of PD-1 gene inactivation in primary human T cells, this strategy was extended to CAR-T cell therapy (326). Hu B. et al., designed CAR-T cells directed against CD133 and KO for the PD-1 gene by using the CRISPR/Cas9 technology, with an average of 91.5% of inactivated gene sites. This disruption enhanced both *in vitro* cytotoxicity against a glioma cell line and *in vivo* antitumor activity in an orthotopic glioma mouse model. No significant toxicity was observed, confirming the safety profile of PD-1 KO CD133-specific CAR-T cells. Moreover, PD-1 KO did not impede cytokine production and CAR-T functionality as PD-1-deficient lymphocytes secreted similar amounts of cytokines (IFN-γ, IL-2, TNF-α, and GM-CSF) as conventional CAR-T cells

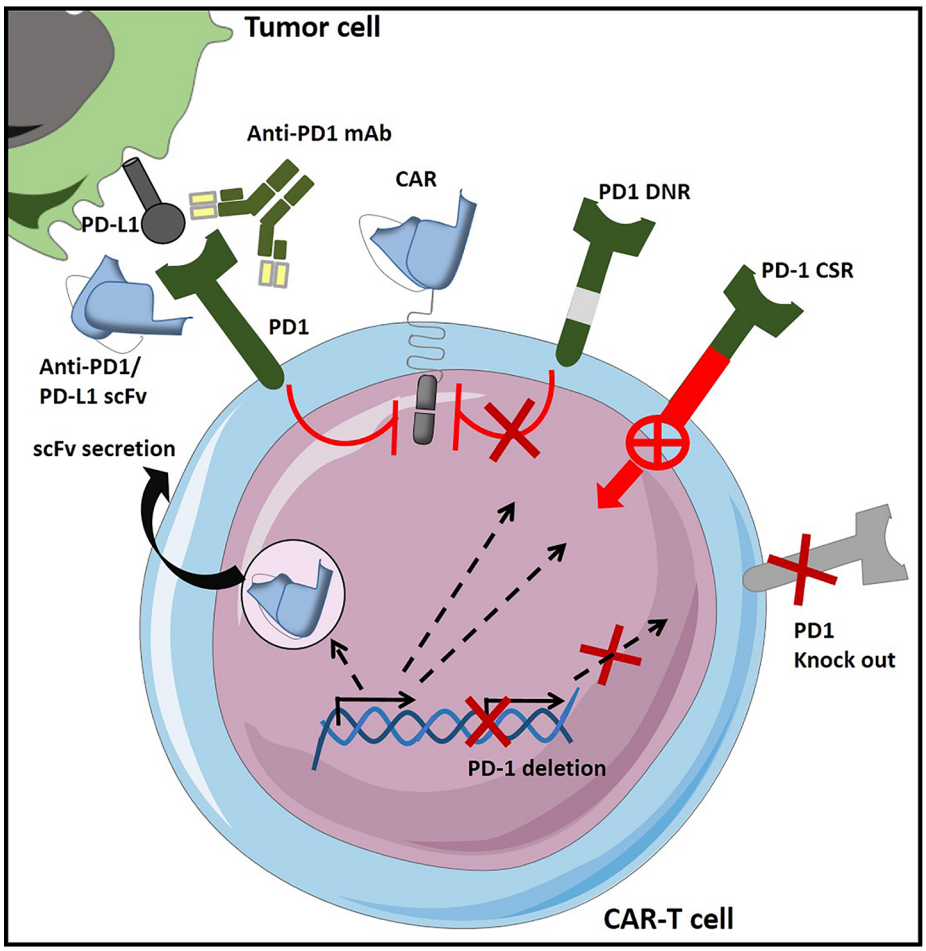

FIGURE 7 | CAR-T cell engineering strategies to overcome inhibition form negative immune checkpoint regulation – Example of PD1/PD-L1 axis targeting in CAR-T cells [Adapted from Rafiq et al. (31)]. In order to prevent CAR-T cell exhaustion and immunosuppression in the TME, different strategies can be used, like combination of CAR-T cells with immune checkpoint inhibitors (ICIs like anti-PD1 or PD-L1 antibodies). PD1-medited inhibition can also be surmounted by designing CAR-T cells that secrete either PD-1-blocking or PD-L1 blocking scFv. Other means of shielding CAR-T cells from the inhibitory effect of the PD1/PD-L1 interaction is to design genetically modified CAR-T cells that express a dominant negative PD-1 receptor (PD-1 DNR) which interferes with PD1 downstream signaling or a PD-1 chimeric switch receptor (CSR), which converts an inhibitory signaling into an activating one. Last type of strategy is based on PD1 expression deletion either by genetic knock-out or by means of shRNA (short hairpin RNA) inhibition.

(286). The team used this technology to disrupt the PD-1 gene in MSLN-specific CAR-T cells also. Despite a significant effect on CAR-T cell proliferation, this strategy greatly increased CAR-T cell cytokine synthesis and cytotoxicity towards PD-L1+ tumor cells *in vitro*. The antitumor activity of PD-1 KO MSLN-specific CAR-T cells was also increased in a TNBC animal model. Moreover, PD-1 KO could improve the CAR-T cells antitumor effect more efficiently than the combination of CAR-T cells with PD-1 blocking (287). Similar results have been published by Guo et al. with PD-1 KO GPC3-specific CAR-T cells in a HCC preclinical study (288), and Choi et al. with PD-1 KO EGFRvIII-specific CAR-T cells in a glioblastoma preclinical study (289). Many ongoing clinical trials are evaluating the efficacy of PD-1 deficient T cells/CAR-T cells in patients with solid tumors or hematological cancers (NCT03545815, NCT03525782, NCT03298828, NCT03525652, NCT02793856, NCT03081715, NCT03399448, NCT02867332, NCT03044743, NCT03030001, NCT02863913, NCT03747965, NCT03706326, ChiCTR1800020306, ChiCTR1800018713, ChiCTR1900022620, ChiCTR-OIC-1701131, ChiCTR-OIN-17012136, ChiCTR1800016023, ChiCTR1900025088, NCT03208556 and NCT04213469) (**Table 10** for clinical tials on solid tumors).

Last but not least, new reports have suggested the feasibly of targeting other inhibitory receptors, such as CTLA-4 (327, 328), LAG-3 (329, 330) or TIM-3 (330, 331). However, additional studies are required to determine whether these novel strategies are as effective as CAR-T cells engineered to overcome PD1 inhibition. Results from ongoing clinical trials with CAR-T engineered to block simultaneously PD-1 and CTLA-4 +/- TIGIT (by antibody or ScFv secretion) will establish the eventual benefit of combinatorial ICI blockade strategies (NCT03179007, NCT03182816, NCT03182803, NCT04842812, NCT03198052).

3 CONCLUSION AND FUTURE PERSPECTIVES

CAR-T cell based cell therapy is a moving field, which showed impressive results in hematopoietic cancer management and brought hope to incurable patients. Unfortunately, success in managing solid cancers was less outstanding. Assiduous research has been done to overcome unexpected roadblocks which impede CAR-T cells trafficking, infiltration, persistence or function in the unwelcoming tumor environment. Indeed, research focused on identifying target antigens and avoiding on-target-off tumor toxicity (206), improving CAR-T cell trafficking and entry into the tumor site, promoting better signaling, less exhaustion, and memory phenotypes in solid tumors. Preclinical models propose various engineering strategies, some of which have already advanced from bench to bedside, with encouraging preliminary results.

As reviewed herein, trafficking and infiltration have been addressed by genetically manipulating chemotaxis and tissue homing. Moreover, tumor stroma targeting emerged as a promising strategy, based either on depletion of stromal cells/immunosuppressive cells or at reprogramming strategies directed at regulating TME plasticity. To this regard, a new generation of CAR-T cells has been designed to directly target stroma components like fibroblasts and immunosuppressive cells (Tregs, TAMs or MDSCs). However, a remaining challenge for the development of both effective and safe CAR-T cell therapies is the insufficient clinical relevance of preclinical mouse models. Indeed, these models sometimes failed to predict clinical level toxicities or, on the other hand, inefficient tumor targeting when translated to the clinic. Further research is still needed to overcome this hurdle and develop advanced preclinical models able to address tumor heterogeneity and TME complexity in order to accomplish a perfect balance between efficacy and safety of CAR-T cell therapies in solid tumors.

Furthermore, exciting new opportunities emerged thanks to gene editing/gene ablation techniques based on the revolutionary, highly specific and efficient CRISPR/Cas9 tools (332, 333), which have been used not only to generate immune-checkpoint knock-outs (PD-1 KO) but also to design "universal" CARs, edited for TCR and/or HLA molecules expression (206, 332, 333), which could pave the road towards cost-effective allogeneic CAR-T cells for an "off-the-shelf" ACT with a broader spectrum (334, 335). This technique can even be used for multiplexed genome editing (336). To this regard, feasibility of targeting multiple genes in T cells by multiplex CRISPR-Cas9 has recently been proven in a small interventional study in patients with advanced, refractory cancer (NCT03399448) (312). Further improvements of this technology are awaited as recent advances seem to insure increased precision and minimized side effects both in case of gene deletion and gene insertion (336). As allogeneic (allo)-CAR-T cells could offer readily available ACT sources that could expand the usage of CAR-cells based immunotherapy, other recent strategies for allo-CAR-T cells generation emerged, like the NKG2D (an NK-based activating receptor) expression. NKG2D expression in allogeneic CAR-T cells offers a non-TCR edited cellular therapy with broad solid tumor targeting, and two clinical trials are ongoing in metastatic colon cancer (NCT04991948 and NCT03692429, Celyad Oncology) with encouraging preliminary results in the second one (2/15 PR and 9/15 SD). Another allo-CAR-T cells product, the CD70-targeting ALLO-316 cells (Allogene Therapeutics) is under evaluation in a clinical trial on renal cell carcinoma (together with anti-CD52 mAb, NCT04696731).

On the other hand, a part from innovations in CAR design addressed in this review, advances in transduction techniques, cell culture and amplification conditions (like IL-7/IL-15 media) as well as identification of the most suitable stage of T cell differentiation (TCM/TSCM) to use for adoptive transfer represent additional steps towards effective CAR-T cell therapy in solid tumors (337, 338). To this regard, the need for large-scale CAR-T manufacturing persists and could limit cancer patients' accessibility to CAR-T cell-based ACT. Therefore, the already engaged transition from academic to industrial manufacturing could ensure increased availability and reproducibility as well as shorter delays thanks to Good Manufacturing Practice (GMP)-compliant automated, closed systems (339–341). Contrary to large scale, commercial *in vitro* manufacturing, Smith and colleagues recently described an *in-vivo* manufacturing technique of CAR-T cells, by programming circulating, bloodstream T cells with DNA-carrying polymer nanoparticles, which efficiently introduced leukemia-targeting CAR genes into T-cell nuclei (342, 343).Accumulating knowledge on efficacy, toxicity and resistance drawn from clinical trials as well as fundamental research data on TILs interaction with the TME will allow for the identification of novel molecular targets in CAR-T cells design (344). To this regard, and pointing out once more the role of the hypoxia response in cancer, the VHL-HIF axis and particularly HIF's activity, has recently been identified as a tool to potentiate tissue residency of CD8+ CTLs, as well as a potential molecular candidate to modulate CAR-T cell therapy efficacy (345). Genetic targeting of precise molecular or metabolic pathways critical for TILs survival in the TME emerge therefore as novel strategies to overcome insufficient amplification and persistence of CAR-T cells in solid tumors (31, 213, 346, 347).

This review focuses less on engineering strategies aiming at enhancing tumor recognition and preventing antigen escape. Such combinatorial targeting strategies employ bispecific/dual CARs or Tandem CARs, trivalent or pool CARs and have already been reviewed by us (206) and others (31, 213, 346, 348). Bispecific CARs have gained an important place in hematologic cancers management, with numerous ongoing clinical trials (NCT04662099, NCT0327115, NCT03919526, NCT03879382, NCT03881761, NCT03706547, NCT04303520, NCT04412174, NCT03825731, NCT04499573, NCT05098613, NCT04034446, NCT04007029, and NCT04215016). However, usage of bispecific CARs in solid tumors is still at its beginning (NCT03672305, NCT04483778, NCT03618381 and NCT04684459) (**Table 10**). Nonetheless, multiple antigen targeting by employing universal immune receptors CAR also gains increasing interest (349) and a universal CAR is being tested in a clinical trial on prostate cancer patients (UniCAR02,

NCT04633148). Moreover, toxicity management strategies and especially prevention of on-target off tumor effects were not thoroughly described here-in but were reviewed previously (206). Switchable CARs for instance emerge as valuable safety strategies, like is the case of the iCas9 safety suicide switch employed in some ongoing clinical trials (NCT04715191, NCT03721068). Moreover, orthogonal switchable CARs or dual-switch CAR-T cells capable of both regulated costimulation/inducible activation to drive CAR-T cell expansion and activity and regulated iCasp9 safety switch for CAR elimination have recently been described (350). Co-activated switchable CAR-T cells also advanced to clinical testing (ongoing clinical trial NCT02744287 of BPX-601 CAR-T cells expressing a PSCA specific CAR and a rimiducid-inducible MyD88/CD40 co-activation switch, Bellicum Pharmaceuticals), with encouraging preliminary results (307) (**Table 10**).

Besides optimization of costimulatory domains discussed here-in, modulation of scFv avidity could be another strategy to increase antigen recognition and CAR-T cell engagement. Surprisingly, lower avidity CAR-T cells (the 8F8-BBz CAR-T cells) could show greater therapeutic potential, by increased resistance to exhaustion and apoptosis in an HCC context (351).

Considering all the aforementioned hurdles in CAR-T cells homing, as well as the diversity and plasticity of cells composing the tumor microenvironment, the best engineering option could be based on a combination of strategies that enhance at the same time trafficking, penetration, persistence and/or CAR-T cell function. Some combinations have already been tested, like it is the case of armored CAR-T cells/TRUCKs engineered to co-express chemokine receptors and secrete vital cytokines. As monotherapeutic approaches are rarely effective, strategies targeting multiple antigens, combinations of different genetic engineering strategies or combinations based on CAR-T cells and innovative immunotherapies (like ICIs) could represent a turning point in a still ongoing revolution in solid cancer management. Nonetheless, CAR-T cells could also be combined with other therapeutic modalities, such as standard chemotherapy and/or radiation therapy, tyrosine kinase inhibitors, epigenetic modulators, other small molecule drugs or vaccines.

All in all, CAR-T cell immunotherapy stands out as a promising, evolving weapon in the fight against solid cancer.

Beside CAR-T cell based ACT, novel genetic engineering techniques, such as gene-editing and cellular reprogramming allowed for the emergence of new ACT strategies employing various innate killer cells (IKC) (like NK cells, NKT cells, and γδ T-cells (CAR-IKC) (352–358), macrophages (CAR-M) (146), and even B lymphocytes (CAR-B cells). A combination of "classic" CAR-T cells and CAR- IKC/CAR-Macrophages as bridging therapy could potentially increase efficiency in solid tumors by increasing the cross-talk between various immune cells or by TME remodeling effects (248, 359, 360). Unfortunately, CAR-NK also have some limitations, as for example high-dosage conditioned efficacy and decreased persistence. On the other hand, co-administration of cord-blood derived-NK cells (CB-NKs) proved to be a potent immunoregulatory strategy, promoting early activation and migration, enhanced fitness and increased anti-tumor efficacy of CAR-T cells (360). Surprisingly, chimeric receptor engineered Tregs (CAR-Tregs), which emerged as potential immune-tolerance inducers in autoimmunity or transplantation (361), also showed potent anti-tumor effect (362). Moreover, CAR-B cells, which could represent safe and controllable vehicles for local delivery of monoclonal antibodies emerged in preclinical studies as potential candidates for infectious diseases and protein deficiencies and might therefore be interesting candidates for cancer therapy as well.

AUTHOR CONTRIBUTIONS

AA and AC contributed equally. All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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CAR T Cell Therapy in Primary Brain Tumors: Current Investigations and the Future

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Chimeric antigen receptor T cells (CAR T cells) are engineered cells expressing a chimeric antigen receptor (CAR) against a specific tumor antigen (TA) that allows for the identification and elimination of cancer cells. The remarkable clinical effect seen with CAR T cell therapies against hematological malignancies have attracted interest in developing such therapies for solid tumors, including brain tumors. Glioblastoma (GBM) is the most common primary brain tumor in adults and is associated with poor prognosis due to its highly aggressive nature. Pediatric brain cancers are similarly aggressive and thus are a major cause of pediatric cancer-related death. CAR T cell therapy represents a promising avenue for therapy against these malignancies. Several specific TAs, such as EGFR/EGFRvIII, IL13Rα2, B7-H3, and HER2, have been targeted in preclinical studies and clinical trials. Unfortunately, CAR T cells against brain tumors have showed limited efficacy due to TA heterogeneity, difficulty trafficking from blood to tumor sites, and the immunosuppressive tumor microenvironment. Here, we review current CAR T cell approaches in treating cancers, with particular focus on brain cancers. We also describe a novel technique of focused ultrasound controlling the activation of engineered CAR T cells to achieve the safer cell therapies. Finally, we summarize the development of combinational strategies to improve the efficacy and overcome historical limitations of CAR T cell therapy.

Keywords: glioma, focus ultrasound, CAR T cell, brain tumor, immunotherapy

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INTRODUCTION

T cells engineered to express chimeric antigen receptors (CAR T cells) have shown remarkable efficacy in treatment of hematological cancer and represent a promising frontier for innovation in their application to treat solid malignancies (1). Chimeric antigen receptors (CARs), which are assembled by the fusion of a recognition domain, single-chain antibody and T cell stimulatory domain, can be engineered to recognize a target antigen without major histocompatibility complex (MHC) presentation (2, 3). These CAR constructs are then transfected into T cells using plasmids, mRNA, or viral vector transduction to ultimately display on the cell surface. CAR T cells can be engineered to target virtually any antigen, such as tumor-associated antigens or microbial antigens.

CAR T cells can become activated without the contribution of antigen presenting cells and MHC molecules, which greatly contributes to their effectiveness in activating the immune system. Other costimulatory receptors such as CD28 or OX40 may be added to further improve the T cell response. Once activated, CAR T cells can individually activate multiple immune cells and additionally secrete cytokines that promote cell trafficking and effector function, amplifying their individual effect.

The first CAR T cells were developed in 1987 by Kuwana et al. (4). Through iterations over the following decades, CAR T cells progressed through second, third, and fourth generation compositions which improved their antitumor activity, effector function and *in vivo* persistence, with expanded modifications allowing for enzymatic degradation of extracellular matrix for solid tumors and costimulation of various receptors with additional ligands (1–3). First generation CARs consist of a single-chain variable fragment antigen recognition domain, transmembrane component, and intracellular T-cell activation domain akin to the CD3 zeta chain (2). Second generation CARs incorporate a costimulatory domain, such as CD28 or 4-1BB, and are utilized in current commercially available CAR T cell therapies. Third generation CARs combine two distinct costimulatory domains into their intracellular structure. Fourth-generation CAR T cells are also known as TRUCKs or "armored CARs" (CAR-redirected T cells that can serve as a delivery platform for transgenic products) (1, 3). These modifications incrementally improved CAR T viability as a therapy for cancers, with particular success in hematological cancer. CAR T therapies have been developed for chronic lymphoblastic leukemia (CLL) and acute lymphoblastic leukemia (ALL), as well as recurrent lymphoma and prostate cancer, and investigation continues for optimizations that prove clinical effectiveness in other malignancies (1).

CAR T CELL THERAPY IN HEMATOLOGIC CANCER

CAR T cell therapy gained its initial foothold for hematogenous cancers, including CLL and ALL, and have rapidly changed the landscape of treatment for acute and chronic B cell leukemias with further indications in lymphoma and myeloma being explored (1, 3, 5). In August of 2017, the first CAR T cell therapy was approved by the Food and Drug Administration (FDA) for treatment of B-cell ALL (1). This therapy, called tisagenlecleucel-T targeting CD19, demonstrated efficacy in preliminary results from the Phase 2 multicenter ELIANA trial (1, 3). Impressively, this study demonstrated a 60% complete remission rate and 81% overall response rate in 75 children and young adults with durable response reported at 80% 6-month relapse-free survival associated with prolonged detection of CAR T cells in peripheral blood samples and persistent B-cell aplasia. Toxicity was common among study participants however, with 73% of patients experiencing severe adverse events (grade 3 and above) (3).

Within the year following the approval of tisagenlecleucel-T, two more therapies were approved by the FDA (2). Based on the Phase 2 multicenter ZUMA-1 trial, the first CAR T cell therapy, known as axicabtagene ciloleucel, was approved in October 2017. Results from this trial demonstrated 83% overall response rate and 58% complete remission rate among 101 participants, with 39% of patients with prolonged durable response at median follow-up of 27.1 months. Toxicity was less common in this trial than the ELIANA trial, with 48% of patients experiencing severe adverse events and 11% with cytokine release syndrome (CRS) compared to 47%, although a greater percentage of patients suffered neurotoxicity (NT) at 32% compared to 13.3% (3).

The success of the JULIET trial led to the approval of tisagenlecleucel for a second indication, relapsed and refractory lymphoma in 2018 (3). The JULIET trial showed 40% CR and 100% durable response at median follow-up of 29.3 months among a study population of 93 patients with diffuse large B-cell lymphoma (DLBCL). Toxicity overall was similar to the ELIANA trial, specifically measuring NT at 12% and CRS at 22% (3).

In another investigation targeting DLBCL, the Phase 2 TRANSCEND trial, lisocabtagene maraleucel was shown to be effective with 80% ORR and 55% CR with a 6-month durable remission of 50% (2). Toxicity in this study was remarkably low with only 1 patient experiencing CRS and 12% of patients experiencing neurotoxicity (3).

The success of CAR T cell therapy against hematogenous cancer is clear and represents tremendous progress in the treatment of these malignancies. Future progress in decreasing the toxicity profile associated with CAR T cell therapy, along with improvements in response rate, durability of remission, and CAR T exhaustion, will be catalytic to more widespread use to greatly improve patient prognosis (1–3, 6).

CAR T CELL THERAPY IN SOLID TUMORS

Inspired by the initial success against hematological cancer, trials of CAR T cells therapy for solid tumors were instigated, though yielded less impressive results (5). The growing number of clinical trials focused on solid tumors include CAR T cells targeting carcinoembryonic antigen (CEA), the diganglioside 2, mesothelin, interleukin 13 receptor alpha (IL-13Ralpha), human epidermal growth factor receptor 2 (HER2), fibroblast activation protein (FAP), and L1 cell adhesion molecule (L1CAM). Although the number of trials here is impressive, the most successful of these trials report complete remission of 3 of 11 patients using GD2 CARs for neuroblastoma, stable disease in 4 of 17 patients using HER2 CARs for sarcoma, and partial response in 2 of 11 patients in HER1 CARs for lung cancer. The differential success of CAR T therapy in solid tumors thus far compared to hematologic cancer has prompted investigation into possible explanations and identification of unique limitations that may not exist for treatment of hematologic cancers (5).

Proposed challenges in the treatment of solid tumors include identifying highly and uniformly expressed tumor antigens

(TAs), CAR T cell trafficking from blood to solid tumor sites, stromal infiltration, TA loss, inherent tumor heterogeneity, and the immunosuppressive tumor microenvironment (TME) (5). Current strategies to optimize CAR T-therapy in solid tumors generally fall within categories of disrupting immunosuppressive axes, autocrine stimulation, remodeling and induction of endogenous immune responses, and enhanced tumor infiltration. Development of armored CARs, or fourth generations CAR T cells, with unique immunostimulatory mechanisms specifically targets the hurdle of the TME. Some examples of fourth generation CAR T cells include candidates that express CD40 ligand, secrete IL-18, and secrete a PD-1-blocking single chain variable fragment (scFvs) (3, 5).

Trafficking of CAR T cells to the tumor site depends on the appropriate matched expression of adhesion molecules and chemokine receptors (such as CXCR3 and CCR5) that allow for endothelial adhesion and transport, along with tumor-specific targeting (3, 5). Appropriate design of CAR T cells to achieve these requirements, particularly as antigen expression greatly varies from tumor-to-tumor, has been notably challenging. Additional physical and anatomical barriers represented by the tumor stroma and high intratumoral pressure, along with unique physical barriers such as the Blood-Brain Barrier (BBB) to treating intracranial malignancies, necessitate further innovation to improve viability of CAR T cell therapy for solid tumors (5). Current investigations to improve trafficking to the tumor site include use of oncolytic viruses armed with chemotactic chemokines to attract CAR T cells and local administration (5, 6). Strategies to adequately disrupt physical barriers include targeted digestion of the dense tumor extra-cellular matrix, which demonstrated success in xenograft models using CAR T cells expressing heparinase, and ultrasonic disruption, such as focused ultrasound (FUS) applied to disrupt the BBB to deliver therapeutics to intracranial malignancies (3, 5, 6).

CAR T THERAPY FOR BRAIN TUMORS

Brain tumors, including primary and metastatic neoplasms, have a great impact on neurological function and quality of life, particularly in cases of more aggressive or malignant neoplasms (7). Advances in imaging instruments, such as computed tomography (CT) and magnetic resonance imaging (MRI), have led to an increase in incidence of brain tumors being diagnosed (8). Within gliomas, glioblastoma (GBM) is most aggressive and malignant with a median overall survival of 14 to 17 months despite standard of care (surgery, radiotherapy, and chemotherapy) (9, 10). Tumor-treating fields, a new FDA-approved therapeutic strategy, has shown promise in extending the overall survival to 20.9 months (11).

Although previously believed to be immune-privileged, the brain is now known to be immunologically dynamic, though quiescent at baseline. The BBB and resident microglia are the first lines of defense in CNS. Immune cells, such as dendritic cells (DCs), lymphocytes, and monocytes, are mostly absent in the CNS during the quiescent period (12). Additionally, the CNS was thought to be lacking conventional lymphatics (13) until 2015 when lymphatic-like structures were discovered along the dural venous sinuses in rodents (14). Both local and systemic immune cells can detect antigens from the CNS; peripheral immune cells are then able to cross the BBB after detection of these danger signals to induce further inflammatory responses, providing also a significant mechanism for targeted immunotherapy against brain tumors (15).

Advances in immunotherapy have increased the therapeutic options for patients with brain cancers. There are 70 clinical trials testing immunotherapy for either primary or secondary brain tumors currently. Although immunotherapy for GBMs is not included in standard of care based on European Association of Neuro-Oncology (EANO) guidelines, ICIs-based immunotherapy is recommended as tumor-specific adjuvant therapy for brain metastases (16). CAR T therapy is a promising strategy to treat hematological malignancies and some kinds of solid tumors. CAR T cells can specifically recognize cancer cells, due to their functionalization with homing surface molecules, and exert targeted cytotoxicity. An ideal TA target should be expressed homogenously on all cancer cells within a primary tumor and metastases. Additionally, in order to avoid killing of normal cells by CAR T cells, the TA should be undetectable or minimally expressed on normal tissues. Non-specific targeting of normal cells leads to toxicity and CRS, which is potential significant side effect of CAR T cell therapy. CAR T cell therapy utilizing specific TAs has been explored to treat GBMs and pediatric brain tumors. In this review, we will examine current clinical and preclinical study of CAR T cell against GBMs and pediatric brain tumors (**Figures 1A, B**).

Glioblastoma

Several molecules have been identified as potential TAs for CAR T cell therapy targeting GBMs through immunohistochemical (IHC) analysis. Here, we will discuss current CARs which have been used in clinical trials and preclinical models of GBM

Completed Clinical Trial of Targets

EGFRvIII

EGRFvIII, a mutated Epidermal Growth Factor Receptor (EGFR) from an in-frame deletion of exons 2 to 7, is the most common variant of this receptor in cancers (17, 18). In GBMs, approximately 40% of newly diagnosed patients have amplification of the EGFR gene, and about 50% of EGFR-amplified GBM patients exhibit constitutively oncogenic EGFRvIII (19, 20). The structure of the extracellular domain altered by this mutation can be targeted as a unique epitope by specific monoclonal antibodies (mAbs) with limited likelihood of on-target/off-tumor toxicity (21). Therefore, both vaccine and CAR T cell therapies targeted to EGFRvIII have been developed (22, 23). In preclinical studies, EGFRvIII CAR T cells demonstrated excellent reduction of tumor growth (24). However, limited efficacy has been shown for CAR T cells specific to EGFRvIII in GBM patients (25). To determine the extent of EGFRvIII CAR T cell trafficking to the tumor, surgical specimens were evaluated in one study for EGFRvIII expression across the TME after EGFRvIII CAR T cell administration. This study found EGFRvIII loss or decreased expression in resected tumors of most patients treated with CAR T cell infusion (23). These data demonstrated that the heterogeneous expression of EGFRvIII in glioma cells limited the efficacy of EGFRvIII CAR T cell therapy, and

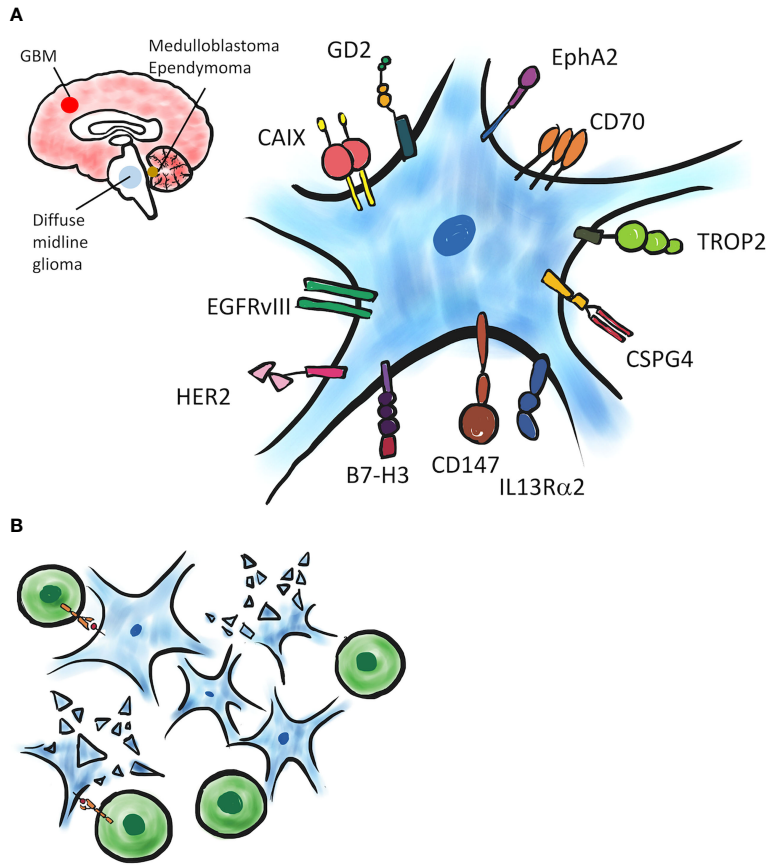

FIGURE 1 | A schematic of CAR T targeting brain tumors. **(A)** Several TAs expressed on tumor cells have been evaluated in preclinical and clinical CAR T studies. **(B)** TA-expressing tumor cells can be recognized and eliminated by TA-specific CAR T cells.

further results in generation of escape variants resistant to the same therapy (21). Regarding the TME, in post-CAR T cell surgical specimens, the phenotypic analyses of non-transduced and polyclonal T cells showed significant infiltration of regulatory T cells (Tregs). Additionally, excessive upregulation of immune checkpoints and immunosuppressive molecules, such as programmed death (PD) ligand 1 (PD-L1), indoleamine 2,3-dioxygenase-1 (IDO-1), transforming growth factor–β (TGF–β), and IL-10, was demonstrated by IHC (23). This suggests that EGFRvIII CAR T cells induced a compensatory immunosuppressive response in the TME, implying EGFRvIII CAR T is possibly more effective when combined with other immunotherapy to enhance immunity or reprogram the TME.

IL13Rα2

The function of IL-13 is to regulate inflammation and the immune response with binding to IL13Rα1. Additionally, IL-13 binds to the high affinity decoy receptor IL13Rα2 (26). IL13Rα2 is expressed in over 75% of GBMs and is related to tumor aggressiveness and poor prognosis. However, it is not expressed significantly in normal brain parenchyma or most normal tissues, except the testes (27). Due to the specificity of IL13Rα2 for GBM, IL13Rα2 has long been used as a potential candidate as a target for CAR T cell therapy (26). In the first-in-human pilot study, twelve intracranial infusions (maximum dose 1 x 10^8) of IL13Rα2 CAR T cells were administrated into three patients with recurrent GBM, showing fair tolerance with delivery of CAR T cells and excellent antitumor response in two of the three treated patients. Some mild adverse events such as headaches and transient neurologic deficits occurred but subsided after management (28). Another trial of IL13Rα2 CAR T cells incorporated positron emission tomography (PET) imaging to check the trafficking of CAR T cells into brain parenchyma (29). Because the uptake of [18F] FHBG (9-[4-[18F] fluoro-3 (hydroxymethyl)butyl]guanine) in HSV1-tk expressing cytotoxic T lymphocytes is higher than in naïve T cells, it can be used as a tracer for IL13Rα2 CAR T cells expressing an HSV1-tk reporter gene. This study demonstrated the approach is safe and feasible with an obvious increase of [18F] FHBG activity demonstrating T-cell trafficking to tumor regions.

HER2

Human epidermal growth factor receptor 2 (HER2), a receptor tyrosine kinase, was found overexpression in many kinds of cancers and approximately 80% of GBMs (30, 31). A recent paper demonstrated that third generation HER2 CAR T cells can target and kill GBM cells *in vitro*. Additionally, significant improvement of

efficacy was found when combined with PD-1 blockade (32). However, safety concerns were raised due to the death of a colorectal cancer patient who received 1 x 10^{10} third-generation HER2 CAR T cells (with a trastuzumab-based antigen-recognition domain and a CD28.4-1BB signaling domain) (33). Following these concerns, up to 1 x 10^{8} second-generation HER2 CAR T cells (an FRP5-based exodomain and a CD28 signaling endodomain) was administrated in GBM patients with no dose-limiting toxic effects observed (34). After infusion, HER2 CAR T cells could be detected by qPCR in all patients with peak timing of 3 hours in 15 of 17 patients. In the remaining 2 patients, peak timing was found to be one week and two weeks respectively. Blood levels declined every month and 2 cases remained positive at 12 months. However, none were positive at 18 months. These findings demonstrated HER2 CAR T cells did not expand after administration but persisted for 1 year. An important point of this study was to utilize CAR-modified T cells which were specific for adenovirus, cytomegalovirus (CMV), or Epstein–Barr virus (EBV). The CAR T cells were generated from virus-specific T cells and potentially provided a co-stimulatory effect under latent virus antigen recognition (34, 35). The major concern of HER2 CAR T cell is risk of on-target off-tumor toxicity because of HER2 expression in some normal tissues of vital organs (33). However, a phase I clinical trial has shown that virus-specific CAR T cells through peripheral infusion in GBM patients are safe and feasible (34), and demonstrated promising results with efficacy.

Ongoing Clinical and Preclinical Studies of CAR T Cell Therapy

CAR T cells targeting several TAs (ex. B7-H3, CD147, GD2) are under investigation in clinical trials currently (**Table 1**). B7-Homolog 3 (B7-H3, as known as CD 276) and PD-L1 (B7-H1) are among the B7 family of immune checkpoint molecules (36). B7-H3 is not only highly expressed on tumor cells in most types of solid cancer (37), but also expressed in vessels and fibroblasts within tumors. This implies that B7-H3 CAR T cells can eliminate tumor cells through direct targeting, disrupt stroma, and inhibit neo-angiogenesis, as well (38, 39). CD147, a 57-kilodalton (kDa) type-I transmembrane protein, is one of the immunoglobulin superfamily of adhesion molecules. It induces metalloproteinases-1, -2, -3, -9, -14, and -15 released by fibroblasts, and further results in extracellular matrix (ECM) degradation, tumor progression, invasion and metastasis (40). CD147 is highly expressed in GBM, associated with poor prognosis in patients, compared to normal brain (41, 42). Disialoganglioside (GD2) is also highly expressed on several cancers including neuroblastoma, retinoblastoma, and melanoma (43). GD2 is an attractive target TA for GBM therapy with high expression demonstrated on GBM cell lines and patient samples (44). There are several ongoing clinical trials of CAR T cell therapy to B7-H3, CD147, and GD2. For B7-H3, a randomized, parallel-arm, phase I/II study (NCT04077866) is in progress to evaluate the safety and efficacy in patients of refractory or recurrent GBM between cycles of temozolomide (TMZ). For CD147, a single-center, single-arm, open label and dose escalation clinical study (NCT04045847) in patients with recurrent GBM is in progress. For GD2, a phase I clinical study (NCT04099797) of CAR T expressing GD2 for treatment of patients with GD2 expressing brain tumors, such as high-grade glioma (HGG) including GBM, diffuse intrinsic pontine glioma (DIPG), medulloblastoma, or other rare brain cancers, is underway.

Recently, several molecules have been investigated as CAR Targeting T cell therapy in preclinical glioma models (**Table 2**). The results are promising, however, the safety concerns and on-target off-tumor toxicity still require further preclinical investigation and clinical trials to determine their severity.

Pediatric Brain Tumor

Pediatric brain cancers remain among the leading causes of cancer-related death in children and thus necessitate urgent development of new therapies (51). CAR T cell therapy represents a promising approach in pediatric brain tumors as they can theoretically be specifically directed to tumor cells with limited cytotoxicity in normal tissues.

Medulloblastoma

Medulloblastoma is malignant brain tumor with highest prevalence in the pediatric population. The incidence rate is approximate 6.0 per million in 1 to 9-year-old patients (59). This tumor develops in the cerebellar vermis and thus are common posterior fossa tumors (60). Based on 2021 WHO classification, medulloblastoma is categorized into four groups based on its distinct molecular subtypes, including WNT-activated, SHH-activated and TP53-wildtype, SHH-activated and TP53-mutant, and non-WNT/non-SHH (61). The classification also directs potential therapeutic targets. The prognosis of medulloblastoma is related with

TABLE 1 | Ongoing clinical trials of CAR-T therapy for glioma.

Molecular target	Clinical trial title	Study phase	CAR-T cell dosage and combination regimen	Sponsor/site
B7-H3	B7-H3 CAR-T for Recurrent or Refractory Glioblastoma	I/II	CAR T cells delivered intratumorally or intracerebroventricularly for three doses between temozolomide cycles	Second Affiliated Hospital of Zhejiang Ningbo Yinzhou People's Hospital, Huizhou Municipal Central Hospital, BoYuan RunSheng Pharma (Hangzhou) Co., Ltd. (China)]
CD147	CD147-CART Cells in Patients With Recurrent Malignant Glioma	I	CAR T cells injected into tumor cavity once a week for three weeks	Xijing Hospital
GD2	C7R-GD2.CART Cells for Patients with GD2-expressing Brain Tumors (GAIL-B)	I	CAR T cells (1x10^{7} -1x10^{8}) delivered *via* intravenous administration with or without lymphodepletion chemotherapy	Baylor College of Medicine (Center for Cell and Gene Therapy, Baylor College of Medicine)

TABLE 2 | Preclinical study of CAR-T therapy for glioma.

Molecular target	Characteristics	Studies
CAIX (Carbonic anhydrases IX)	• induced under hypoxic conditions • overexpressed in solid tumors including GBMs (45)	• CAR T cells induced cytotoxicity in GBM cells with survival benefit in mice (46)
CD70	• type II transmembrane protein binding to CD27 • expressed on activated T cells and mature DCs • expressed on certain solid tumors including GBMs • constitutive CD70 expression on GBM cells cause an immune escape by promotion of T cell death (47)	• CAR T cells target and lead CD70$^+$ GBM cells to death *in vitro*, and no toxicity in xenograft and syngeneic models (48) • effective in glioma and head and neck cancer by CD70-specific CAR T cells (48)
CSPG4 (Chondroitin Sulfate Proteoglycan 4)	• related with cell proliferation/migration *in vitro*, and metastatic spread *in vivo* • expression level correlated inversely with survival period in glioma patients (49)	• highly expressed in GBM tissue and tumor associated vessels, without detection in healthy brain tissues (50) • CAR T cells with intracranial delivery could inhibit tumor progression in orthotopic GBM neurosphere xenograft models (50)
EphA2 (Erythropoietin-producing hepatocellular carcinoma A2)	• Eph family of receptor tyrosine kinases (RTKs) • correlated with tumorigenesis, invasion, angiogenesis and metastasis (44, 51)	• kill differentiated GBM cells and GBM cancer stem-like cells *in vitro* with survival benefit in orthotopic xenograft SCID mouse models (52) • Great anti-glioma activity (53)
TROP2 (Trophoblast cell surface antigen 2)	• 36 kDa transmembrane glycoprotein • highly expression over certain solid tumors (54, 55), • a stem cell marker (56) • high TROP2 expression on GBM cells, however, low expression on normal brain parechyma (57)	• highly expressed in breast, pancreas and prostate cancer cells (58) • the recognition and elimination of GBM cells by CAR T cells is under investigation.

histology and molecular diagnosis, metastatic status, and age (61, 62). Thus far, the current standard of care consists of surgery, chemotherapy, and craniospinal irradiation. CAR T cell therapy provides an alternative strategy for treating medulloblastoma, with several target antigens currently under investigation.

HER2 expression can be estimated approximately 40% of medulloblastomas (63) but there is no expression in normal brain tissues (64). Therefore, it is likely an ideal target antigen for CAR T cell therapy for medulloblastoma. First generation CAR T cells targeting HER2 (without co-stimulatory domain) demonstrated good tumor targeting and tumor regression in orthotopic xenogeneic medulloblastoma mice model (65). Second generation CAR T cells (with 4-1BB co-stimulatory domain) exhibit increased T cell activation and down-regulate T cell exhaustion with improved persistence of CAR T cells (66, 67). B7-H3 CAR T cells also demonstrated benefit in xenograft models of medulloblastoma, pediatric osteosarcoma, and Ewing sarcoma (68). Due to heterogeneity of antigen expression, multivalent CAR T cell targeting EPHA2, HER2, and IL13Rα2 were created, showing positive results in preclinical models of recurrent medulloblastoma and GBM (69, 70).

Pediatric Ependymoma

Ependymomas are the third most common (about 5.2%) in pediatric brain tumors (30). Ependymomas are classified into molecular groups based on pathological histology, molecular features, and anatomic site (supratentorial, posterior fossa, and spinal compartments) (61). Posterior fossa ependymomas are categorized into 2 different groups, group A (PFA) and group B (PFB), each having distinct characteristics, epigenetics, and prognosis. PFA tumors are typically only diagnosed in infants, while PFB tumors are found equally in adults and adolescents (71). PFA patients suffer from higher recurrence after treatment and worse overall survival (72). Standard of care includes surgical resection and radiotherapy. Aggressive gross total resection is important to prevent recurrence, but it is sometimes difficult due to local infiltration. The 5-year overall survival is as low as 37% for recurrent tumors (73). It is clear that new strategies, including immunotherapy, are needed for treatment of ependymoma. EphA2, IL13Rα2, HER2, and Survivin molecules are expressed specifically in ependymomas (74, 75), and have been shown potentially target for CAR T cell therapy. Trivalent CAR T cells targeting to HER2, IL13Rα2, and EphA2 demonstrated efficacy in xenograft ependymoma models (69).

Pediatric High-Grade Gliomas

Pediatric High-Grade Gliomas (pHGGs) make up less than 20% of pediatric brain tumors. Based on 2021 WHO classification, they are classified to four types, including Diffuse midline glioma (DMG) H3 K27-altered, Diffuse hemispheric glioma H3 G34-mutant, Diffuse pediatric-type high-grade glioma H3-wildtype and IDH-wildtype, and infant-type hemispheric glioma (61). DMG H3 K27-altered arise in midline regions such as thalamus, brainstem, and spinal cord, lending to their inoperability. Thus far, no standard therapy for DMG has been proven to be beneficial, though radiotherapy, targeted chemotherapy, and several strategies with mechanism of cell cycle inhibitor or anti-angiogenesis are treatment options (76–80). CAR T cell therapy for pHGGs is emerging as a result of translational research from adult GBM. In preclinical models of H3K27M-mutated DMG, GD2 CAR T cells intravenous (IV) administration could cleared most of engrafted tumors (81). Autologous GD2 CAR T cells for H3K27M$^+$ DMG is ongoing as a phase I clinical trial (NCT04196413). GD2 CAR T cells at dose level 1 (1 million cells/kg IV) demonstrated not only improved or subsided neurological deficits and improved radiographic images, but also no evidence of on-target off-tumor toxicity. In this clinical trial, CAR T cells could be detected in CSF and blood, demonstrating successful trafficking to the CNS (82). In addition, B7-H3 also serves as a target for CAR T cells in clinical trials (NCT04185038, NCT0409979) due to high expression in DMG. CAR T cells targeting GD2 investigated in brain tumors, including neuroblastoma, proved to be well tolerated (83). B7-H3

CAR T cells significantly improved survival in preclinical medulloblastoma and DIPG xenograft mice models (68). Due to the heterogeneity of glioma, multivalent CAR T cell therapy designed to prevent antigen escape in pHGGs is of considerable benefit (84).

FOCUSED ULTRASOUND TO MEDIATE CAR T FUNCTION

The most common complication of CAR T cell therapy is CRS, which usually occurs 1-2 weeks after initial administration (85, 86). Large-scale activation of CAR T cells leads to excessive inflammatory cytokines release, subsequently resulting in hypotension, fever, tachycardia, and even death from multiple organ failure (87). Administration of CAR T cell into the brain also introduces potential risk of neurotoxicity. Therefore, in addition to selection of optimal antigens for specific CAR T binding, mediating the functions of CAR T cells is another potential way to decrease toxicity of CAR T cell therapy.

Recently, focused ultrasound (FUS) has proven to be an innovative tool widely applied clinically to tumor thermo-ablation, brain-blood barrier opening with microbubbles for enhanced drug delivery, neuromodulation, and transgene expression (88–92). Notably, a FUS-based approach utilized acoustogenetics technology to activate CAR T cells with high precision control at the confined location of solid tumors (92) by transducing ultrasound signals into cellular activations and even genetic activation for therapeutic practices. This technique can decrease on-target off-tumor toxicity of CAR T cell therapy. Through the heat generated by short pulses of FUS, heat-induced reporter genes can be activated with high efficiency. In this study, the Cre-mediated gene was employed as a switch to deliver outputs of genetic activities from FUS inputs. Based on this mechanism, heat-inducible CAR expression and further functional reaction were proved *in vitro* Jurkat and primary T cells. Furthermore, MRI-guided FUS was utilized to induce gene activation *in vivo*. The FUS-inducible cytotoxicity of engineered CAR T cell has been shown significant tumor regression but significant less cytotoxicity in non-FUS-treated sites. Once CAR T cells leave the tumor site without further FUS stimulation, they will lose their CAR expression gradually. This leads to less on-target off-tumor toxicity of FUS-CAR-T-cells than with standard CAR T cells. Thus, this modular acoustogenetic approach with switchable target CAR genes can aimed at different cancers. Acoustogenetics, with advantages of direct and non-invasive control, may provide a broadly applicable method for genetically engineered cell therapeutics.

STRATEGIES TO ENHANCE CAR T CELLS THERAPY

Although CAR T cell therapy has showed remarkable clinical response toward CD19 hematological malignancies, the benefit for solid tumors has been modest due to several challenges, such as insufficient trafficking to the tumors, defective recognition of the targeted TA, on-target off-tumor toxicity due to expression of the targeted TA in normal tissues, limited persistence and low proliferation in the TME, and the immunosuppressive TME. For CNS malignancies, penetration through BBB into tumor sites represents an additional hurdle. The BBB is a physiological barrier consisted of specialized endothelial cells joined by tight junction, with pericytes and astrocytes forming additional hurdles. Systemic administration of CAR T cells have shown limited accumulation in CNS tumors (79), necessitating further innovation in the delivery of CAR T cells to treat GBM. In several preclinical models of brain tumors, locoregional administration of CAR T cells increased T cell infiltration in tumor site with better tumor control (93–95). The findings support intratumoral or intracavitary injection of CAR T cells into the tumor or the resected cavity of tumor, or intracerebral/intraventricular injection into the brain parenchyma or cerebral ventricle, as viable strategies to mediate historical limitations.

Alternatively, enhancing the function of CAR T cell constructs and targeting multiple TAs may also mitigate barriers to effective treatment (**Figure 2**). The strength and potency of CAR T antitumor activity has been already enhanced in previous studies *via* addition of costimulatory domains and functional moieties. Further engineering of the CAR to induce or secrete cytokines could additionally increase activity and persistence of CAR T cells (96). To enhance T cell trafficking into tumor site, engineered CAR T cells can also express chemokine receptors. For example, CD70-specific CAR T cells with CXCR1 and CXCR2 modification have demonstrated improvement of T cell trafficking and efficacy in tumor control in preclinical models of GBM (97). Additionally, disruption of immune checkpoint signaling within CAR T cells has been investigated. CAR T cells were engineered to release PD-L1 antibody (98) or to knock down PD-1 and Lag3 genes by CRISPR/Cas9 technology (99, 100). In addition, the hypoxia transcription factor HIF-1a subdomain can be incorporated in a CAR construct to reduce on-target off tumor toxicity, ensuring CAR T cells only activate under hypoxic conditions such as within the TME (101).

TA expression is frequently heterogeneous on many solid tumors, including GBMs, thus tumors may escape CAR T cell recognition and elimination due to antigen escape with increased risk of recurrence (79). In order to overcome antigen escape, CAR T cells targeting multiple TAs were developed (102). Trivalent CAR T cells targeting HER2, IL13Rα2, and EphA2 could cover more TAs and have showed significant survival benefit in mice bearing patient-derived GBM xenografts (70).

Combination therapy may also improve CAR T cell therapeutic viability in GBM (**Figure 2**). Radiotherapy and chemotherapy are usually standard therapeutic options for solid tumors and could be theoretically combined with CAR T cell therapy to target multiple mechanisms of oncogenesis. Radiotherapy can shape the TME to boost CAR T cell efficacy. First, radiation facilitates cytotoxic $CD8^+$ T cells to recognize and eliminate cancer cells by increasing the expression of TAs (48). Then, tumor necrosis and apoptosis caused by radiation release more danger signals, and further increase infiltration of immune

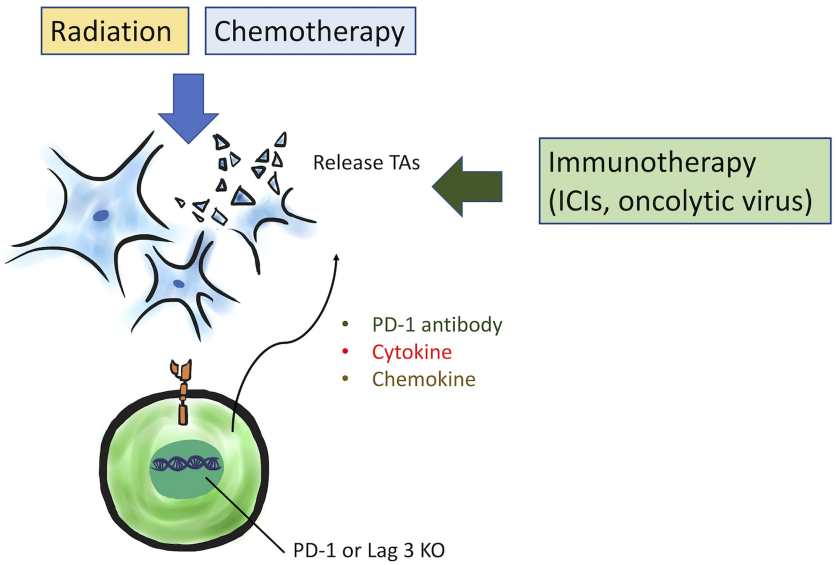

FIGURE 2 | Several strategies to enhance CAR T cell functions are under investigation, including expression of cytokine and chemokine, induction of PD-1 antibody release, or PD-1 and Lag3 genes knockdown by CRISPR/Cas9 technology. Additionally, combination therapy with radiation or chemotherapy can cause tumor necrosis or apoptosis to increase TAs. Immunotherapy, such as ICIs and oncolytic viruses, could overcome the immunosuppressive TME to increase CAR T cell efficacy synergistically.

cells in TME *via* release of proinflammatory cytokines (IFNγ) or chemokine ligands (103, 104). Therefore, combination with radiotherapy demonstrated improved efficacy of CAR T cell therapy in some solid tumors including GBMs (48, 105, 106). Chemotherapy also has a similar ability to shape the TME to enhance CAR T cell efficacy by upregulation of TAs (107) and elimination of immunosuppressive cells (108). Exploration into suitable combinations of chemotherapy and CAR T cell therapy is underway.

Immunotherapy is another exciting candidate for combination therapy with CAR T cells, such as immune checkpoint inhibitors (ICI) or oncolytic viruses. Anti-PD-1/PD-L1 and anti-CTLA4 antibodies have demonstrated to increase CAR T cell efficacy in preclinical models (109). A clinical trial of IL13Rα2 CAR T cells with nivolumab (anti-PD-1) and ipilimumab (anti-CTLA4) showed synergic effect for recurrent GBMs (NCT04003649). Oncolytic viruses can cause immunogenic cell death with induction of a type I IFN response in the TME, and systemic innate and adaptive immune responses are activated consequently (110). The response in the TME to oncolytic viral therapy is promising as a concurrent enhancement of CAR T cell activity (111). For example, IL-7-loaded oncolytic adenovirus (oAD-IL7) combined with B7-H3 CAR T cells for the preclinical GBMs mice model is under investigation and has shown synergic survival benefit with tumor regression (112).

CONCLUSION

CAR T cell therapy is a promising strategy for treatment of solid tumors. TA selection is important to target cancer cells specifically with preservation of normal tissues, with minimal on-target/off-tumor toxicity. CAR T cells as a monotherapy in clinical trials against various solid tumors have proven non-efficacious due in large part to immune escape (113). Combinatorial strategies with radiotherapy, chemotherapy, and other immunotherapies are promising to overcome the limitations of the immunosuppressive TME, while further investigation is warranted to optimize CAR T cell therapy for solid tumors.

AUTHOR CONTRIBUTIONS

Y-JL, LM, and ML wrote and revised the manuscript. Y-JL drew the figures. ML initiated the concept and supervised the writing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Special Chimeric Antigen Receptor (CAR) Modifications of T Cells: A Review

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Chimeric antigen receptor (CAR) -T cell therapy has become one of the hot topics in tumor immunity research in recent years. Although CAR-T cell therapy is highly effective in treating hematological malignancies, there are numerous obstacles that prevent CAR-T cells from having anti-tumor effects. Traditional CARs, from the first to the fourth generation, are incapable of completely overcoming these challenges. Therefore, identifying ways to boost the efficacy of CAR-T cells by utilizing the limited tumor surface antigens has become an urgent area of research. Certain special CARs that have special structures, special systems, or are greatly improved on the basis of traditional CARs, such as tandem CAR, dual-signaling CARs, AND-gate CARs, inhibitory CAR, AND-NOT CARs, CARs with three scFvs, ON/OFF-switch CARs, and universal CARs have been introduced. This study aims to use these special CARs to improve the anti-tumor ability, accuracy, and safety of CAR-T cells. In addition to summarizing various special CARs of T cells, this paper also expounds some of our own conjectures, aiming to provide reference and inspiration for CARs researchers.

Keywords: special CARs, improve, efficacy, safety, conjectures

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INTRODUCTION

CAR-T cell therapy is a type of tumor immunotherapy that has developed rapidly in recent years. Currently, CAR-T cell therapy is mainly used to treat hematological malignancies, and it has had considerable success. However, the biological characteristics of solid tumors are more complex, posing many obstacles to CAR-T cells (1–4), including CAR-T cells homing barriers, tumor microenvironment (TME) inhibition, CAR-T cells trogocytosis (5), tumor antigen heterogeneity (6), CAR-T cells toxic responses, among others. These obstacles inevitably influence the anti-tumor effects of CAR-T cells in solid tumors. At present, CAR has developed from the first generation to the fifth generation. The intracellular structure of first-generation CARs only has one signal structure domain. The second-generation CARs add one co-stimulatory molecule to the first-generation CAR. The third-generation CARs add 2 costimulatory molecules. The fourth-generation CARs are modified by adding the cytokine inducer or suicide genes based on the second-generation or the third-generation CARs. The fifth-generation CARs added a "third party" intermediate system in the extracellular domain. None of the traditional CARs, from the first-generation to the fourth-generation, can completely overcome the aforementioned obstacles. Nowadays, in addition to

searching for tumor-specific antigens (TSAs), constructing new special CARs or optimizing traditional CARs to minimize their toxicity and kill tumor cells more efficiently has become the main research focus of CAR-T cell therapy. This review will be elaborated from the following four aspects, including optimizing the recognition ability of CARs, improving the accuracy of CARs, improving the killing ability of CARs and improving the safety of CARs.

OPTIMIZE THE RECOGNITION ABILITY OF CARS

Double scFvs

Double scFvs are designed with two corresponding scFvs for two different tumor surface antigens. Examples of CARs that used double scFvs include "tandem CARs", "dual-signaling CARs", "AND-gate CARs", and "inhibitory CARs".

The Tandem CAR (TanCAR)

TanCAR adopts a design concept of the "OR" gate. Two different scFvs are connected in the extracellular domain of a CAR. Grada et al. (7) constructed TanCAR using gene-editing technology to connect two different series of scFvs to a single transgenic receptor. The two scFvs of TanCAR were connected outside the cell (in series) by a Gly-Ser linker and had good flexibility (**Figure 1A**) . The TanCAR, which is activated when any one of the scFvs binds to a target antigen, can enable a CAR-T cell to synchronously recognize two types of tumor surface antigens. When two scFvs bind to their respective target antigens, the TanCAR will not only be activated but will also produce synergistic effects to further improve the activation of CAR-T cells and their tumor killing ability (7–9). The synergistic effect may be caused by binding two or more antigens simultaneously, which may enhance the signal transduction of immune synapses (10). When compared to traditional CARs with only one scFv, TanCAR-T cells have a higher anti-tumor effect and can limit tumor cell immune evasion (10–13).

A plasmid is very small, which makes it impossible to add gene elements to it without restriction. Therefore, Grada et al. (7) constructed the TanCAR based on a second-generation CAR, whereas Zhao et al. (14) successfully constructed a TanCAR using human trophoblast cell surface antigen 2 (Trop 2) and programmed death-ligand 1 (PD-L1) as targets, based on the third-generation CAR (**Figure 1B**). Preclinical experiments revealed that (Trop2/PD-L1)-CAR-T cells significantly enhanced the killing effect on gastric cancer cells when compared to Trop2-CAR-T cells or PD-L1-CAR-T cells.

Conjectures

A TanCAR constructed based on the fourth-generation CAR, i.e. a TanCAR that has the function of a fourth generation CAR, can

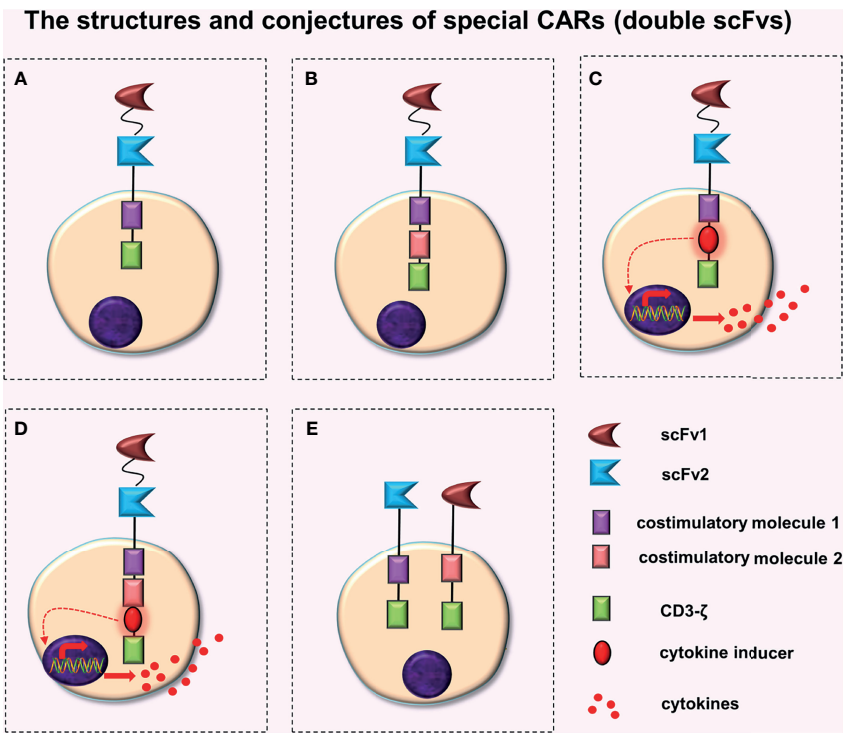

FIGURE 1 | The structures and conjectures of special CARs (double scFvs). Construction of a TanCAR based on the **(A)** second-generation CAR, **(B)** third-generation CAR, **(C)** fourth-generation CAR (the intracellular domain contains a costimulatory molecule), and **(D)** fourth-generation CAR (the intracellular domain contains two costimulatory molecules). **(E)** Construction of the dual-signaling CARs based on the second-generation CAR.

secrete additional anti-tumor cytokines when activated. Extracellular domains of these CARs have an extra scFv compared to traditional fourth-generation CARs (**Figures 1C, D**). These two designs (**Figures 1C, D**) will further complicate plasmid construction. These designs may increase the antigen recognition range of CAR-T cells and may produce synergistic effects (7–9), which will enhance the anti-tumor activity of CAR-T cells. Although no relevant literature has been retrieved, the structure and principle of these two CAR-T cells are relatively simple, indicating that they may already exist or are being developed.

Dual-Signaling CARs

Dual-signaling CARs refer to the expression of two separate CARs on the same T cell respectively and simultaneously, with each CAR cell having its own intracellular domains (**Figure 1E**). Ruella et al. (15) constructed dual-signaling CARs (targeting CD19 and CD123) by first creating two kinds of plasmids (CD19-CAR and CD123-CAR) respectively, and then transfecting the same T cell successively with lentivirus one by one. Finally, dual-signaling CAR-T cells, which express two types of CARs simultaneously, were screened out. Preclinical trials indicated that dual-signaling CAR-T cells had stronger anti-tumor activity than single-expression of CAR-T cells (CD19-CAR-T cells or CD123-CAR-T cells) or the mixed combination of CAR-T cells (CD19-CAR-T cells and CD123-CAR-T cells), and could better prevent disease recurrence caused by downregulation or loss of target antigens on the tumor cell surface.

Triple scFvs (Arranged in Tandem)

The antigen binding domain of CAR is composed of three scFvs in tandem (**Figure 2A**). Bielamowicz et al. (16) created a trivalent-tandem CAR with a single universal tricistronic transgene to treat glioblastoma. The trivalent-tandem CAR could target and recognize three different TAAs on the surface of glioblastoma cells, including human epidermal growth factor receptor 2 (HER2), interleukin-13 receptor subunit alpha-2 (IL13Rα2), and ephrin-A2 (EphA2). The results showed that trivalent-tandem CAR-T cells have greater anti-tumor activity and can overcome tumor antigen heterogeneity than nonspecific or bispecific CAR-T cells. Balakrishnan et al. (17) also conducted a similar study, constructing a trivalent-tandem CAR by connecting three scFvs in tandem with designed ankyrin repeat proteins (DARPins). The results demonstrated that these CAR-T cells have potent anti-tumor effects and can better cope with tumor antigen heterogeneity and immune escape.

The trivalent-tandem CARs were constructed by Bielamowicz et al. (16) and Balakrishnan et al. (17) using intracellular

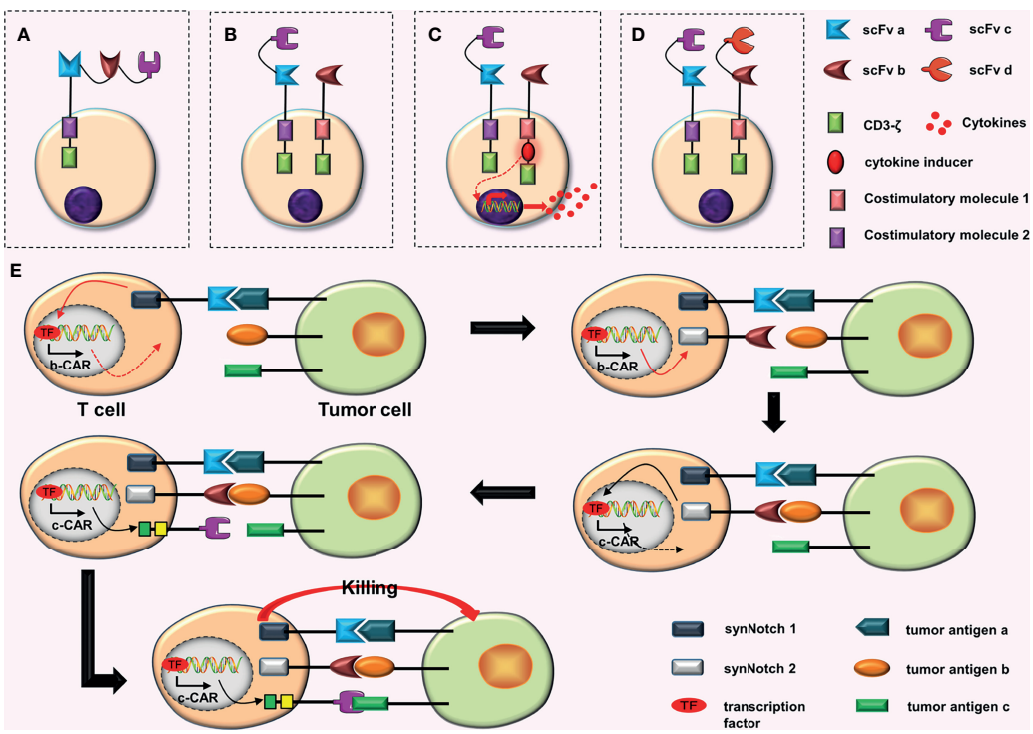

FIGURE 2 | Some conjectures about CARs containing three scFvs. **(A)** Connecting three scFvs in tandem. **(B)** In dual-signaling CARs, one CAR is replaced by a TanCAR. **(C)** One of the dual-signaling CARs is replaced with a TanCAR, and the other is upgraded to a fourth-generation CAR. **(D)** Both CARs in dual-signaling CAR are replaced with tandem CARs. **(E)** At first, synNotch 1 receptor is activated after binding to tumor antigen (a), and the activated synNotch 1 receptor induces the expression of synNotch 2 receptor. Then synNotch 2 receptor binds to tumor antigen (b), which induces the expression of c-CAR; c-CAR can bind to the tumor antigen (c); finally, the CAR-T cell is activated.

costimulatory molecules CD28 and 4-1BB, respectively, based on second-generation CARs. Because it has been extremely difficult to build a trivalent-tandem CAR, making additional improvements based on it, such as adding a costimulatory molecule or constructing a tetravalent-tandem CAR, is more challenging. The vector volume is also extremely high, hindering the transmission of efficient genes, and the subsequent transfection rate may be very low. Furthermore, determining whether the genes can be successfully translated into proteins is a great challenge.

Conjectures

The following three combinations can be used in combining dual-signaling CARs with the TanCAR.

a. Replacing one of the CARs in dual-signaling CARs with a TanCAR (**Figure 2B**). This design will increase the recognition range of CAR-T cells for targets to three different TAAs.
b. Replacing one CAR among the dual-signaling CARs with a TanCAR, and upgrading the other to a fourth-generation CAR (**Figure 2C**). This design will increase the recognition range of CAR-T cells for targets, which can recognize three different TAAs. Furthermore, the inclusion of four-generation CAR may further enhance anti-tumor effects of CAR-T cells.
c. Replacing both CARs in dual-signaling CAR with TanCARs (**Figure 2D**). This design will further increase the recognition range of CAR-T cells for targets, resulting in the recognition of four different TAAs.

The advantage of these CAR-T cells is that they can be activated when one scFv binds to the corresponding tumor antigen. When all scFvs bind to the corresponding tumor antigens, they may have synergistic effects, enhancing the anti-tumor ability of CAR-T cells (7–9). Moreover, certain designs, such as trivalent-tandem CARs, can effectively cope with tumor antigen heterogeneity and immune escape (11, 17–19).

IMPROVING THE ACCURACY OF CARS

AND-gate CARs

AND-gate CARs adopt the "AND" gate design concept, and can only be activated when two scFvs bind to corresponding tumor antigens simultaneously. Roybal et al. (20) invented the synNotch receptor (a novel modular receptor), which is the core design of AND-gate CARs. The synNotch receptor binds to tumor antigen (a), inducing the expression of b-CAR, which selectively binds to tumor antigen (b). Finally, the T cell is activated (**Figure 3A**). SynNotch AND-gate T cells can only kill tumor cells that express double target antigens. Consequently, various studies (21–24) have proved that SynNotch AND-gate T cells are considerably safe and accurate, because they are ineffective against tumor cells

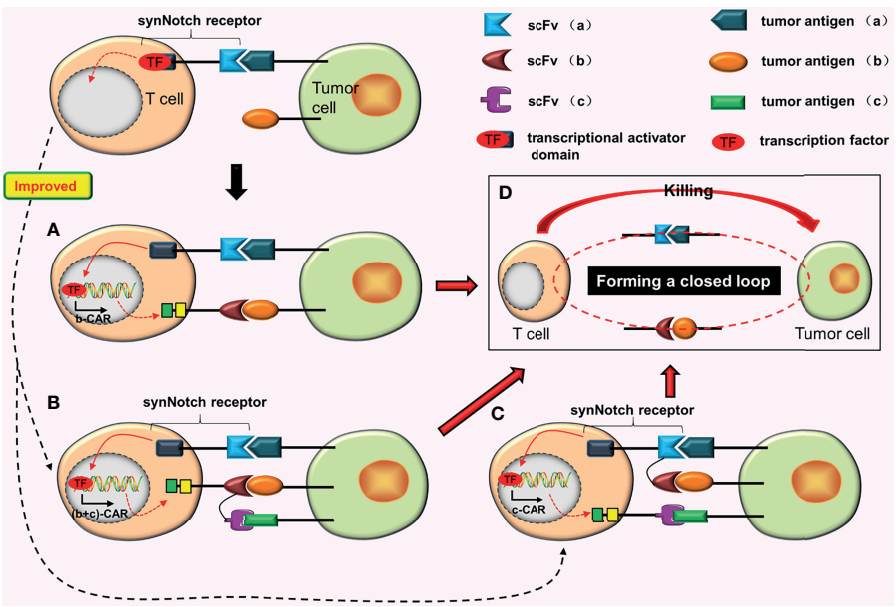

FIGURE 3 | AND gate CARs. **(A)** When the synNotch receptor binds to tumor antigen (a), the transcriptional activator domain of the receptor is released, which can enter the nucleus and drive the expression of b-CAR genes. Subsequently, b-CAR will be expressed on the surface of the T cell, and the newly expressed b-CAR specifically binds to tumor antigen (b) to form a closed loop. The CAR-T cell is finally activated. **(B)** The synNotch receptor binds to tumor antigen (a) and induces TanCAR (b + c) expression. At this point, as long as one or two scFvs of this TanCAR bind to the corresponding tumor antigens, the T cell will be activated. **(C)** Adding a TanCAR to the synNotch receptor. As long as one of the scFv or two scFvs of this TanCAR bind to the corresponding tumor antigens, the expression of c-CAR will be induced. **(D)** Once the closed loop is formed, CAR-T cells will be activated.

expressing a single antigen while efficient against tumor cells expressing double target antigens.

The purpose of synNotch AND-gate CARs is to improve the precision of CAR-T cells. To further improve the antigen recognition range and anti-tumor effects of synNotch AND-gate CAR-T cells, Sabahi et al. (25) proposed the construction of tandem AND-gate CARs by combining TanCAR with AND-gate CARs. Tandem AND-gate CARs are activated as follows: the scFv (a) on the synNotch receptor induces the tandem CAR (b + c) after binding with tumor antigen (a) and the T cell is activated soon after as long as one scFv of the TanCAR is attached to the target antigen (**Figure 3B**). Recently, Williams et al. (26) successfully constructed the tandem AND-gate CARs. The experimental results showed that these CAR-T cells not only have high accuracy, but also have the potential to increase the recognition range of CAR-T cells and improve their ability to kill tumor cells. Furthermore, they linked the synNotch receptor with a TanCAR to construct another type of AND-gate CARs (26). The formation of c-CAR is induced as long as one scFv of the TanCAR is attached to the corresponding tumor antigen (**Figure 3C**). CAR-T cells with this structure are more easily activated.

Conjectures

a. Combining dual-signaling CARs with AND-gate CARs may have two forms of construction. In the first structural form, two CARs in dual-signaling CARs add a synNotch receptor in the intracellular domain; as long as one of the CARs is attached to the target antigen, the formation of the third CAR will be induced (**Figures 4A, B**). Furthermore, two CARs combine with target antigens while inducing the formation of the third CAR (**Figure 4C**), resulting in the release of more transcription factors (TF), which may further increase the production of the third CAR.

b. The second structural form is mainly to further optimize the synNotch receptor. When the synNotch receptor is combined with the corresponding tumor antigen, dual-signaling CARs (or two independent CARs) are formed (**Figure 4D**). In theory, CARs with this structure should have equivalent anti-tumor impact as tandem AND-gate CARs, but it complicates plasmid construction due to the addition of an extra set of intracellular elements of CAR.

Triple scFvs (the In-Series Three-Input Cascade Circuit)

Williams et al. (26) constructed the more complex 3-input AND-gate CARs, which have three CARs, on the basis of the original AND-gate CARs, which have two CARs. They used the physical series circuit concept and introduced two separate synNotch receptors. These CARs are activated in a similar way to a cascade reaction. First, the combination of synNotch 1 receptor and the tumor antigen (a) induce the expression of synNotch 2 receptor.

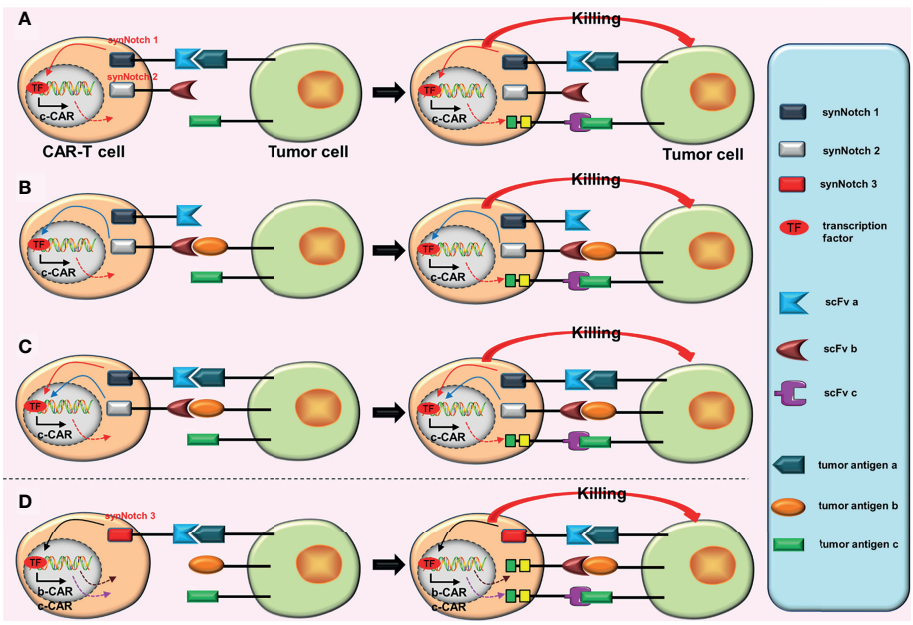

FIGURE 4 | Some conjectures on the construction of "dual-signaling CARs" combined with "AND gate CARs". **(A)** a-CAR of dual-signaling CARs binds to the corresponding tumor antigen (a), which can induce the expression of c-CAR. After c-CAR binds to tumor antigen (c), the CAR-T cell will be activated. **(B)** b-CAR of dual-signaling CARs binds to the corresponding tumor antigen (b), which can induce the expression of c-CAR. After c-CAR binds to tumor antigen (c), the CAR-T cell will be activated. **(C)** The expression of c-CAR can be induced by the combination of a-CAR and b-CAR with corresponding tumor antigens. After c-CAR binds to tumor antigen (c), the CAR-T cell will be activated. **(D)** The combination of a-CAR with corresponding tumor antigen (a) can induce the expression of b-CAR and c-CAR. As long as one of b-CAR and c-CAR binds to the corresponding tumor antigen, the CAR-T cell will be activated.

Subsequently, the expression of c-CAR is induced by the combination of synNotch 2 receptor and the tumor antigen (b). Finally, c-CAR binds to the tumor antigen (c) and the CAR-T cell is activated (26) (**Figure 2E**). *In vitro* experiments showed that (26) "the in-series three-input cascade circuit" not only improve the accuracy of CAR-T cells, but also enhance the activation and tumor-killing ability of CAR-T cells.

The activation of 3-input AND-gate CAR-T cells requires special conditions, which includes the simultaneous expression of three different tumor antigens by tumor cells. Therefore, this design greatly improves the accuracy of CAR-T cells. However, because four stages are involved in the activation of 3-input AND-gate CAR-T cells, it takes longer than other traditional CAR-T cells. Additionally, although this design improves the accuracy of CAR-T cells, it is ineffective against tumor cells that have lost the target antigen, which is a great disadvantage.

Inhibitory CARs (iCARs)

The iCAR was constructed by Fedorov et al. (27) to distinguish between tumor cells and non-tumor cells and to suppress the T cell reaction once activated. There are two kinds of CARs on the surface of iCAR-T cells: traditional CARs, which target tumor cell surface antigens, and iCARs, which target non-tumor cell surface antigens. The iCAR contains a surface antigen recognition region for non-tumor cells and an acute inhibition signal region. CAR-T cells are activated when traditional CARs bind to the target tumor antigens, killing tumor cells. However, when iCARs bind to the target non-tumor antigens, they generate inhibitory signals that inactivate traditional CARs, protecting non-tumor cells from damage (**Figure 5A**). ICARs can effectively reduce the off-target effects of CAR-T cells. However, one drawback of this design is that finding related surface antigens, which are missing or downregulated in tumor tissues but highly expressed in non-tumor tissues, is difficult.

Conjectures

The iCAR can be upgraded to tandem iCARs, which will enhance the recognition range of iCARs for non-tumor cell surface antigens and hence reduce the effects of off-target more effectively (**Figure 5B**). Furthermore, iCARs can be combined with tandem CARs to increase the range of tumor cell surface antigens recognized by CAR-T cells (**Figure 5C**).

AND-NOT CARs

To improve accuracy and tumor-killing ability of CAR-T cells while reducing toxic reactions, Williams et al. (26) constructed the OFF-Notch receptor by innovatively combining the "AND gate" with the "NOT gate". Combining OFF-Notch receptors with the corresponding tumor antigens may promote the expression of proapoptotic factor truncated BH3-interacting domain death agonist (tBID), which may eventually induce CAR-T cells apoptosis (26). The mechanism of AND-NOT CARs is as follows: synNotch receptors bind to the corresponding tumor antigens (a), inducing the expression of b-CARs; subsequently, b-CARs bind to tumor antigens (b); and finally, CAR-T cells are activated (**Figure 6A**). However, as long

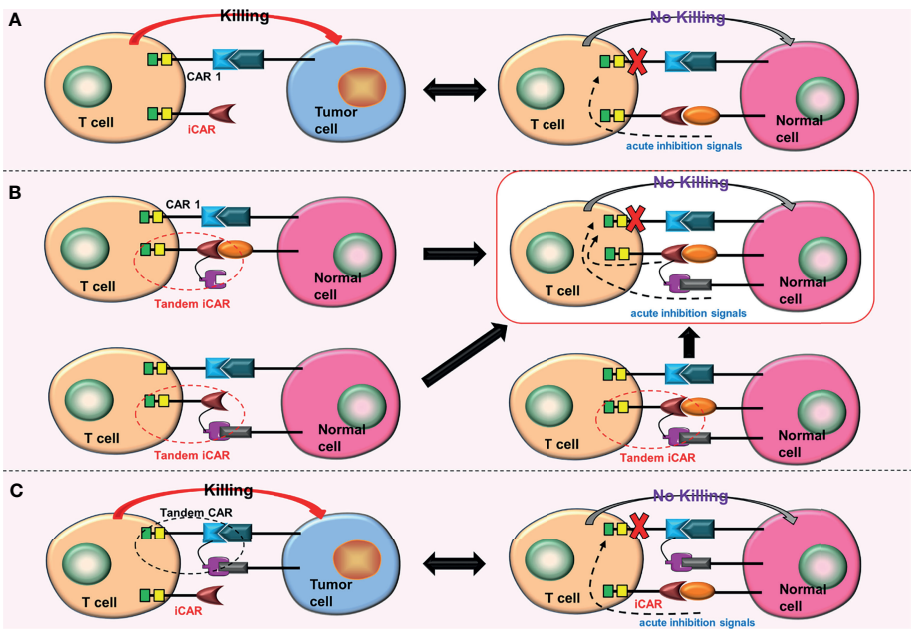

FIGURE 5 | Some conjectures about the iCARs. **(A)** When iCAR and the non-tumor cell surface antigen are combined, acute inhibitory signals are produced, inhibiting the function of CAR 1. **(B)** Replacing the iCAR with a tandem iCAR. As long as one of the scFvs binds to the corresponding non-tumor cell surface antigen, acute inhibitory signals will be generated. These acute inhibitory signals will inhibit the function of the CAR 1. **(C)** Combining an iCAR with a TanCAR. The iCAR will also inhibit the function of the TanCAR after being activated.

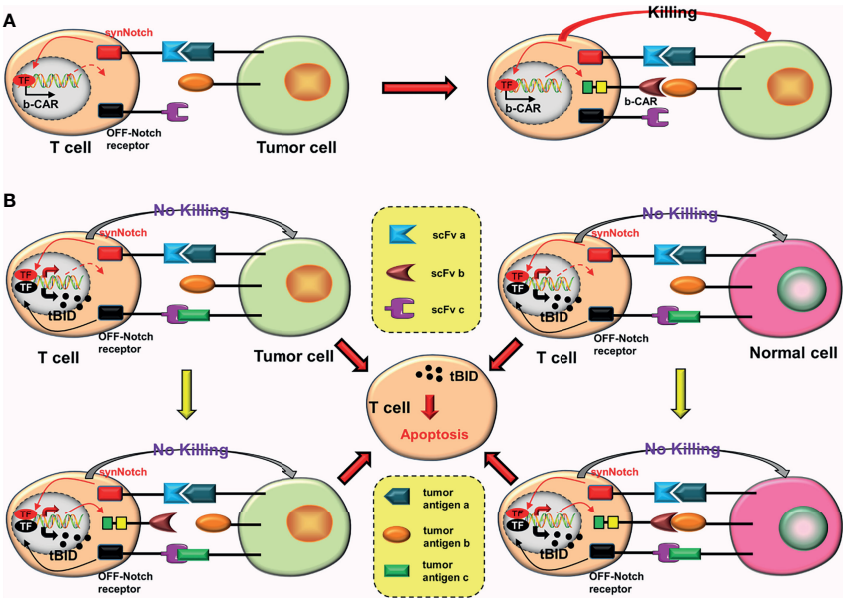

FIGURE 6 | AND-NOT CARs. **(A)** The synNotch receptor binds to the corresponding tumor antigen (a), causing the expression of b-CAR genes. Subsequently, the newly expressed b-CAR binds to the target antigen (b). Finally, the CAR-T cell is activated. **(B)** The binding of the OFF-Notch receptor to the corresponding antigen (c) induces the expression of tBID, regardless of whether b-CAR is formed and whether it binds to the corresponding tumor antigen. Apoptosis of the CAR-T cell can be induced by the tBID.

as the OFF-Notch receptors bind to the corresponding antigens (c), the expression of tBID will be induced, and eventually cause apoptosis of the CAR-T cells (**Figure 6B**).

This design is equivalent to a complex version of iCARs. When compared to the iCARs, AND-NOT CARs improve accuracy (by adding the AND-gate system) and anti-tumor effect (double targets) of CAR-T cells. However, AND-NOT CAR-T cells face a significant challenge: OFF-Notch receptors must be timely activated to promptly promote apoptosis of these effector CAR-T cells.

IMPROVING THE KILLING ABILITY OF CARS

Special CARs Based on the Traditional Fourth-Generation CARs

The intracellular domain of traditional fourth-generation CARs have only one cytokine receptor. Adach et al. (28) constructed 7 × 19 CAR-T cells that expressed IL-7 and CCL19 chemokines synchronously. When compared to the traditional fourth-generation CAR-T cells, 7 × 19 CAR-T cells not only have stronger proliferation ability, endurance, and anti-tumor ability, but also improve the ability to recruit immune cells.

Conjectures

a. As aforementioned, constructing the fourth-generation TanCARs (**Figures 1C, D**).

b. Based on AND-gate CARs, a cytokine inducer is added to the intracellular domain of the second CAR, which upgrades a second-generation CAR to a fourth-generation CAR (**Figure 7A**). Now that tandem AND-gate CARs (**Figure 3B**) can be successfully constructed, this design should also be feasible.

c. Based on tandem AND-gate CARs, a cytokine inducer is added to the intracellular domain of the second CAR, upgrading the second-generation CAR to a fourth-generation CAR (**Figures 7B, C**). Addition of a fourth-generation CAR may enhance the activation and anti-tumor effects of these CAR-T cells. Similarly, it is more difficult to construct these CAR-T cells.

CARs That Can Reverse the Inhibition of Immune Checkpoints or Tumor Microenvironment

Chimeric-Switch Receptor (CSR)

Combining programmed cell death protein-1 (PD-1) on the surface of T cells with PD-L1 on the surface of tumor cells produces inhibitory signals that prevent the activation and proliferation of T cells. Prosser et al. (29) were the first to design a novel PD-1/CD28 chimeric-switch receptor (CSR), which could reverse (rather than block) the PD-1 immunosuppression. Chen et al. (30) combined CSR with the third-generation CAR (CD28, 4-1BB) to construct cMet-PD1/CD28-CAR-T cells for treating gastric cancer. In their experiment, they combined the extracellular structure of PD-1 with the transmembrane and intracellular domains of CD28 to form CSR. The PD-1/CD28

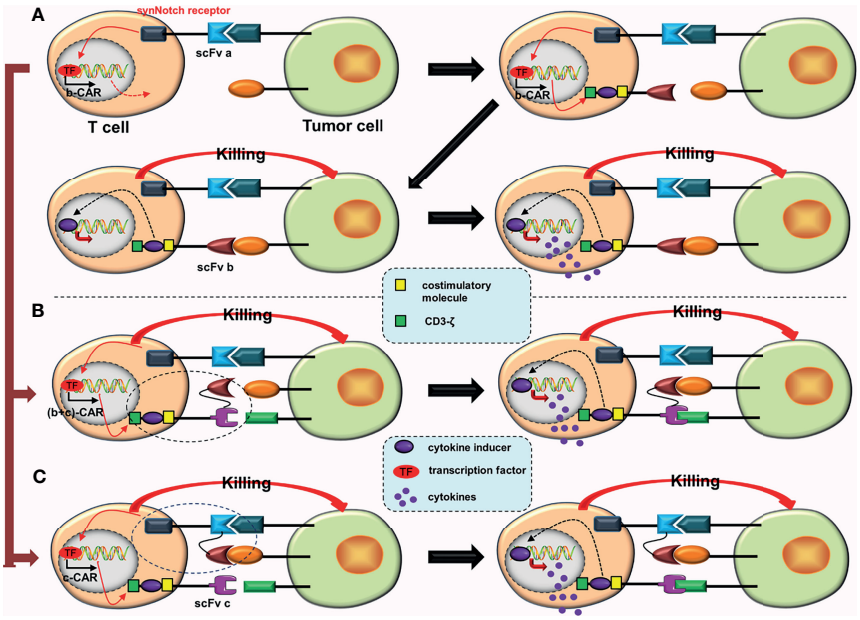

FIGURE 7 | Some conjectures about special CARs based on the fourth-generation CARs. **(A)** The SynNotch receptor binds to the corresponding tumor antigen (a), inducing the expression of b-CAR, which is a fourth-generation CAR. When activated, the CAR-T cell will release extra cytokines. **(B)** The SynNotch receptor binds to the corresponding tumor antigen (a), which induces the expression of (b + c)-CAR (a tanCAR based on the fourth-generation CAR). When activated, the CAR-T cell releases extra cytokines. **(C)** The SynNotch receptor has two scFvs connected in tandem, and as long as one scFv binds to the corresponding tumor antigen, it will induce the expression of c-CAR (a fourth generation CAR); when activated, the CAR-T cell will also release extra cytokines.

CSR could convert the immunosuppression transmitted by PD-1 into activation signals in cells (**Figure 8A**). Preclinical experiments revealed that cMet-PD1/CD28-CAR-T cells had higher anti-tumor effects and safety than traditional c-Met CAR-T cells (30).

Although the cMet-PD1/CD28-CAR-T cells belong to the third-generation CAR-T cells group in principle, they are better third-generation CAR-T cells. Despite the fact that the cMet-PD1/CD28-CAR has only one scFv, it can simultaneously have another target (PD-1). Therefore, the function and anti-tumor effect of this CAR may be equivalent to that of a TanCAR.

Conjectures

a. Adding other immune checkpoints. More inhibitory signals can be reversed by combining the extracellular structures of two different immune checkpoints with intracellular CD28.

b. Replacing the intracellular 4-1BB of the cMet-PD1/CD28 CAR with a cytokine receptor (upgrading from the third-generation CAR to the fourth-generation CAR). Although the total number of components of this CAR remains unchanged when compared to cMet-PD1/CD28-CAR, this type of CAR-T cells can also release some anti-tumor cytokines.

Inverted Cytokine Receptor (ICR)

The tumor microenvironment (TME) is rich in IL-4. It has been proven that IL-4 promotes tumor growth while also protecting tumor cells from autoimmune destruction (31). To reverse the inhibitory effect of IL-4, Leen et al. (32) innovatively constructed the IL4/7 chimeric receptor (IL4/7 ChR), which is also known as IL4/7 inverted cytokine receptor (IL-4/7 ICR), by fusing the extracellular domain of the IL-4 receptor with the intracellular domain of the IL-7 receptor. The downstream signal generated by the combination of IL-4/7 ICR and IL-4 would eventually be sent out through the intracellular domain of the IL-7 receptor, where it would be converted into an activation signal. The results showed that IL-4/7 ICR could reverse the inhibitory effect of IL-4, and enhance the persistence and anti-tumor activity of T cells (maintaining Th1 phenotype). Mohammed et al. (33) used IL-4/7 ICR to construct CAR-T cells that target prostate stem cell antigen (PSCA) for treating pancreatic cancer. The preclinical study showed that these CAR-T cells could not only survive in IL-4-rich TME, but that IL-4 could also boost their activity and anti-tumor ability (**Figure 8B**). Wang et al. (34) constructed the IL-4/21 ICR-CAR-T cells, which were shown to be potentially safer than IL-4/7 ICR-CAR-T cells. Moreover, IL-4/21 ICR-CAR-T cells could only be activated when IL-4 and target antigen coexisted.

These two designs enable CAR-T cells to play a potent anti-tumor role in the IL-4-rich TME. Although both designs can reverse inhibitory signals, there are essential differences between them. The PD-1/CD28 CSR reverses inhibitory signals transmitted by tumor cells, whereas the IL-4/7 ICR reverses inhibitory effects of IL-4.

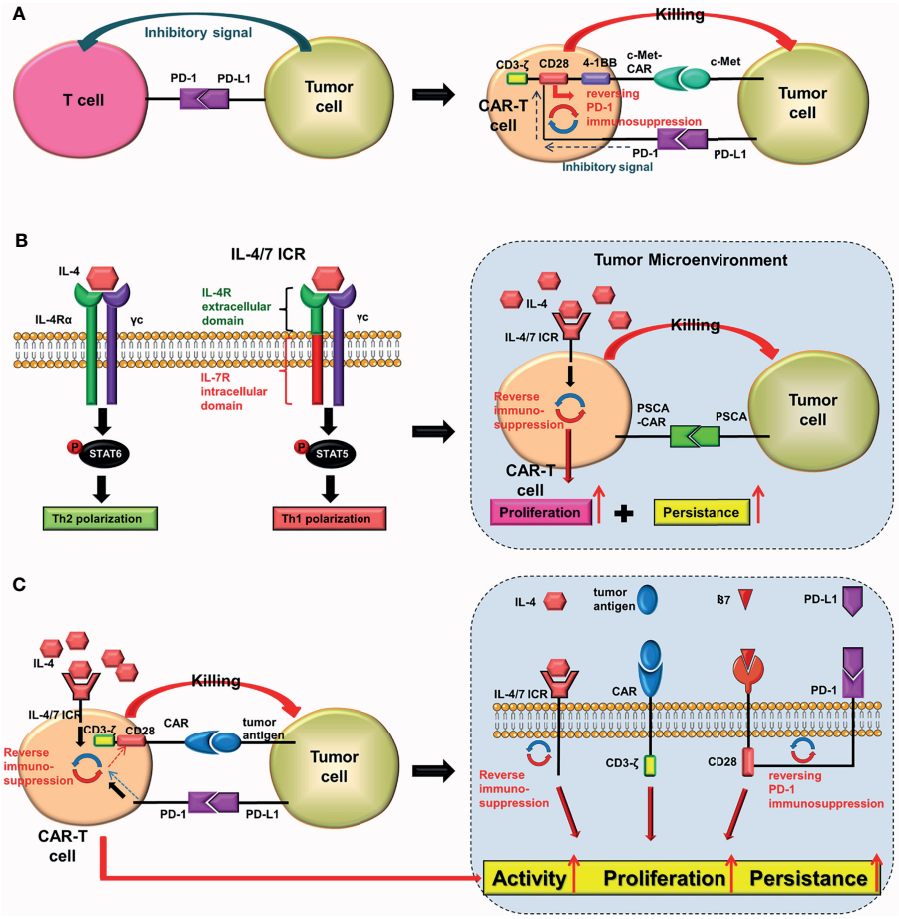

FIGURE 8 | PD-1/CD28 CSR and IL-4/7 ICR. In T cells: **(A)** the PD-1/CD28 CSR converts inhibitory signals delivered by PD-1 into activation signals, whereas **(B)** the IL-4/7 ICR converts inhibitory signals delivered by IL-4 into activation signals. The IL-4/7ICR can improve the proliferation and persistence of CAR-T cells in an IL-4-rich tumor microenvironment. **(C)** The PD-1/CD28 CSR and the IL-4/7 ICR are both injected into CAR-T cells simultaneously.

Conjectures

Both the PD-1/CD28 CSR and the IL-4/7 ICR should be combined (**Figure 8C**). After being activated, CAR-T cells expressing these two receptors may be able to reverse the inhibitory signal transmitted by tumor cells as well as the inhibitory effect of IL-4 simultaneously (29, 32, 34–37).

Universal CARs

One of the biological hallmarks of malignant tumors is antigen heterogeneity. Because traditional CARs can only target one tumor antigen, it is possible for tumor cells that do not express or underexpress these antigens to elude the immune system during treatment. To improve the flexibility of CARs and expand the range of antigen recognition, researchers have developed universal CARs, also known as fifth-generation CARs. Universal CARs can overcome tumor antigen heterogeneity better than traditional CARs. Unlike traditional CARs, universal CARs have a "third party" intermediate system between the transmembrane domain and the scFv. Currently, universal CARs mainly include BBIR CAR and SUPRA CAR. As an example, the SUPRA CAR system consists of two parts: scFv with leucine zipper adaptor (zipFV) and T cell universal receptor with leucine zipper adaptor (zipCAR) (38). Combining zipFV and zipCAR will induce T cell activation (**Figure 9A**). Universal CARs are currently in the preclinical research stage.

Synthetic T Cell Receptor and Antigen Receptor (STAR)

CAR-T cell therapy has shown a high response rate and lasting disease control in hematological malignancies. Although TCR is stronger than CAR in signal transduction (39), co-expression of the two on the surface of T cells is not recommended. One of the principles of producing universal T cells is to knockout TCR genes and HLA class I genes of T cells, preventing the occurrence of graft-versus-host response (GvHD). Although knocking out the TCR genes improves anti-tumor effects of CAR-T cells, it reduces their persistence (40).

To overcome these defects, Liu et al. (39) did not co-express TCR and CAR on the surface of T cells, but instead used another innovative idea to fuse their structures and successfully

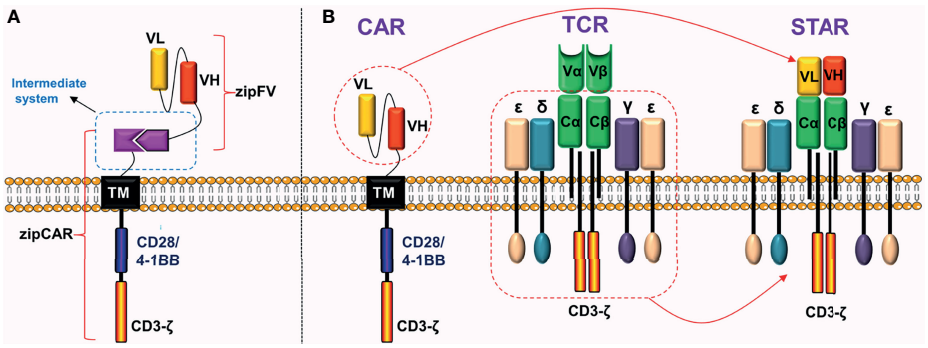

FIGURE 9 | Universal CAR and STAR. **(A)** Adding a "third party" intermediate system to the extracellular domain of CAR. **(B)** STAR is mainly composed of two parts: the scFv of CAR and the constant region of TCR.

constructed a novel chimeric receptor. This is a double-chain chimeric receptor referred to as STAR. The STAR contains a specific scFv of the CAR, which recognizes the tumor antigen, and the constant region of TCR, which participates in endogenous signal transduction (**Figure 9B**). Therefore, STAR combines the advantages of the CAR and the TCR. Preclinical experiments revealed that (39) the anti-tumor ability of STAR-T cells in a variety of solid tumor models was obviously superior to traditional CAR-T cells. The advantages were mainly manifested in the fact that STAR further improved the antigen sensitivity, persistence, and proliferation ability of T cells without causing obvious toxic reactions.

IMPROVING THE SAFETY OF CARS

If the proliferation of CAR-T cells is not controlled once they are inserted into patients, significant toxic reactions may occur. According to meta-analyses, the incidence of cytokine release syndrome (CRS) is about 55.3% (41), and the incidence of immune effector cell-associated neurotoxicity syndrome (ICANS) is approximately 37.2% (41) or 21.7% (42) in patients with hematological malignancies receiving CAR-T cell therapy. In recent years, certain non-traditional CARs that can reduce toxic reactions have emerged.

ON/OFF-Switch CAR

To control the activation of CAR-T cells more accurately and prevent them from overreacting, Wu et al. (43) constructed a split synthetic receptor system. They used the system to divide the intracellular signal domain of a CAR into two parts (costimulatory domain and CD3ζ), and construct a special CAR called ON-switch CAR. ON-switch CAR-T cell is initially in an inactivated state, because the intracellular costimulatory domain and CD3ζ are in separate states. The intracellular costimulatory domain and CD3ζ can be reassembled and the intracellular domain restored to a complete state only with the use of a specially designed drug (rapamycin analog AP21967). Finally, the ON-switch CAR-T cell can be activated after combining with the corresponding tumor antigen (**Figure 10A**). Local administration of activating drugs can better reduce off-target effects for on-switch CAR-T cells.

Jan et al. (44) constructed the ON-switch CAR (Lenalidomide ON-switch split CAR) and the OFF-switch CAR (Lenalidomide OFF-switch degradable CAR). The ON-switch CAR uses the same design principle as Wu et al. (43), and a special drug is also needed to reassemble the intracellular domain of the CAR. The OFF-switch CAR uses "targeted protein degradation technology", which could degrade the labeled CAR proteins in the presence of Lenalidomide, preventing CAR-T cells from recognizing tumor cells. After stopping using Lenalidomide, T cells produce new CAR proteins and gradually restore anti-tumor function (**Figure 10B**). This design can limit the short-term toxicity of CAR-T cells (the time depends on the metabolic time of Lenalidomide) but has no effect on their long-term anti-tumor efficacy.

Conjectures

The aforementioned designs are mainly aimed at alleviating toxic reactions, and the next step should be to further improve their anti-tumor effects.

a. Constructing the ON/OFF-switch CAR based on a fourth-generation CAR.
b. Combining the ON/OFF-switch CARs with TanCARs to expand their recognition range of tumor antigens.

CARs That Require Special Conditions (Light or Ultrasound) to be Activated

The light-switchable gene systems can regulate the expression of target genes by adjusting light intensity and duration (45). These systems not only realizes temporal and spatial control of gene expression, but they also improve the accuracy and safety of anti-tumor therapy, which has great potential in the treatment of malignant tumors (45–47). Huang et al. (48) constructed a light-inducible nuclear translocation and dimerization (LINTAD) system that enabled them to control the expression of CAR genes by blue light and the activation of CAR-T cells by regulating genes (**Figure 10C**). They found that light

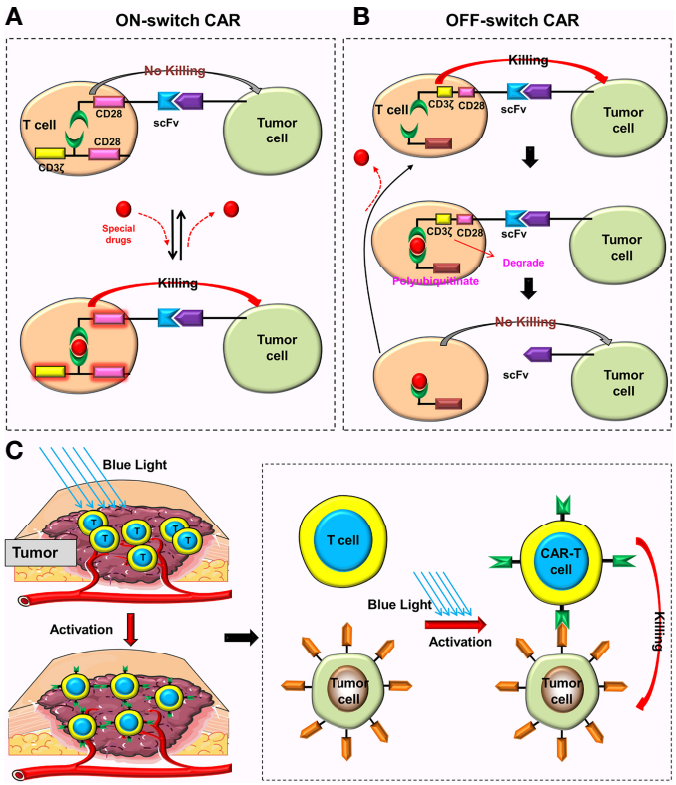

FIGURE 10 | Special CARs that can reduce toxic reactions. **(A)** Only when special drugs exist can the ON-switch CAR return to its complete state and be activated. When these drugs are removed, the ON-switch CAR will gradually return to the inactive state. **(B)** In the absence of lenalidomide, the OFF-switch CAR-T cell can be normally activated. In the presence of lenalidomide, the labeled CAR proteins will be degraded, preventing the CAR-T cell from recognizing the tumor cell. CAR proteins will gradually be generated after these drugs are removed. **(C)** These CAR genes can only be activated and translated into CAR proteins under the irradiation of blue light.

stimulation for 12 hours could achieve the maximum induction, and that this induction ability could last for about two days. Therefore, the activated CARs could only last for two days after turning off the illumination. With the help of the LINTAD system, the activation of CAR-T cells at the appropriate place and time can precisely be controlled, and be limited to the tumor site. The results showed that these CAR-T cells could minimize off-target toxicity and improve safety while ensuring anti-tumor activity (48). Additionally, Allen et al. (49) constructed the TamPA-Cre system, a novel genetic AND-gate switch that can induce the activation of CAR genes only when special drugs (tamoxifen) and blue light were simultaneously present. Their results revealed that the TamPA-Cre system could accurately control the local expression of CARs and subsequent activation of CAR-T cells while further reducing off-target toxicity (49).

Nevertheless, each of these designs has its own limitations. Due to the limited capacity to transmit blue light, these CAR-T cells are more suited to treat superficial tumors. Although red light or the red light system has a stronger penetration ability, it appears less effective than the blue light system (50), and requires additional auxiliary factors (51).

Ultrasound is a form of sound wave with a frequency higher than 20,000 Hz that can transport mechanical energy into the body (tens of centimeters from the body surface) safely and non-invasively (52, 53). On that account, the penetration ability of ultrasound far exceeds that of light (52, 53). Focused ultrasound (FUS) has thermal effects (54) that can make biological tissues to heat up locally. Wu et al. (55) constructed a heat-induced system, which was an eGFP reporter vector with a heat-shock proteins (Hsp) promoter. Additionally, they integrated Cre-lox gene switch into the system. Under heating conditions (43°C), T cells with this heat-induced system could express corresponding CAR proteins. In mice experiments, FUS was used to control the local temperature *in vivo* under the guidance of magnetic resonance imaging (MRI), and significant expression of CAR genes was observed in FUS-CAR-T cells with only two 5-minute FUS stimulation (Nalm-6 cells and double luciferase reporter gene were used to judge FUS-induced gene activation) (55).

To minimize the use of exogenous components, Wu et al, further optimized the FUS-CAR-T cells, in which the CARs expression was directly driven by Hsp without the use of a Cre-lox switch. Six hours after the first round of heat induction (43°C, 15 minutes), 43.9% of Hsp-CAR-T cells expressed CARs, and their expression level returned to the original basal level after 24 hours. During the second round of heat induction, 44.2% of Hsp-CAR-T cells expressed CARs, and the same degradation kinetics appeared.

The experimental results showed that (55) Hsp-driven FUS-CAR-T cells were not only safe and effective, but also reversible.

Conjectures

Huang et al. (48) also proposed several improved conjectures to enhance the limited penetration ability of blue light, including the use of up-conversion nanoparticles, which can convert near-infrared (NIR) light to stimulate blue light-responsive proteins, and implantable light-emitting diodes that were wirelessly controlled *via* radio frequency or NIR light for those solid tumors located deeper,.

Adding Suicide Genes

Suicide genes are added to CAR-T cells (56, 57), and when the suicide genes are active, they induce irreversible apoptosis of CAR-T cells that cause toxic reactions or over-activation, thus reducing the toxic reaction. Common suicide genes include herpes simplex virus thymidine kinase (HSV-tk), the caspase 9 (iCasp9) suicide genes, and CD20 and truncated epidermal growth factor receptor (EGFRt). According to certain preclinical experiments, CAR-T cells containing suicide genes could reduce their toxic reactions and improve their safety (58–61). While the suicide genes regulate toxicity, they also inevitably trigger irreversible apoptosis of certain CAR-T cells, reducing the anti-tumor effect.

Designing the Optimal Length of CD8α Hinge and Transmembrane Domain

The CD8α hinge and transmembrane domain play an important role in anti-tumor function and safety of CAR-T cells (62, 63). To reduce the adverse reactions of CAR-T cell therapy, Ying et al. (64) used the tertiary structure prediction program (Phrye2) to construct a group of CD19-BBz variants mainly by changing the length of CD8α hinge and transmembrane domain. Ying et al. found that CD19-BBZ (86) in this group of variants not only guaranteed the robust anti-tumor activity of CAR-T cells, but

TABLE 1 | Summary of some CAR types.

Year	Authors	References	Journals	Types of CARs
2013	Grada et al.	TanCAR: A Novel Bispecific Chimeric Antigen Receptor for Cancer Immunotherapy (7)	Molecular Therapy Nucleic Acids	Tandem CAR (TanCAR)
2016	Ruella et al.	Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies (15)	The Journal of Clinical Investigation	Dual-signaling CARs
2018	Bielamowicz et al.	Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma (16)	Neuro-oncology	Trivalent-tandem CAR
2016	Roybal et al.	Precision Tumor Recognition by T Cells With Combinatorial Antigen-Sensing Circuits (20)	Cell	synNotch AND-gate CAR
2020	Williams et al.	Precise T cell recognition programs designed by transcriptionally linking multiple receptors (26)	Science	Tandem AND-gate CAR
2020	Williams et al.	Precise T cell recognition programs designed by transcriptionally linking multiple receptors (26)	Science	3-input AND-gate CAR
2013	Fedorov et al.	PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses (27)	Science Translational Medicine	Inhibitory CAR (iCAR)
2020	Williams et al.	Precise T cell recognition programs designed by transcriptionally linking multiple receptors (26)	Science	AND-NOT CARs
2018	Adach et al.	IL-7 and CCL19 expression in CAR-T cells improves immune cell infiltration and CAR-T cell survival in the tumor (28)	Nature Biotechnology	7 × 19 CAR (the fourth generation)
2012	Prosser et al.	Tumor PD-L1 co-stimulates primary human CD8(+) cytotoxic T cells modified to express a PD1:CD28 chimeric receptor (29)	Molecular Immunology	PD-1/CD28 CSR
2014	Leen et al.	Reversal of tumor immune inhibition using a chimeric cytokine receptor (32)	Molecular Therapy	IL-4/7 ICR
2019	Wang et al.	An IL-4/21 Inverted Cytokine Receptor Improving CAR-T Cell Potency in Immunosuppressive Solid-Tumor Microenvironment (34)	Frontiers In Immunology	IL-4/21 ICR
2018	Cho et al.	Universal Chimeric Antigen Receptors for Multiplexed and Logical Control of T Cell Responses (38)	Cell	Universal CAR
2021	Liu et al.	Chimeric STAR receptors using TCR machinery mediate robust responses against solid tumors (39)	Science Translational Medicine	STAR
2021	Jan et al.	Reversible ON- and OFF-switch chimeric antigen receptors controlled by lenalidomide (44)	Science Translational Medicine	ON/OFF-switch CAR
2020	Huang et al.	Engineering light-controllable CAR T cells for cancer immunotherapy (48)	Science Advances	Needing special light (LINTAD system)
2021	Wu et al.	Control of the activity of CAR-T cells within tumors *via* focused ultrasound (55)	Nature Biomedical Engineering	Needing focused ultrasound (FUS)
2019	Warda et al.	CML Hematopoietic Stem Cells Expressing IL1RAP Can Be Targeted by Chimeric Antigen Receptor-Engineered T Cells (58)	Cancer Research	Adding suicide genes
2019	Ying et al.	A safe and potent anti-CD19 CAR T cell therapy (64)	Nature Medicine	Designing the optimal length of CD8α hinge and transmembrane domain
2021	Singh et al.	Antigen-independent activation enhances the efficacy of 4-1BB-costimulated CD22 CAR T cells (65)	Nature Medicine	Optimizing the length of the linker

also significantly reduced their toxic reactions. Twenty-five patients with B lymphomas received CD19-BBZ (86)-CAR-T cells. The results of the study showed that anti-tumor effects of these CAR-T cells were not compromised, and the most encouraging thing was that none of the 25 patients had CRS (> Grade 1) or ICANS. Recently, Singh et al. (65) found that shortening the length of the linker can better activate CAR-T cells and improve their anti-tumor effects. Although the underlying mechanism is not clear, it is feasible to improve the anti-tumor activity and safety of CAR-T cells by optimizing the length of CD8α hinge, transmembrane domain, and linker.

SUMMARY

Chimeric antigen receptor-T (CAR-T) cell therapy has been proven to be a promising immunotherapy for hematological malignancies. Compared to hematological malignancies, CAR-T cells need to overcome more obstacles to play a better anti-tumor role in solid tumors. To improve the efficacy and safety of CAR-T cells in malignant tumors, numerous researchers are focusing on designing new CARs and optimizing the CAR framework structures (**Table 1**). Several new designs and optimizations of CARs have shown promising anti-tumor effects in preclinical and clinical trials. However, as the complexity of CAR framework structures and the increasing number of CAR components increases, constructing corresponding plasmids is becoming more difficult, and transfection efficiency is decreasing. Therefore, the concept of CAR construction in the future will still be to further streamline the components of CARs, optimize the structure of CARs, or build new CARs in order to ensure anti-tumor ability and safety. Some of the conjectures in this paper may be realized only partially or not at all. As gene-editing technology advances, we anticipate that these conjectures will be verified. Five CAR-T cell therapies have been approved for marketing so far, and we expect that an increasing number of malignant tumor patients will benefit from CAR-T cell therapy in the future.

AUTHOR CONTRIBUTIONS

LM: Writing-Original draft preparation, manuscript, investigation, and figure preparation. JZ and BH: manuscript, investigation, and figure preparation. ZZ, SW, and FT: Investigation. MT: Investigation, methodology, supervision. YL: Conceptualization, methodology, supervision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Update for Advance CAR-T Therapy in Solid Tumors, Clinical Application in Peritoneal Carcinomatosis From Colorectal Cancer and Future Prospects

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Latest advances in the field of cancer immunotherapy have developed the (Chimeric Antigen Receptor) CAR-T cell therapy. This therapy was first used in hematological malignancies which obtained promising results; therefore, the use of CAR-T cells has become a popular approach for treating non-solid tumors. CAR-T cells consist of T-lymphocytes that are engineered to express an artificial receptor against any surface antigen of our choice giving us the capacity of offering precise and personalized treatment. This leaded to the development of CAR-T cells for treating solid tumors with the hope of obtaining the same result; however, their use in solid tumor and their efficacy have not achieved the expected results. The reason of these results is because solid tumors have some peculiarities that are not present in hematological malignancies. In this review we explain how CAR-T cells are made, their mechanism of action, adverse effect and how solid tumors can evade their action, and also we summarize their use in colorectal cancer and peritoneal carcinomatosis.

Keywords: CAR (chimeric antigen receptor) T cells, solid tumor, peritoneal carciomatosis, colorectal (colon) cancer, immunotherapy

INTRODUCTION

Conventional treatment with chemotherapy, radiotherapy or surgery is the therapy of choice most cancer patients (1). However, conventional treatment is not sufficient in many cases of cancer, thus the use of immunotherapy has acquired an important role for treatment of relapse or refractory tumors and its use has increased, being currently a common approach in cancer treatment (2). In the same way, investigations with new immunotherapy treatments are being developed. Particularly the use of chimeric antigen receptor (CAR)-T cell has become a popular approach and, in the last decade, many studies about CAR-T cell efficacy have been published. Their results, especially in

hematological malignancies, have been more than surprising, achieving complete remission in refractory or relapsed disease (3). This evidence has resulted in the approval between 2017 to 2021 of four CAR-T cell therapy by the FDA (4, 5). Most of the studies about CAR-T cells have investigated the safety and efficacy of this treatment in hematological malignancies, however only few clinical trials have studied their effect in solid tumors.

The success of CAR-T cells is due to the possibility of targeting any antigen on the cell surface of our choice, generating an immune response against the tumor cell in an MHC-independent manner for subsequent tumor elimination. CAR-T cells are made by extracting the leukocytes from the patient whole blood. Then these leukocytes are genetically modified to express chimeric receptors, offering the patient a precise, individual, unique and personalized treatment.

However, to fully understand their success we must comprehend their manufacture, how these modified cells carry out their anti-tumoral effect, which adverse effects they may produce and how they can develop tumor resistance. In this review, we summarize the current CAR-T cell therapy evidence regarding to the basis of this therapy, starting from their production and structure, their mechanism of action, adverse effects and mechanism of resistance to CAR-T therapy. We also show the current challenges in CAR-T cell therapy and in particularly in solid tumors. And finally, we delve further into their evidence in colorectal cancer and discuss the future perspectives in peritoneal carcinomatosis.

GENERAL INFORMATION ABOUT CAR-T CELLS

Definition

Chimeric antigen receptor (CAR)-T cells are lymphocytes that have been genetically modified to express chimeric receptors that enable them to target specific surface antigens in a major histocompatibility class-independent manner. This type of modified T cells was first described by Gross et al. in 1989, though only in the last decade has this technology become more widely developed, particularly for treating hematologic malignancies (6).

Immunotherapy has become a more widely used approach in cancer treatment with the application of CAR-T cells, as they give T cells the ability to express synthetic receptors against surface antigens of our choice and thereby destroy tumor cells (7). These antigens are not limited to proteins, but rather are able to bind to carbohydrates, glycolipids, and proteoglycans (8, 9). CAR-T cells have shown promising results in the treatment of hematologic malignancies and are being used mainly to treat cancers for which the primary target is CD19, i.e., B-cell lymphoma, childhood acute lymphoblastic leukemia (ALL), and adult-onset ALL. The FDA has approved the use of 4 CAR-T cell therapies that target CD19 in the last 5 years: axicabtagene ciloleucel (trade name, *Yescarta*), tisagenlecleucel (trade name, *Kymriah*), lisocabtagene maraleucel (trade name, *Breyanzi*) and brexucabtagene autoleucel (trade name, *Tecartus*) (4, 5, 10, 11).

However, the use of CAR-T cells in solid tumors has been less widely researched, and current evidence is insufficient to determine how these cells may be used in the clinic, so this type of therapy is now limited to clinical trials (12).

CAR-T cells comprise the following (13–16) (**Figure 1**):

-A single chain variable fragment (scFv) tumor-targeting domain, which enables the T cell to bind to the target antigens on the cell surface.

-Hinge or spacer domain: The portion that binds the scFv to the transmembrane domain. The function of this domain is to improve scFv flexibility so that it may bind to the target. This portion is created using sequences derived from immunoglobulins. IgG1 and IgG4 are the most commonly used immunoglobulins for this purpose (9, 17).

-A transmembrane domain (TD): CD3 ζ, CD28, and CD8α have been used as membrane domains in CAR constructs. They are the link between the extracellular and intracellular portion. They also play a role in CAR-T cell efficacy and stability (17).

-Costimulatory molecules that improve CAR-T cell proliferation and persistence.

-CD3 ζ: An intracellular signaling domain that activates T cells after binding to the antigen.

Development of CAR-T cells began in the late 1980s with research into these modified lymphocytes. Eshhar et al. observed that combining a variable fragment of an antibody with the constant region of the T-cell receptor endows the T-cell of the specificity of an antibody and the effector function of a cytotoxic T-cell; this resulted in the first generation of CAR-T cells, which consisted of the scFv region and the CD3ζ intracellular domain only. These cells were found to be unsuccessful in clinical trials: they were able to activate but did not proliferate, which indicated low efficacy (18–21). Second-generation CAR-T cells include costimulatory molecules in the intracellular domain, and the third generation contains 2 such molecules in the intracellular domain (12, 22) (**Figure 2**). This costimulatory molecule can be CD28, CD134 (OX40), CD137 (4-1BB), or CD27, which improve the efficacy and enhance the action, proliferation, and persistence of CAR-T cells (21–26). The fourth generation of CAR-T cells is currently being designed and have an inhibitory effect on the tumor microenvironment. These are the so-called TRUCKs (T cells redirected for universal cytokine-mediated killing), which are designed to secrete proinflammatory cytokines and recruit other immune cells, thereby improving their antitumor activity in an immunosuppressive microenvironment (12, 21, 22).

Production

CAR-T cell therapy begins by obtaining a sample of whole blood from the patient. This blood will be used to extract and modify T cells so that we may produce CAR-T cells. This is a complex, work-intensive process comprising several steps (**Figure 3**). The total cost of the process is estimated at 300 000 USD.

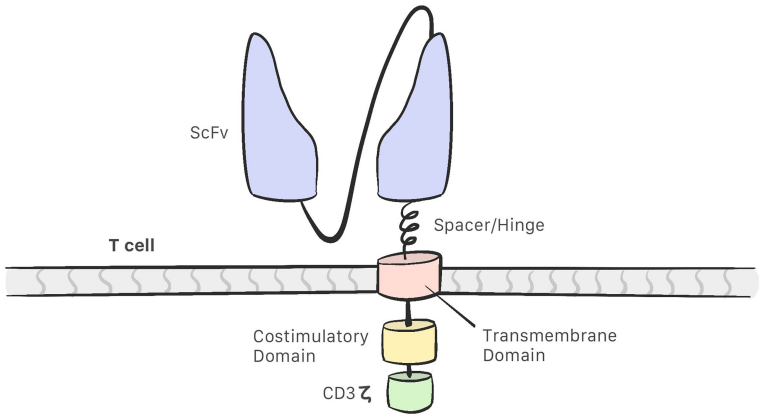

FIGURE 1 | The structure of a CAR consist of a single chain variable fragment (scFv) that enables the CAR-T cell to recognise the target; a hinge/spacer that binds the scFv to the transmembrane domain and improves the scFv flexibility; a transmembrane domain that act as a link between the extracellular and intracellular portion; Coestimulatory molecules that improvesCAR-T cell efficacy and a CD3z intracelullar domain that activates the CAR-T cell.

Leukapheresis and Cell Washing

Patients whole blood is centrifugated and white blood cells are extracted. Anticoagulants are added throughout the process to prevent clotting (27).

Activation

Under physiological conditions, T cells are activated by antigen-presenting cells (APCs) and depends on the interaction of the T-cell receptor (TCR) and the major histocompatibility complex. This means of activation is a complex process requiring participation by dendritic cells, meaning that CAR-T cells are activated differently. In CAR-T cells, the primary method used to activate T cells is through anti-CD3/CD28 coated magnetic beads which are used as artificial antigen presenting particles. After T cell activation, they are removed with a strong electromagnet (27, 28). Monoclonal antibodies or artificial antigen-presenting cells can be also used to activate T cell (27).

Gene Transfer

When the T cell is activated, the gene containing the information for producing the chimeric antigen receptor has to be inserted in the T cell genome. Retroviral transduction, particularly with the use of lentiviral vectors, is the most commonly used method of gene transfer used in CAR-T cells (28, 29). Transposon/transposase system in plasmids can be also used as a non-viral method (30).

Expansion

These cells, which already express CAR, expand in a culture medium containing interleukins. This process takes between 10 days and 3 weeks to complete (9, 28).

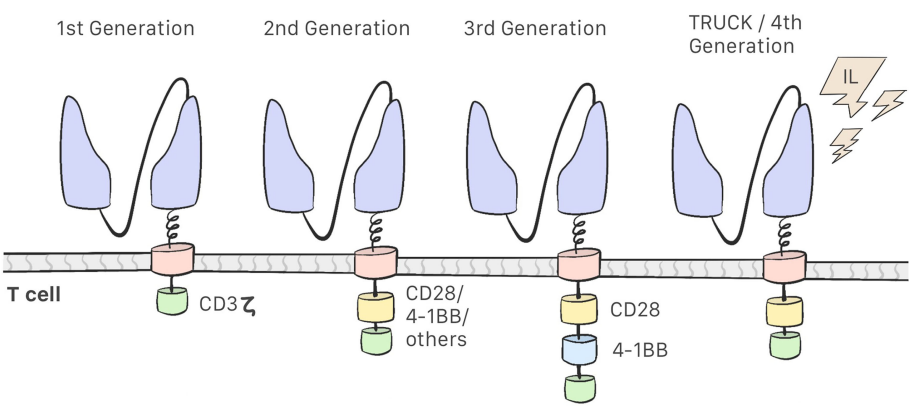

FIGURE 2 | First Generation CAR-T cells consist of a scFv region and the CD3ζ intracellular domain. Second-generation CAR-T cells include a costimulatory molecules in the intracellular domain. Third-generation contains two costimulatory molecules. Fourth-generation CAR-T cell or TRUCKs secretes proinflamatory cytokines.

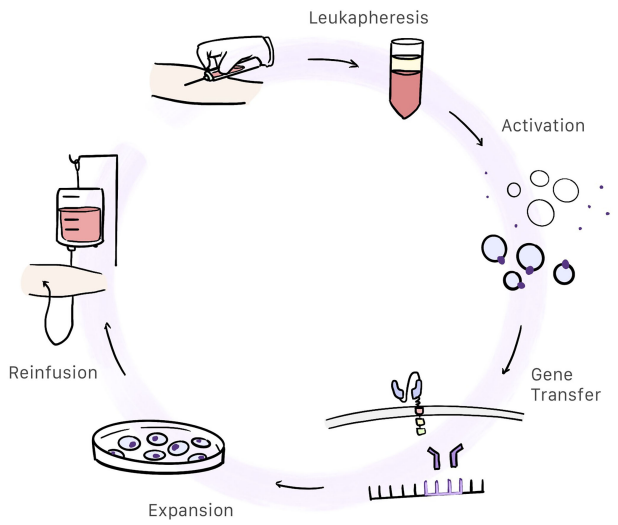

FIGURE 3 | Patient whole blood is extracted and centrifugated in order to obtain the white blood cells. Then T-cells are selected and activated. Afterwards, gene transfer containing the CAR information is inserted into the activated T-cells genome resulting in the creation of CAR-T cells. These cells are expanded and after a quality control, are reinfunsed into the patient.

Quality Control

Once the production process has completed, CAR-T cells undergo quality control, mostly to evaluate sterility (using cultures and Gram staining), purity (confirmed by the absence of replication competent viruses by means of quantitative PCR, and the absence of other type cells or endotoxins), and effectivity measured *in vitro*. Also, in this stage, the quantity of CAR-T cells produced is measured (27, 31).

Mechanism of Action

When the scFv has been bound to the antigen, cell-destruction mechanisms are set off within the cell through the intracellular domain. T cells use 2 main pathways to mediate cytolytic effector function (14):

Fast-Acting. Exocytosis of Cytotoxic Granules Containing Perforin and Granzymes

This is the primary mechanism of target cell death (32). Activated T cells release cytotoxic granules containing perforins, which trigger the formation of pores in the membrane of the target cell, which will facilitate the entry of pro-apoptic granzymes inducing cell death (33). Separation of the CAR-T cell from the target cell depends specifically on cell destruction, meaning that in the absence of cell death, CAR-T cells may cause cytokine hypersecretion, leading to adverse effects such as cytokine-release syndrome (14).

Slow-Acting. Expression of Membrane Bound Tumor Necrosis Factor (TNF) Family Ligands

The Fas ligand (Fas-L) belongs to the TNF family. Antigen recognition by the T cell will induce Fas-L expression on the surface. Ligation between Fas and Fas-L activates a caspase cascade that initiates an induced apoptosis in the target cell (14, 34). This mechanism also has an effect on tumor cells that do not express the target antigen, though it does require previous CAR-T cell activation (35).

Independently of the direct action of T cells on tumor cells, activated CAR-T cells produce cytokines that will stimulate antitumor activity in neutrophils, macrophages, and natural killer cells. These cytokine-activated cells will trigger lysis of cells that are invisible to CAR-T cells thanks to the activation of innate immunity (9, 14). Furthermore, destruction of tumor cells will liberate tumor antigens within an immunostimulatory microenvironment, which will allow for cross-presentation by dendritic cells and generation of endogenous CD8 responses against tumor antigens which were not originally targeted (36).

Adverse Effects Associated With CAR-T Cell Therapy

As with any treatment, use of CAR-T cells is not entirely free of adverse reactions, some of which may be life-threatening. The incidence of this toxicity varies considerably between published studies based on the types of CAR-T cells and costimulatory elements used (37).

Cytokine-Release Syndrome *(CRS)*

Cytokine-Release Syndrome is the main adverse effect in patients who receive CAR-T cell therapy and can result in lethal outcomes. CRS occurs in between 50% and 90% who undergoes anti-CD19 CAR-T cell therapy, mostly in the first week following the infusion (37). This effect is related to treatment response and tumor burden more so than the dose administered (21, 38). In fact, an absence of this adverse effect raises doubts as to the efficacy of the treatment and the expansion and elimination of tumor cells (12, 39). This syndrome is consequence of an over-activated immune response caused by stimulated CAR-T cells which stimulates other cells of the immune system (39, 40).

Signs and symptoms of CRS can vary widely. The most frequent symptoms are general malaise and nausea, though fever is the first symptom to present. Nonetheless, the disease may progress clinically to acute respiratory distress, acute renal failure, kidney failure, disseminated intravascular coagulation, cardiomyopathy, or even arrhythmia (12, 40). On laboratory analysis, this syndrome manifests as an increase of ferritin in serum and C-reactive protein and elevated cytokines (39, 41).

Mild cases of CRS are treated with acetaminophen and fluid therapy. Treatment with the IL-6 antagonist, tocilizumab, is indicated in patients presenting hemodynamic instability refractory to intensive fluid management and inotropic drug treatment (42). Corticosteroids may also be considered.

CAR T-Cell-Related Encephalopathy

Neurotoxicity can appear in 30% to 90% of patients who receive CAR-T cell therapy against CD19. Patients may suffer from headache, mild confusion or, in severe cases, cerebral edema (37). Patients with encephalopathy may exhibit disorientation, focal neurological deficit (dysphasia or motor deficits), and

seizures (40, 43, 44). Encephalopathy appears between 5 and 7 days following infusion (37). Its cause is somewhat unknown, though it may be related to high levels of cytokines released, to direct neurological damage or to an off-tumor response caused by the expression of CD19 in mural cells of the brain (12, 45). A possible explanation is that CRS and the systemic inflammation may alter the permeability of the blood brain barrier allowing the arrival of cytokines and immune cells including CAR-T cells. Presence of CAR-T cells has been demonstrated in the cerebrospinal fluid of some patients (40). Neurotoxicity has only been reported in CAR-T cell therapy targeting CD19 and CD20 and until now, there is no evidence of neurotoxicity in solid tumors.

On-Target, Off-Tumor Response

CAR-T cells can be activated by heathy tissues that express the target antigen resulting in the destruction of non-tumoral cells. This response is more frequent in solid tumors. The intensity and frequency of this response varies according to the target and route of administration used (9, 46). For example, CD19 is widely expressed in B cells (47). The use of CD19-redirected CAR-T cells destroy not only CD19-positive neoplastic cells, but also health B cells, thus causing B-cell aplasia (48). This response can be avoided by selecting a highly specific tumor antigen but also, CAR-T cells must have high affinity and specificity for the tumor antigen chosen and an appropriate dose of CAR-T cells must be administered (9, 22).

Barriers

Obstacles Related to Production

Producing CAR-T cells requires a supply of T cells. This first hurdle in the process is related to the raw material and concerns both the quality and quantity of the cells harvested. Unfortunately, CAR-T cells are currently approved only for hematologic malignancies, and some patients lack a sufficient amount of T cells from this source to create CAR-T cells. Moreover, many patients receive previous chemotherapy, which has been associated with lower quantity and quality of these T cells (49).

The remaining obstacles concern the design of CAR-T cells, particularly regarding variations in their structure, production and the time required to produce these cells (50). CAR-T cell exhaustion refers to a dysfunctional state in which the T cells lose their effector function and in which there is an increase in the number of inhibitory receptors induced by chronic stimulation such as that observed in cancer. This exhaustion may lead to treatment failure (51).

The main limitation of CAR-T cell therapy involves limited access to treatment despite the fact that this therapy has been approved by the FDA. Treatment must be carried out in specialist facilities capable of adhering to product manufacturing and administration protocols that can absorb the high cost of this therapy (50).

The toxicity-related factors involved in this treatment are the result of the adverse effects commented on previously in this review.

Tumor-Related Factors

-Antigen-negative tumor relapse: The presence of antigen-negative tumor cells from the beginning leads to the selection of cells that will not be destroyed. To develop resistance to CAR-T cells, complete antigen loss is not necessary; rather, tumor cells may become resistant with diminished numbers of antigens (50). Tumor escape may also occur due to alternative splicing events, in which the antigen continues to be present, although in an isoform that CAR-T cells are unable to recognize (52).

-Antigen-positive tumor relapse: Antigen-positive relapse may be due to defects in CAR-T cells or may result from tumor-dependent factors. The antitumor effect of CAR-T cells does not only depend on antigen recognition. Rather, it may depend on the activation of cell-destruction mechanisms. The mechanism that causes antigen-positive tumor resistance is primarily based on changes in survival and apoptosis of the tumor cell (21). A signal that induces tumor apoptosis is the TNF–related apoptosis-inducing ligand (TRAIL). The absence of TRAIL in tumor cells implies antigen-positive tumor resistance (53). Another cause of relapse concerns CAR-T cell destruction, as tumor cells may express FAS-L, which induces CAR-T cell apoptosis (54). Recurring antigen-positive tumors may respond to a second treatment with CAR-T cells. However, re-infusion of CD19-redirected CAR-T cells has not resulted in a clinical response in a number of published studies (55–57).

Factors Related to the Tumor Microenvironment

This factor affects particularly to solid tumors and will be explained more deeply afterward. Tumor microenvironment confers to the tumor a physical barrier composed by the tumor stroma and the extracellular matrix, a metabolic barrier and an immunological barrier contributing to an immunosuppressive state.

TREATMENT OF SOLID TUMORS WITH CAR-T CELLS

In light of the surprising results obtained in CAR-T cell therapy in hematologic malignancies, it comes as no surprise that research on their use in solid tumors is now under way. Multiple targets in solid tumors are currently being investigated for CAR-T cell therapy, including human epidermal growth factor receptor 2 (HER-2) for breast, ovarian, lungs, and pancreas pancreas; carcinoembryonic antigen (CEA) for digestive tumors and tumors of the lung; disialoganglioside 2 (GD2) for neuroblastoma; IL-13Rα for glioblastoma; epidermal growth factor receptor (EGFR) for pancreatic cancer and glioblastoma (58); MUC for pancreatic, gastric, and ovarian cancer; mesothelin for mesothelioma; and prostate-specific membrane antigen (PMSA) for prostate cancer. However, current evidence is lacking to transfer this approach to the routine clinical practice, and the results obtained to date are less promising when compared to applications in hematologic malignancies.

Solid tumors respond differently owing to the presence of barriers that do not affect liquid tumors. Directing CAR-T cells against solid tumors requires presence of a specific antigen on the tumor surface. Once administered, CAR-T cells must migrate toward the tumor and infiltrate it. Subsequently, CAR-T cells must reach the tumor cell and recognize it in within a hostile microenvironment characterized by oxidative stress, acidic pH, hypoxia, nutritional depletion, presence of inhibitory factors and cytokines and also immunosuppressive cells (59). Regarding to the adverse effects of CAR-T cell therapy in solid tumors, they are the same as in hematologic malignancies aforementioned taking into account that on-target, off-tumor response varies depending on the target and if it is administered regionally, local reaction may appear. Therefore, current challenges in CAR-T cell therapy in solid tumors focus on target selection, CAR-T cells migration and tumor microenvironment.

Current Challenges in Solid Tumors

Target-Antigen Selection

To reduce on-target, off-tumor toxicity, the target antigen must be present on the surface of all tumor cells and be absent from healthy cells. Locating a specific tumor-associated antigen (TAA) poses a challenge in solid tumors. Surface oncofetal antigens are a good target for CAR-T cell therapy, as their expression is limited to tumor cells (7).

Additionally, TAAs on solid tumors are more heterogeneous than those of hematologic malignancies (60, 61). Use of CAR-T cells for treating hematologic malignancies derived from B cells have the advantage that practically all the B-cell line (whether they are tumor cells or not) expresses CD19, which makes this a perfect therapy target. However, CD19-redirected CAR-T cells also destroy healthy cells, thus causing B-cell aplasia, which makes the host prone to opportunistic infections (48). Therefore, finding a marker with these characteristics in solid tumors is fundamental.

Migration

Once CAR-T therapy has been administered, these engineered cells must penetrate the tumor and reach tumor cells. Hematologic malignancies are free of this difficulty, as both CAR-T cells as well as their target cells are hematopoietic, which makes them tend to migrate to similar locations (62). Chemokines secreted by solid tumor cells, such as CCL2, frequently do not match the chemokine receptors in CAR-T cells. Induction of expression of these receptors such as CCR2b favors migration to the tumor (63). Tumor cells also express chemokines such as CXCL5, which attracts myeloid-derived suppressor cells (MDSCs), which have an immunosuppressive effect (64). One possible solution to this is to administer CAR-T cells locally.

Tumor Microenvironment

The tumor microenvironment provides the tumor with a series of barriers that hinder the action of CAR-T cells.

Physical Barrier

Even after the CAR-T cells have migrated properly, effector cells encounter a physical barrier as the tumor stroma prevents them from infiltrating the tumor. Destroying this stroma by generating CAR-T cells that are able to secrete enzymes that degrades the stroma (65); or as an alternative, local collagenase may be administered, favoring this way CAR-T cell infiltration (66).

Metabolic Barrier

Glucose is the preferred energy source of tumor cells, causing an increase in lactic acid production. This causes nutritional depletion, lactate elevation (and, thus, acidic pH), reduced glucose and increased oxidative stress, thus inhibiting T-cell proliferation and cytokine production (7, 62, 67).

Immunologic Barrier

Multiple soluble inhibitory factors exist in the tumor microenvironment. Netwick et al. describe presence of prostaglandin E2 (PGE2), a molecule produced by tumor cells and macrophages, as well as high levels of adenosine, which are further increased during hypoxia. Both PGE2 and adenosine inhibit T-cell proliferation (7). TGF-β (transforming growth factor β) and IL-10 are secreted by tumor cells and immunosuppressive cells (62). TGF-β favors tumor progression and metastasis and has a direct negative effect on T-cell differentiation and cytotoxic function (68). IL-10 inhibits the activation of T cells (69). Blocking of TGFβ signaling or the use of TRUCKs may favor a response by CAR-T cells.

In addition to soluble inhibitory factors, the tumor microenvironment contains immunosuppressive cells such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and tumor-associated neutrophils (TANs), which inhibit the function of effector T cells. Tregs inhibit T-cell function through cell-cell contact and soluble factors such as TGFβ and IL-10 (70). MSDCs, TAMs, and TANs inhibit the immune response by producing TGF-β and PGE2 (71).

Another inhibitory factor involves the expression of programmed death-ligand 1 (PD-L1) by tumor and immunosuppressive cells and the expression of cytotoxic T lymphocyte associated antigen-4 (CTLA-4; also known as B7-1/B7-2) by these cells; both reduce T-cell function. The PD receptor is located on the membrane of T cells. PD-L1 expression by health cells prevents them from being destroyed by T cells. Cancer cells and Tregs express PD-L1, which enables them to inhibit the action of CAR-T cells and also favors CAR-T cell apoptosis. CTLA4 is a receptor expressed by T cells, though when stimulated by B7-1/B7-2 inhibits the effector functions of T cells (72). Lastly, Fas-L expression by tumors induce T-cell apoptosis (54). Adjuvant CAR-T cell administration with monoclonal antibodies that block PD1 and CTLA4 may increase the antitumor effect of CAR-T cells (59, 73).

Future Prospects

Many studies have shown that when used as monotherapy, CAR-T cells have limited efficacy against solid tumors. However, these results may be biased by the sample of patients who have received previous failed treatments and who then initiate CAR-T cell therapy in a poor physical state and with aggressive tumors (74).

Chemotherapy

Combined therapy consisting of chemotherapy and CAR-T cells has a synergistic effect. Chemotherapy reduces tumor burden and plays an immunomodulatory role when administered at low doses; this benefits the inhibition of suppressant immune cells, as they are more sensitive that cytotoxic T cells (75) and reduce autoimmunity by prolonging the persistence of CAR-T cells (74). Chemotherapy with low-dose carboplatin also sensitizes tumor cells to immunotherapy, thereby increasing the antitumor effect of CAR-T cells (76). Lastly, the cytotoxic effect of chemotherapy facilitates tumor-antigen recognition and presentation (74).

Radiation Therapy

Radiotherapy induces tumor necrosis and apoptosis, which favors the maturation and activation of dendritic cells and the presentation of antigens (77). Following radiation, INF-ϒs and damage-associated molecular patterns (DAMPS) are released, attracting immune effector cells, which boosts migration and infiltration of the tumor, and also increases MHC class-I molecules expression (78).

Radiation is also followed by an immune-mediated antitumor response targeting distant tumors, which affects primary-tumor metastasis; this phenomenon is referred to as the abscopal effect (79).

Combination With Other Types of Immunotherapies

Optimization of CAR-T cells allows for enhanced antitumor function.

The effect of CAR-T cells is enhanced when administered in conjunction with TRUCKs. TRUCKs were designed to secrete proinflammatory cytokines that increase their action in an immunosuppressive tumor microenvironment; in particular, TRUCKs that produce IL-12 improve the cytotoxicity of T cells and favor the expansion and secretion of cytokines, thus conferring resistance to Tregs (80).

Creating CAR-T cells capable of recognizing 2 antigens would prevent tumor escape. Dual CAR-T cells are T cells that express 2 CARs against different antigens and are only activated when both antigens are expressed on the tumor surface (81). Tandem CARs (TanCARs) are T cells that express a CAR that is capable of recognizing two different antigens, which are activated by either receptor (82).

It has been shown that adding anti-PD1 monoclonal antibodies or CAR-T cells capable of secreting anti-PD-L1 can block the inhibitory effect of these receptors and allow CAR-T cells to function, thus improving their efficacy and persistence (83, 84). Anti-CTLA-4 antibodies have also been shown to increase T-cell activity (85).

Local Treatment With CAR T Cells

Most studies using CAR-T cells in solid tumors have been carried out by means of systemic administration of these T cells. This body of research has reported limited efficacy owing to the low capacity of CAR-T cells to migrate to the tumor site. As a result, local application of CAR-T cells would likely increase tumor penetration. However, this approach is limited by its high technical complexity.

Brown et al. showed that local infusion of anti-IL13Ra2 CAR-T cells into the resection cavity of 3 patients with glioblastoma was both safe and feasible (86). Brown and her coauthors later published a case report of a single patient with recurrent multifocal glioblastoma who received multiple local infusions of CAR-T cells targeting IL13Ra2, observing that administration into the resected cavity controlled local relapse and progression of glioblastoma in distal sites; on the other hand, the authors indicated that intraventricular infusions led to regression of all tumors of the central nervous system (58).

Local CAR T-cell administration may provide a solution to the problem of CAR T-cell migration to the tumor site, thus improving penetration. Doing so would prevent adverse effects associated with on-target, off-tumor responses and lower the occurrence of CRS (87).

TREATMENT OF COLORECTAL CANCER WITH CAR-T CELLS

Colorectal cancer (CRC) is the third most common type of tumor and the second leading cause of cancer-related death (88). As a result of screening initiatives, many patients with colorectal tumors are diagnosed at an early stage of the disease, for which curative treatment is available. However, approximately 20% of these patients present metastatic disease on diagnosis, and many cases of CRC may recur following conventional therapy (89). In these patients with distant tumors, chemotherapy can make it possible for these patients to have a mean survival of 20-30 months (90). Despite the many available treatment lines, the survival rate continues to be low. Therefore, CAR-T cells hold potential as therapy for these patients.

To date, all studies on CAR-T cell therapy in CRC patients have been performed in patients with metastatic disease, as conventional approaches to localized tumors have demonstrated good outcomes. The first such study was conducted in patients with CRC and metastasis of the liver. The investigation consisted of 2 trials using first-generation CAR-T cells targeting tumor-associated glycoprotein-72 (TAG-72), an oncofetal mucin overexpressed by most human epithelial adenocarcinomas, with expression predominantly restricted to tumor cells. In one of the trials, cells were administered intravenously in escalating-dose and in the other infusion was administered through the hepatic artery. The study concluded that CAR-T cell therapy is safe despite the migration difficulties found for these cells (91).

Carcinoembryonic antigen (CEA), another attractive target for CAR-T cell therapy, is a marker for gastrointestinal cancer that is widely expressed in CRC. This marker is not detected in most normal adult tissues and is only expressed at very low levels in the luminal epithelia of the gastrointestinal tract and lung tissue, which causes it to go undetected by immune cells, while in tumor cells this antigen loses its polarity, causing it to be expressed on the entire cell surface (92).

The first therapy to use CAR-T cells redirected against CEA employed hepatic transarterial administration to deliver second-

generation cells to patients with liver metastases. The authors of the study found that this type of therapy is safe and also provided evidence of the presence of CAR-T cells in liver and tumor tissue (93).

Another clinical trial studied systemic administration of CAR-T cells directed against CEA in patients with metastatic CRC. The authors reported that the patients tolerated the treatment well, even at high dose levels (up to 10^8 cells/kg), and that the treatment helped control the disease. The study consisted of administering second-generation CAR-T cells in 10 patients with progressive metastatic CRC. Following treatment, 7 patients had stabilized disease, 2 of whom remained stable for over 30 weeks, and another 2 patients had a reduction in the size of their tumors (38). The effect of these cells depends on the ability of the CAR-T cells to expand and persist; cell loss may lead to tumor relapse (94). The antitumor effect and expansion of CAR-T cells is determined by the presence of immunosuppressive factors; these factors can be attenuated with lymphodepletion by cyclophosphamide/fludarabine chemotherapy (38). CAR-T cells that persist in the body may be capable of eliminating tumor cells in the event of rechallenge (95). Although use of TCRs redirected against CEA has been associated with colitis, none of the patients studied developed this adverse effect, even at high doses (96).

This indicates that anti-CEA CAR-T cell therapy is safe. The adverse effects related to CRS are mild and easily manageable (38, 93, 97). Therapy consisting of CEA-specific CAR-T cells administered simultaneously alongside IL-12 has been shown to increase antitumor activity and favor CAR-T cell proliferation (98).

Meanwhile, other targets are being investigated as approaches in CRC therapy. Epithelial-cell adhesion molecule (EpCAM) CAR-T cells may have antitumor effects. EpCAM is expressed on most carcinomas and is associated with E-cadherin–mediated adhesion to support tumor dissemination (99). EGFR CAR-T cells (*epidermal growth factor receptor*) have shown antitumor activity *in vivo* (100). HER-2 is another target expressed on many tumors and in approximately 15% of CRCs (101). Animal models have shown good results (95), though in one clinical trial caused acute respiratory failure syndrome (102). A phase-I clinical trial used CD133-directed CAR-T cells in patients with hepatocellular carcinoma, pancreatic carcinoma, or CRC, observing an antitumor response (103).

Since early stages of CRC can be managed by conventional treatment, the use of CAR-T cells in CRC could only offer benefits in metastatic disease. Thus, all effort must be focused in treating distant metastasis. To date, CEA is the most promising target for treating disseminated CRC. However, multiples targets are being investigated that have showed promising results.

TREATMENT OF PERITONEAL CARCINOMATOSIS WITH CAR-T CELLS

The peritoneum is a common site of dissemination in CRC, forming part of the natural evolution of the disease in up to 40% of CRC patients (104). Between 5% and 10% of peritoneal metastases are detected on diagnosis (105). This entity has a high mortality rate and a mean survival of 6 months if left untreated (106).

As aforementioned, solid tumors may encounter some obstacles that are not present in hematological malignancies. CAR-T cells migrate to similar locations as hematological malignancies due to their similar origin. This situation does not happen in solid tumors, thus CAR-T migration is suboptimal. To improve CAR-T migration to the peritoneal metastases, local treatment with intraperitoneal instillation might be a possible solution.

Katz et al. used second-generation CAR-T cells targeting CEA to treat peritoneal carcinomatosis in a murine model, demonstrating that local peritoneal infusion of CAR-T cells was superior to systemic administration. Intraperitoneal injection of CAR-T cells was associated with greater tumor reduction when compared to intravenous infusion and showed a lasting effect, protecting the host from rechallenge and from tumors in extraperitoneal sites (107).

In a similar study, another group used second-generation TAG72-CAR-T cells in an animal model of peritoneal ovarian carcinomatosis. Regional intraperitoneal delivery showed better results than systemic delivery, with increased tumor reduction and overall survival, which were higher after repeated infusion (108).

Another study of peritoneal carcinomatosis observed that repeat intraperitoneal delivery of EpCAM CAR-T cells using mRNA-mediated transfection produced an inhibitory effect on tumor growth and, given that these cells express CAR-T cells *via* mRNA, their expression is temporary, which increases their degree of safety (109). Occasionally, CAR-T cells may fail to recognize tumor targets due to an absence of the target epitope caused by gene deletions or alternative splicing; in these cases, a second infusion of CAR-T cells may not have any added effect (52, 103).

Another challenge present in the treatment of solid tumors is due to the tumor microenvironment components that offers a resistance preventing CAR-T cells to reach the tumor cells. One of these components is the tumor stroma that confers a physical barrier. Particularly in the case of peritoneal metastases, they present high levels of collagen in their extracellular matrix. Thus, the destruction of these collagen fibers using intraperitoneal collagenase followed by intraperitoneal CAR-T cells instillation may favor CAR-T cells infiltration to the tumor and enhance their activity (66).

Therefore, when compared to systemic administration, regional intraperitoneal delivery of CAR-T cells to treat peritoneal carcinomatosis produces a greater antitumor effect, added protection against rechallenge, and protection in extraperitoneal tumor sites. This effect may be amplified with repeat infusions. Peritoneal administration allows increased local concentration of effector cells, which triggers a local immune response in the peritoneal cavity in addition to minimizing the adverse systemic effects caused by CAR-T cells (109, 110). This, however, does not influence the effect of CAR-T cells on distant tumors, as it is not a result of the direct action of CAR-T cells, but

rather a phenomenon resembling the abscopal effect (107). This mechanism, which is seen in radiotherapy, consists of tumor-antigen secretion by the cancer cells destroyed by CAR-T cells, allowing for cross-presentation by dendritic cells, which mount an immune response against antigens other than those targeted by the CAR-T cell (14). Furthermore, activation of CAR-T cells releases cytokines, which stimulate the innate immune response (36).

CONCLUSIONS AND FUTURE PROSPECTS

Despite that CAR-T cells therapies are being widely investigated and had reached excellent result in hematological malignancies; solid tumors have not achieved the expected effect and its efficacy is still unclear. This lack of efficacy is due to some important hurdles that are present in solid tumors and need to be resolved. The tumor microenvironment entails the main difficulty for CAR-T cell to carry out their function due to the physical barrier and immunosuppressive microenvironment. Numerous studies are trying to improve CAR-T cell efficacy by prolonging their persistence, trafficking, tumor infiltration and tumor elimination by means of using different costimulatory molecules, CAR-T capable of secreting proinflammatory cytokines or capable of detecting two different antigens. Also, combination therapy with other immunotherapies, chemotherapy or radiotherapy may improve their results.

Recent data show that peritoneal carcinomatosis can be treated with local instillation of CAR-T cells with promising result and less systemic adverse effects. We suggest treating peritoneal carcinomatosis with combination therapy using local instillation of collagenase for treating the tumor stroma followed by intraperitoneal CAR-T cell instillation. We believe that this approach could improve the efficacy of CAR-T cell therapy in peritoneal carcinomatosis. But also, its combination with other immunotherapy such as anti-CTLA-4 or anti-PD1 monoclonal antibodies offers a wide field of investigation.

AUTHOR CONTRIBUTIONS

SQ contributed in writing the original draft preparation. SQ, PV-C, IG, and CQ wrote de manuscript. SH-V, SG-S, HG, SJ-G, DG-O, and CQ reviewed the manuscript. CQ corrected the manuscript. SQ and PV-C edited the manuscript before submission. CQ supervised the manuscript. All authors contributed to the article and approved the submitted version.

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The Chemokine Receptor CCR8 Is a Target of Chimeric Antigen T Cells for Treating T Cell Malignancies

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Chimeric antigen receptor (CAR) T cells have been successfully used in the therapy of B cell leukemia and lymphoma, but still have many challenges in their use for treating T cell malignancies, such as the lack of unique tumor antigens, their limitation of T cell expansion, and the need for third party donors or genome editing. Therefore, we need to find novel targets for CAR T cell therapy to overcome these challenges. Here, we found that both adult T-cell leukemia/lymphoma (ATLL) patients and ATLL cells had increased CCR8 expression but did not express CD7. Moreover, targeting CCR8 in T cells did not impair T cell expansion *in vitro*. Importantly, anti-CCR8 CAR T cells exhibited antitumor effects on ATLL- and other CCR8-expressing T-ALL cells *in vitro* and *in vivo*, and prolonged the survival of ATLL and Jurkat tumor-bearing mouse models. In conclusion, these collective results show that anti-CCR8 CAR T cells possess strong antitumor activity and represent a promising therapeutic approach for ATLL and CCR8$^+$ tumors.

Keywords: CCR8, TAX, ATLL, T cell malignancy, CAR T cells

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INTRODUCTION

Although chimeric antigen receptor (CAR) T cell therapies lead to high clinical response rates in patients with certain B cell malignancies (1, 2), their use for treatment of T cell malignancies has still proven challenging because of limitations such as the disruption of target antigen expression on CAR-modified T cells, the need to target antigens with limited expression on T cells, and the need

for third party donor cells that are either non-alloreactive or have been genome edited at the T cell receptor α constant (TRAC) locus (3). Previous reports suggested CD7-knockout T cells expressing a CD7-specific CAR exhibited antitumor activity in preclinical and clinical trials (3–5). However, the use of anti-CD7 CAR T cells requires knockout CD7 and TRAC expression in cells from third-party donors, and most recipients subsequently relapse. Therefore, we need to find more potential targets for CAR T cell treatment of T cell malignancies.

Adult T-cell leukemia/lymphoma (ATLL) and peripheral T-cell lymphoma (PTCL) are major subtypes of T-cell lymphoma (6). ATLL is a malignancy of mature T lymphocytes that is triggered by human T-cell lymphotropic virus type I (HTLV-1) (7, 8). ATLL was proposed to have four clinical subtypes from ATLL patient databases: acute, lymphoblastic, chronic and smoldering. The 4-year overall survival (OS) rates of ATLL patients are 11%, 16%, 36% and 52% for patients with the acute, lymphoblastic, chronic and smoldering subtypes, respectively (9). Despite the prognosis and recent progress in treatment modalities for patients with acute and lymphoblastic ATLL, their 4-year OS rates are still poor. Moreover, further prolonging the overall survival of ATLL patients with chemotherapy is difficult (10). In addition, ATLL cells express CD3, CD4, and CD25 but lack CD7 (11, 12). Therefore, anti-CD7 CAR T cells are not suitable for ATLL patients.

Expression of the chemokine receptor CCR8, a G protein-coupled receptor (GPCR), is induced by the CC chemokine CCL1/I309 (13, 14). Pervious study suggested that tumor infiltrating Treg expressed high expression of CCR8, whereas NK cells, $CD8^+$ T cells, myeloid cells, γδT cells, the bulk of $CD4^+$ Tconv cells, and Treg cells found in peripheral blood did not express CCR8 (15). CCR8 controls the immunosuppressive function of tumor infiltrating Treg cells (16), and blocking CCR8 depletes Treg cells and improves antitumor immune responses (17). CCR8 is expressed on ATLL-derived cells and inhibits ATLL cell apoptosis (18). Therefore, we investigated whether targeting CCR8 with CAR T cells significantly suppresses tumor progression in ATLL models. Here, we found that ATLL patients and ATLL cells both expressed CCR8 but not CD7. Moreover, we generated two anti-CCR8 CAR T cell lines and found that anti-CCR8 CAR T cells did not exhibit impaired expansion *in vitro*. In addition, anti-CCR8 CAR T cells exhibited antitumor activity against ATLL cells and CCR8-expressing T-ALL cells, and prolonged the survival of ATLL and Jurkat tumor-bearing mouse models.

MATERIALS AND METHODS

RNA Sequence Analysis

The GEO data set (GEO33615, logFC> 1) used is from GEO database (https://www.ncbi.nlm.nih.gov/geo/), and the download data format is MINIML. Box plots are drawn by boxplot; PCA graphs are drawn by R software package ggord; The box plot is implemented by the R software package ggplot2; the heat map is displayed by the R software package pheatmap (19).

Chimeric Antigen Receptor Constructs and Lentivirus Production

Third-generation anti-CD19, anti-CCR8(10) (PCT/JP2019/051603) and anti-CCR8 (19) (PCT/JP2019/051603) CAR vectors incorporating CD28, TLR2 (20, 21) and CD3ζ signaling domains were constructed. The sequence of TLR2 domains: CAGGCCAAAAGGAAGCCCAGGAAA GCTCCCAGCAGGAACATCTGCTATGATGCATTTGTTTC TTACAGTGAGCGGGATGCCTACTGGGTGGAGAACCTTA TGGTCCAGGAGCTGGAGAACTTCAATCCCCCCTTCAAG TTGTGTCTTCATAAGCGGGACTTCATTCCTGGCAAGTG GATCATTGACAATATCATTGACTCCATTGAAAAGAGCC ACAAAACTGTCTTTGTGCTTTCTGAAAACTTTGTGAAG AGTGAGTGGTGCAAGTATGAACTGGACTTCTCCCATTT CCGTCTTTTTGATGAGAACAATGATGCTGCCATTCTCA TTCTTCTGGAGCCCATTGAGAAAAAAGCCATTCCCCAG CGCTTCTGCAAGCTGCGGAAGATAATGAACACCAAGAC CTACCTGGAGTGGCCCATGGACGAGGCTCAGCGGGAA GGATTTTGGGTAAATCTGAGAGCTGCGATAAAGTCC. Lentiviral particles were produced in HEK-293T cells following polyethyleneimine (Polysciences, Inc., USA)-mediated transfection with the pWPXLd-based transfer plasmid and the packaging and envelope plasmids psPAX2 and pMD2.G. Lentivirus-containing supernatant was harvested at 24, 48, and 72 h post transfection and filtered through a 0.22-μm filter.

Isolation, Transduction, and Expansion of Primary Human T Lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult donors using Lymphoprep (Stem Cell Technologies, Vancouver, Canada). T cells were negatively selected from PBMCs using a MACS Pan T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and activated using microbeads coated with anti-human CD3, anti-human CD2 and anti-human CD28 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) at a bead:cell ratio of 1:2 and a density of 2.5×10^6 cells/ml for two days in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml recombinant human IL-2, 10 mM HEPES, 2 mM glutamine and 1% penicillin/streptomycin. On Day 2 post activation, T cells were transduced with CAR vector lentiviral supernatants in the presence of 8 μg/mL polybrene at a multiplicity of infection (MOI) of 2.0 (Sigma–Aldrich, St. Louis, USA). Twelve hours after transduction, T cells were cultured in fresh medium containing IL-2 (300 U/mL); subsequently, fresh medium was added every 3 days to maintain the cell density at approximately 1×10^6 cells/ml. T cells were transduced with the CAR-expressing lentiviral vectors for 24 h. The healthy PBMC donors provided informed consent for the use of their samples for research purposes, and all procedures were approved by the Research Ethics Board of the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences (GIBH).

Cells and Culture Conditions

HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA). Cell lines such as Jurkat/Jurkat-GL (T-acute lymphoblastic leukemia), Molt-4 (T-acute lymphoblastic leukemia), MT-4/MT-4-GL (adult T-cell leukemia/lymphoma), and C8166/C8166-GL (adult T-cell leukemia/lymphoma), were maintained in RPMI-1640 medium. The medium was supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, NY, USA), 10 mM HEPES, 2 mM glutamine (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). All cells were cultured at 37°C in an atmosphere of 5% carbon dioxide.

GFP-2A-Luciferase (GL) Generation of Tumor Cells

The GFP-2A-Luciferase (GL) vector contains EGFP, Luciferase and 2A sequence and clones by Sangon Biotech company (Shanghai, China). GL-Lentiviral particles were produced in HEK-293T cells following polyethyleneimine (Polysciences, Inc., USA)-mediated transfection with the pWPXLd-based transfer plasmid and the packaging and envelope plasmids psPAX2 and pMD2.G. Lentivirus-containing supernatant was harvested at 24, 48, and 72 h post transfection and filtered through a 0.22-μm filter. 1×10^6 Tumor cells transduced with 10 ml GL-lentiviral particles for 12 h, and the GFP percentage of GL-tumor cells were detected through flow cytometry for 48 h. GL-tumor cells were sort by FACS Aria. The purity of GL-tumor cells were >95% for killing assay.

Flow Cytometry

Flow cytometry was performed on a Fortessa cytometer (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA). The antibodies used, including anti-human CD3-PE (UCHT1), anti-human CD4-APC-Cy7 (OKT4), anti-human CD8-PE_Cy7 (OKT8), anti-human CCR8-APC (SA214G2), and anti-human CD7-FITC (4H9/CD7), were purchased from Biolegend (San Diego, USA). Staining was performed on ice for 30 minutes, and cells were then washed with PBS containing 2% FBS before cytometric analysis. For intracellular staining, cells were fixed and permeabilized with a Foxp3/Transcription factor staining kit (Cat#421403, Biolegend), followed by staining with transcription factor-specific antibodies, such as Tbet, Gata3 and Foxp3. Cells were gently vortexed and were then incubated in the dark for 30 minutes at room temperature. Afterward, the cells were washed once more with cold flow buffer and were then analyzed immediately.

Cytotoxicity Assays

MT-4-GL, C8166-GL and Jurkat-GL target cells were incubated with 1928z, C1028z or C1928z T cells at the indicated ratio in triplicate wells of U-bottom 96-well plates. Target cell viability was monitored 18 hours later by adding 100 μl/well of the substrate D-luciferin (potassium salt) (Cayman Chemical, Michigan, USA) at 150 μg/mL. Background luminescence was negligible (<1% of the signal from wells containing viable target cells alone). The percentage of viable target cells (%) was calculated as (experimental signal- blank signal)/(targeted signal-blank signal) ×100, and the percentage of cytotoxicity was calculated as 100 – percentage of viable target cells.

Cytokine Release Assays

T cells were cocultured with target cells at an E:T ratio of 4:1 for 24 hours, and supernatants were analyzed for cytokine release by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' protocols. ELISA kits for IFN-γ and Granzyme-B were purchased from Thermo Fisher Scientific Inc., USA.

Xenograft Models and *In Vivo* Experiment

Animal experiments were performed in the Laboratory Animal Center of GIBH, and all animal procedures were approved by the Animal Welfare Committee of GIBH. All protocols were approved by the relevant Institutional Animal Care and Use Committee (IACUC). NSI mice (22) were maintained in specific pathogen-free (SPF)-grade cages and provided autoclaved food and water. Mice were randomized into experimental groups of ≥ 4. Direct intravenous (tail vein) injection of the indicated leukemia cells in 200 μl of PBS was performed to establish tumors. At the indicated time for each experiment, 2×10^6 transduced human T (GFP^+ or CAR^+) cells in 200 μL of PBS were adoptively transferred into tumor-bearing mice systemically by tail vein injection. Peripheral blood was obtained by retro-orbital bleeding. Body weight was measured every 2–3 days as indicated. Tumors were measured every 3 days with a caliper. Tumor volume was calculated using the following formula: $(\text{length} \times \text{width}^2)/2$. *In vivo* whole-body imaging of luciferase-labeled cells was performed using a cooled CCD camera system (IVIS 100 Series Imaging System, Xenogen, Alameda, CA, USA). D-luciferin Firefly, potassium salt was injected at 75 mg/kg. Mice were imaged 5 minutes after injection of the substrate. Quantification of the total and average emission was performed using Living Image software.

Statistical Analysis

Statistical significance was determined using Student's t test (two groups) or ANOVA with Tukey's multiple comparison test (three or more groups). All statistical analyses were performed using Prism software, version 7.0 (GraphPad, Inc., San Diego, CA, USA). The gene distribution of GEO database was analyzed using wilcox tests. Kaplan-Meier survival curves of vivo experiments were analyzed using log-rank. P values < 0.05 were considered statistically significant, and the following annotations were used: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. For assessment of differential gene expression, a minimum fold change of 2 was used, and a false discovery rate-corrected P value of < 0.05 (Fisher's combined p value method) was considered significant (23).

RESULTS

CCR8 Was Highly Expressed in ATLL Patients and Cell Lines

To evaluate CCR8 and CD7 expression in primary ATLL patients, we downloaded GSE33615 from GEO database to

compare CCR8 and CD7 expression between ATLL patients and normal CD4 T cells. After standardized the GEO data (GSE33615) (**Figure S1A**), We performed principal component analysis (PCA) to confirm the data for subsequent analysis (**Figure S1B**). Interestingly, we found that cells from ATLL patients had higher expression of CCR8 (**Figure 1A**) and lower expression of CD7 (**Figure 1B**) than normal CD4 T cells. In addition, we detected CCR8 expression in two ATLL cell lines, MT-4 and C8166 cells, through flow cytometry. These two ATLL cell lines expressed CCR8 (**Figure 1C**) but did not express CD7 (**Figure 1D**). Given that CCR8 is a new target, we further confirm that CCR8 whether expressed in normal human tissues/cells. Based on publicly available GEPIA database, CCR8 expression levels in human normal tissue are undetectable or very low, compared with the corresponding tumor tissues (**Figures S2A, B**). A previous study suggested that overexpression of TAX, an oncogene in ATLL, promoted primary PBMC expansion *in vitro* (24). Therefore, we further examined the relationship between CCR8 and ATLL. We established a series of TAX-expressing lentiviral vectors and transduced them into primary T cells. We found that compared with EGFP-expressing T cells, TAX-expressing T cells upregulated CCR8 expression (**Figure 1E**). In addition, tazemetostat, an inhibitor of EZH2, suppressed CCR8 expression

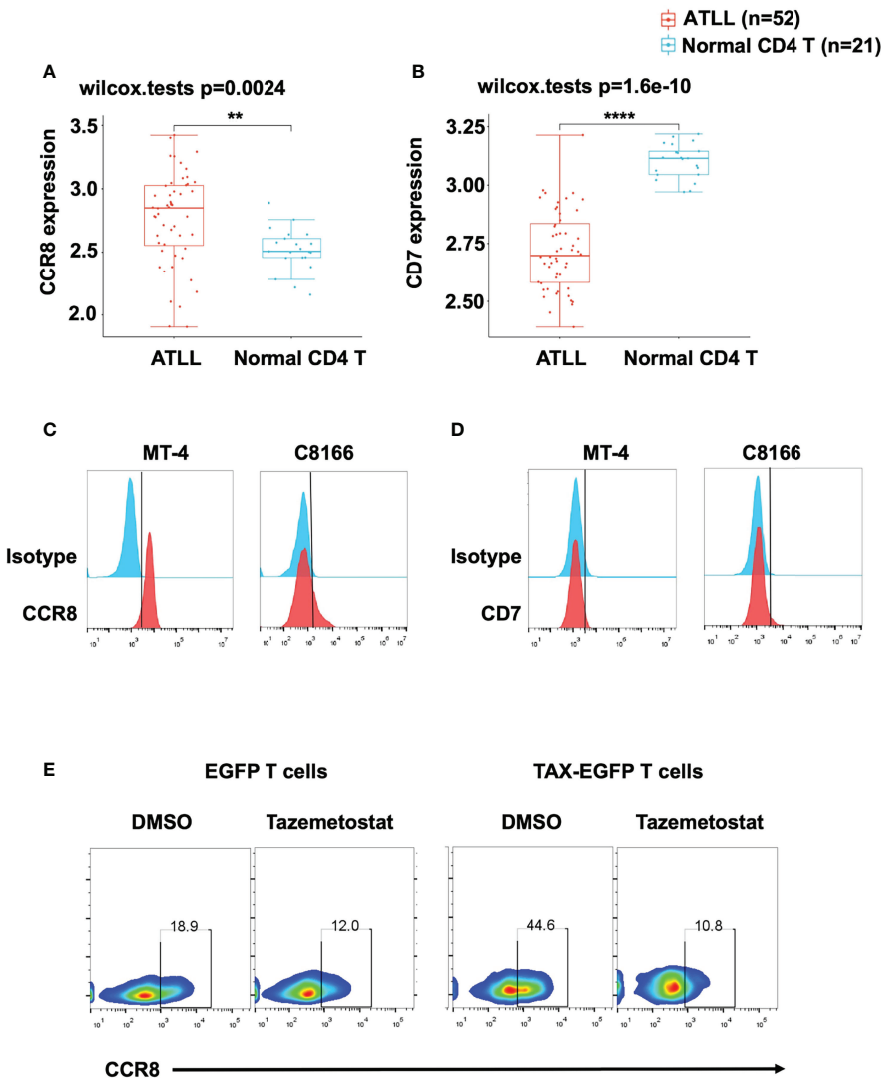

FIGURE 1 | CCR8 was highly expressed in ATLL patients and cell lines. **(A, B)** The expression distribution of CCR8 **(A)** and CD7 **(B)** gene in in ATLL patients (*N* = 52) and normal CD4 T cells (*N* = 21) from healthy donors were analyzed in the publicly datasets (www.aclbi.com) (19) and the data were obtained from GEO database (GSE33615), where the horizontal axis represents different groups of samples, the vertical axis represents the gene expression distribution, where different colors represent different groups, and the upper left corner represents the significance p-value test method (wilcox.tests); **(C, D)** The levels of CCR8 **(C)** and CD7 **(D)**, as detected by flow cytometry, in MT-4 and C8166 cells; **(E)** The level of CCR8 was determined by flow cytometry in TAX-expressing and EGFP T cells (Day 9) upon treatment with DMSO or the EZH2 inhibitor tazemetostat (1 μM, MCE) for 24 h. **P < 0.01, ****P ≤ 0.0001.

in TAX-expressing T cells (**Figure 1E**), which was consistent with the finding that EZH2 was associated with ATLL cell development and interacted with TAX (24). Therefore, these results suggest that CCR8 is upregulated in ATLL primary tissues and cell lines and that CCR8 may be a potential therapeutic target for the patients with ATLL.

Anti-CCR8 CAR T Cells Did Not Impair T Cell Expansion *In Vitro*

To investigated whether anti-CCR8 CAR T cells suppress T cell function *in vitro*, we constructed two third-generation CAR vectors, namely, anti-CCR8 (10A11) C1028z and anti-CCR8 (19D7) C1928z, and anti-CD19 CAR (1928z) served as negative control, which contain the scFv, the human CD28 transmembrane domain (CD28TM) and endodomain, the human CD3ζ T cell activating domain, a human TLR2 domain (T2) and EGFP; these vectors were introduced into human T cells through lentiviral transduction (**Figures 2A, B**). We found that compared with that in the 1928z group, the CCR8 levels in the C1028z and C1928z groups were decreased (**Figure 2C**). Furthermore, we found that the T cell expansion and relative CAR expression in the C1028z and C1928z groups were similar to those in the 1928z group (**Figures 2D, E**), which was consistent with pervious study that T cells expressing CD5 CAR undergo only limited fratricide and can be expanded long-term *in vitro* (25). In addition, we found the CD4/CD8 ratio and TH cell classification in the C1928z and C1028z groups were similar to those in the 1928z group (**Figures S3A–C**). These results suggest that anti-CCR8 CAR T cells do not impair T cell expansion *in vitro*.

Anti-CCR8 CAR T Cells Exhibited Antitumor Efficacy Against ATLL Cells *In Vitro*

To further investigate whether anti-CCR8 CAR T cells suppress ATLL cell growth *in vitro*, we performed a cytotoxicity assay with C1028z and C1928z and 1928z T cells in two ATLL cell lines that did not express CD19 (**Figure S4A**), and showed that C1028z and C1928z T cells exhibited higher cytotoxicity than 1928z T cells after coculture with MT-4-GFP-2A-Luciferase (GL) and C8166-GL cells at the indicated effector:target (E:T) ratio *in vitro* (**Figures 3A, B**). Moreover, the C1028z and C1928z groups had higher expression of Granzyme-B (**Figures 3C, D**) and IFN-γ (**Figures 3E, F**) than the 1928z group. In addition, we found that the expression of CD107a (26), a sensitive marker for cytotoxic activity determination, was increased in both the C1028z and C1928z groups compared with the 1928z group (**Figure 3G**). Notably, C1028z T cells exhibited better antitumor efficacy than C1928z T cells against the two ATLL cell lines (**Figures 3A–G**). These collective results suggest that C1028z and C1928z T cells exhibit a considerable antitumor effect on $CCR8^+$ ATLL cells *in vitro*.

Anti-CCR8 CAR T Cells Exhibited Antitumor Efficacy Against ATLL Cells *In Vivo*

We next evaluated the antitumor effects of anti-CCR8 CAR T cells *in vivo*. We infused T cells expressing C1028z, C1928z and 1928z into immunodeficient NSI mice (22) that had been inoculated with MT-4-GL cells (**Figure 4A**). C1928z and C1028z T cells induced significant regression of tumors formed from MT-4-GL cells, while the tumors in the 1928z group continued to progress, as detected by bioluminescence imaging (BLI) (**Figures 4B, C**, **S5A**). In addition, C1928z and C1028z T cells prolonged the survival of MT-4 tumor-bearing mice, compared with 1928z T cells (**Figure 4D**). Interestingly, C1028z T cells had higher cytotoxic activity than C1928z T cells against MT-4 cells *in vitro* (**Figure 3**), but the anticancer response of C1028z T cells was similar to that of C1928z T cells *in vivo* (**Figures 4B–D**). Therefore, these results demonstrate that C1028z and C1928z T cells exhibit antitumor effects on $CCR8^+$ ATLL cells and prolonged the survival of MT-4-GL tumor-bearing mice models *in vivo*.

Anti-CCR8 CAR T Cells Exhibited Antitumor Efficacy Against CCR8-Expressing T-ALL Cells

Although anti-CD7 CAR T cells were found to exhibit a good safety profile and achieve a high complete remission rate, the presence of CD7-negative tumor cells may lead to relapse. We examined whether anti-CCR8 CAR T cells inhibit the growth of T-ALL cells in addition to ATLL cells. First, we detected CCR8 and CD19 expression on T-ALL cells, including Jurkat and Molt-4 cells. We found that CCR8 was expressed on Jurkat cells but not Molt-4 cells (**Figure S6A**), and CD19 did not expressed on Jurkat and Molt-4 cells (**Figure S6B**). Moreover, we found that C1028z and C1928z T cells exhibited higher cytotoxicity than 1928z T cells after coculture with Jurkat cells at the indicated effector to target (E:T) ratio *in vitro* (**Figure 5A**), but C1028z and C1928z T cells did not suppress the growth of Molt-4 *in vitro* (**Figure S6C**). In addition, we found that T cells expressing C1028z and C1928z produced higher levels of cytotoxic cytokines, such as Granzyme-B (**Figure 5B**) and IFN-γ (**Figure 5C**), than T cells expressing 1928z. We further evaluated the antitumor effects of anti-CCR8 CAR T cells in the Jurkat NSI mouse model. We infused T cells expressing C1028z, C1928z and 1928z into Jurkat NSI model mice. We found that compared with 1928z T cells, C1928z and C1028z T cells significantly suppressed the growth of Jurkat tumors *in vivo* and improved the overall-survival of Jurkat tumor-bearing mice (**Figures 5D–F**, **S7A–C**). Therefore, these collective results suggest that C1928z T cells and C1028z T cells can suppress the growth of CCR8-expressing T-ALL tumors and improve the overall survival of tumor-bearing mice.

DISCUSSION

Adult T-cell leukemia/lymphoma (ATLL) patients have an extremely poor prognosis that cannot be prolonged through chemotherapy alone. Here, we show that activated human T cells that express the CCR8 CAR can specifically recognize and kill ATLL cells *in vitro* (**Figures 3A, B**). Moreover, anti-CCR8 CAR T cells produced higher levels of IFN-γ, Granzyme-B and

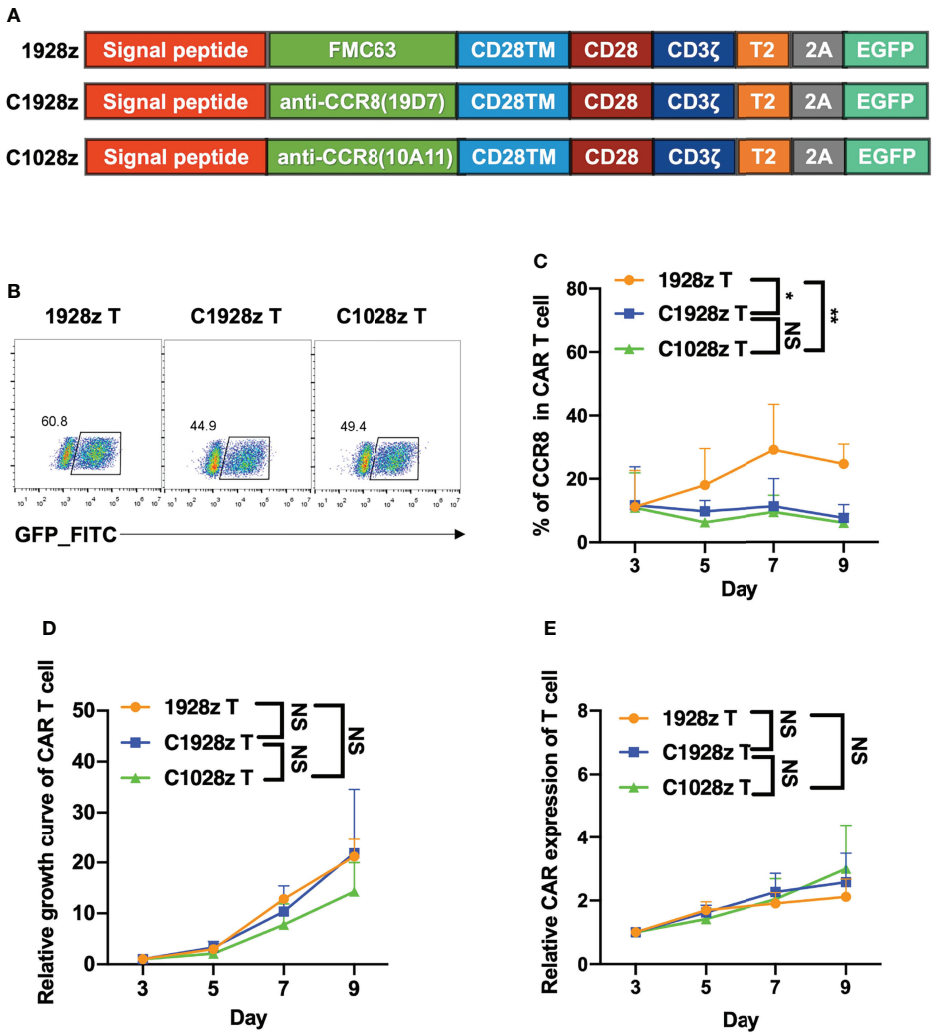

FIGURE 2 | Anti-CCR8 CAR T cells did not impair T cell expansion *in vitro*. **(A)** Anti-CD19 CAR vector based on an anti-CD19 scFv (FMC63, 1928z) and two anti-CCR8 CAR vectors based on an anti-CCR8 scFv (10A11, C1028z) and an anti-CCR8 scFv (19D7, C1928z). All vectors contained expression cassettes encoding a human CD8 leader signal peptide, the CD28, CD3ζ, TLR2 signaling domains, and EGFP fused to the sequences described in **Figure 2A**; **(B)** CAR expression on CAR T cells was detected by flow cytometry; **(C)** CCR8 expression on CAR T cells was detected by flow cytometry at the indicated time for three different donors; **(D)** The relative growth of CAR T cells was analyzed by flow cytometry at the indicated time for three different donors; **(E)** The CAR level of CAR T cells were detected by flow cytometry at the indicated time for three different donors. **(C–E)** Data are shown as the mean ± SEM values; two-way ANOVA with Tukey's multiple comparisons test; *P < 0.05, **P ≤ 0.01. n.s >0.05.

CD107α than 1928z T cells after coculture with ATL cells (**Figures 3C–F**). In addition, anti-CCR8 CAR T cells significantly suppressed MT-4 tumor progression *in vivo* and prolonged the survival of MT-4 tumor-bearing mice (**Figure 4**). Similar to T cells transduced with a CD5 CAR, anti-CCR8 CAR T cells did not significantly suppress T cell expansion *in vitro*. In addition, CCR8 was largely expressed on tumor infiltrating Treg cells, indicating that anti-CCR8 CAR T cells did not impair their anticancer immune response. Therefore, these results suggest that anti-CCR8 CAR T cells exhibit stronger antitumor immune responses in $CCR8^+$ ATLL or T-ALL.

Although the success of CD19 CAR T cell therapy in B-ALL has revolutionized anticancer therapy, the high rate of complete response is sometimes limited by the emergence of CD19-negative leukemia cells (27). Here, we found that CCR8 is also expressed on T-ALL cells such as Jurkat cells (**Figure S6A**). Furthermore, we showed that anti-CCR8 CAR T cells inhibited the growth of Jurkat cells *in vitro* (**Figure 5A**). In addition, the production of IFN-γ and Granzyme-B was improved in the C1928z and C1028z groups compared with the 1928z group after coculture with Jurkat cells (**Figures 5B, C**). Notably, anti-CCR8 CAR T cells reduced Jurkat tumorigenesis and prolonged

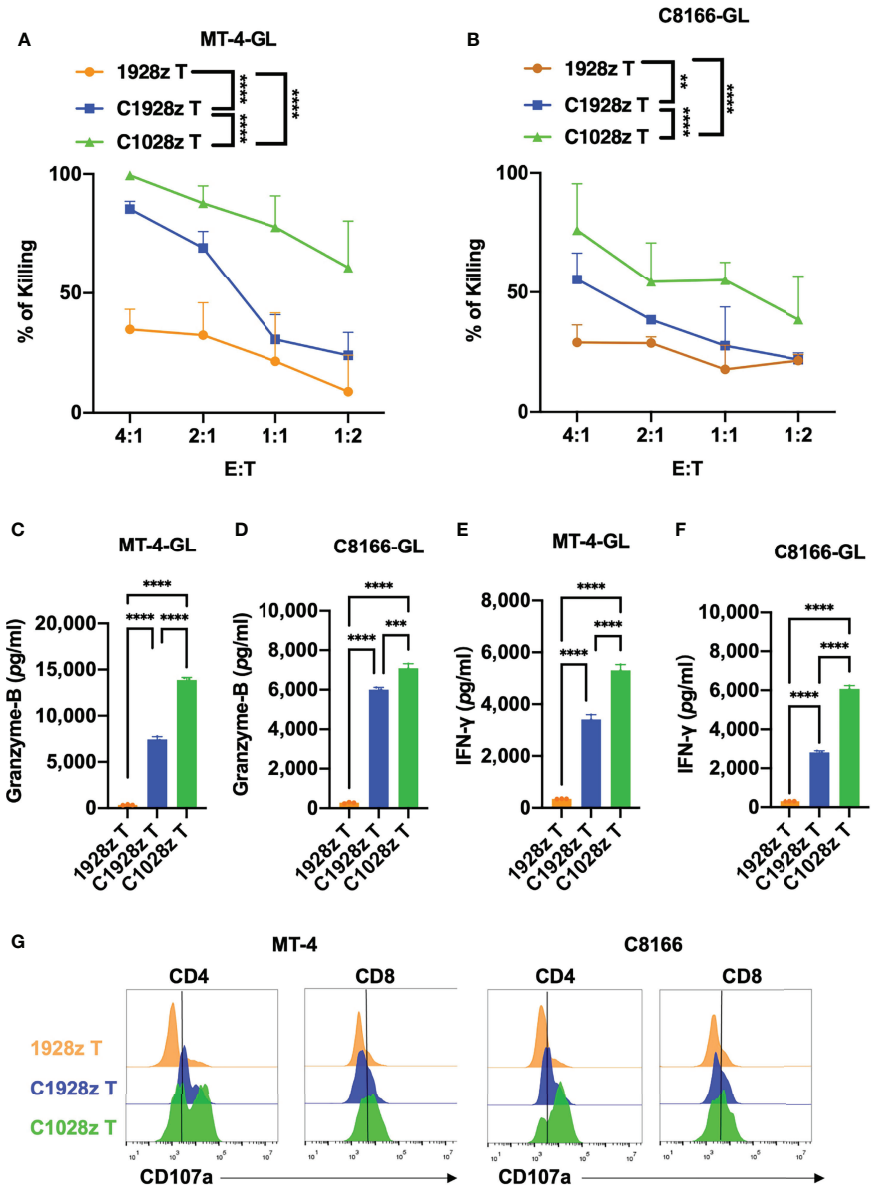

FIGURE 3 | Anti-CCR8 CAR T cells exhibited antitumor efficacy against ATLL cells *in vitro*. **(A, B)** The percentages of MT-4 **(A)** and C8166 cells **(B)** whose lysis was induced by 1928z, C1928z and C1028z T cells; **(B–F)** 1928z, C1928z and C1028z T cells were incubated with MT-4 or C8166 cells at a 4:1 effector **(E)** target (T) ratio in 96-well round-bottom plates for 24 hours at 37°C. Supernatants were then harvested and analyzed with a multiplex immunoassay to determine the concentrations of the indicated cytokines. The concentrations of Granzyme-B **(C, D)** and IFN-γ **(E, F)** were measured by ELISA; **(G)** The level of CD107a, as detected by flow cytometry, in 1928z, C1928z and C1028z T cells after coculture with MT-4 or C8166 cells for 24 h; **(A, B)** Data are shown as the mean ± SEM values; two-way ANOVA with Tukey's multiple comparisons test; $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$; **(C–F)** Data are shown as the mean ± SEM values; one-way ANOVA with Tukey's multiple comparisons test; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

survival of Jurkat tumor-bearing mice (**Figures 5D–F**). Therefore, anti-CD7 and anti-CCR8 dual CAR T cells may be a good choice to prevent antigen escape and further improve the antitumor effect of CCR8$^+$ T-ALL treatment. In addition, CCR8 is a driver of Treg cells that secrete immunosuppressive cytokines, such as TGFβ1, to inhibit MSLN CAR T cell function in solid tumors (28). Therefore, anti-MSLN and anti-CCR8 dual CAR T cells could impair Treg cell differentiation and increase the MSLN CAR T cell population in solid tumors.

In conclusion, we demonstrated that anti-CCR8 CAR T cells exhibit a stronger anticancer response than other CAR T cells against CCR8$^+$ ATLL cells and T-ALL cells and provided a novel treatment for patients with CCR8$^+$ T cell malignances.

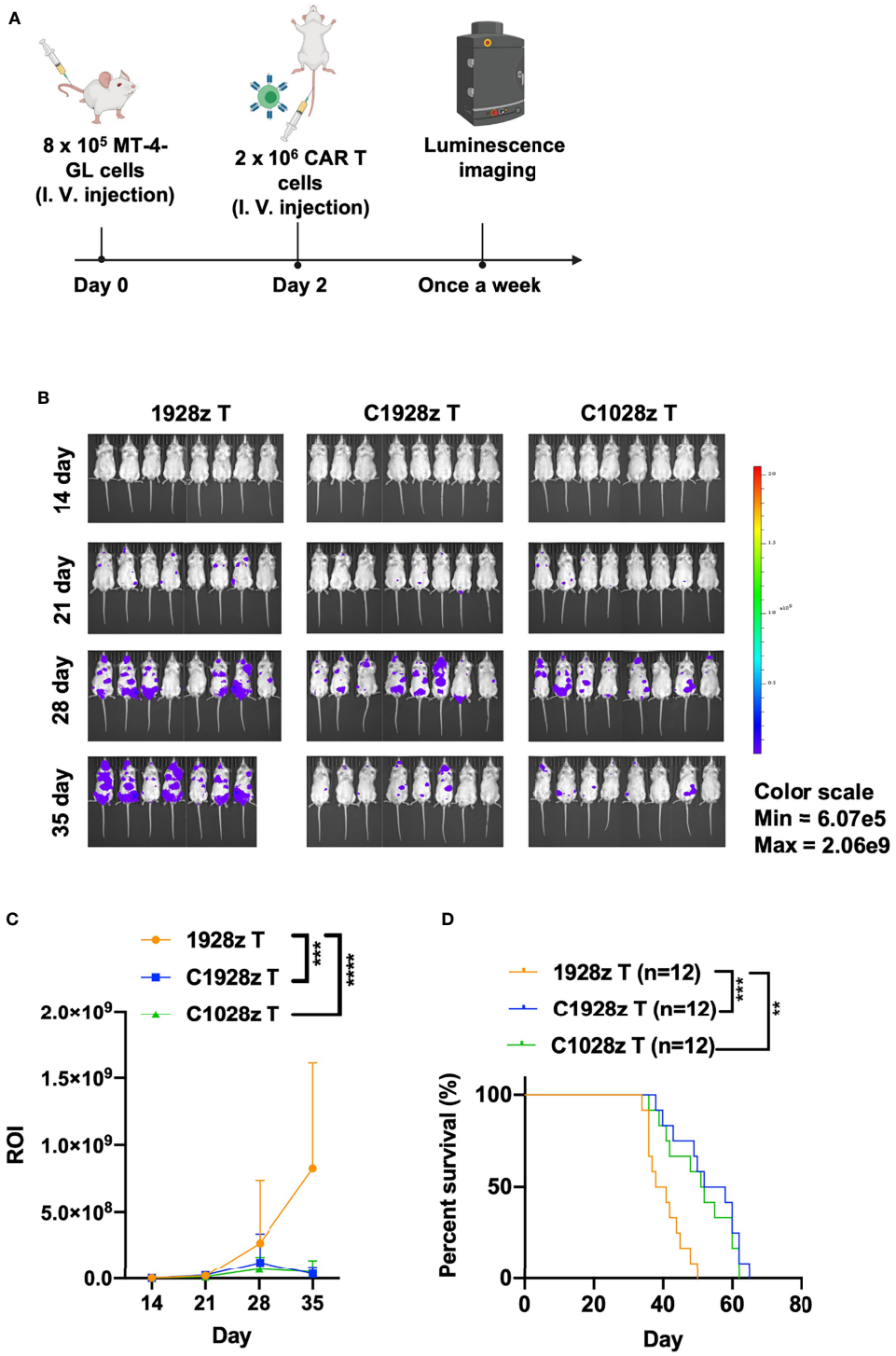

FIGURE 4 | Anti-CCR8 CAR T cells exhibited antitumor efficacy against ATLL cells *in vivo*. **(A)** Schematic representation of the experiments; **(B)** BLI of MT-4-GL mice intravenously injected with MT-4-GL cells and then treated with 1928z, C1928z or C1028z T cells. Briefly, NSI mice received an i.v. injection of 1 × 10^6 MT-4-GL cells. After 2 days, 2 × 10^6 1928z, C1928z or C1028z T cells were intravenously injected into the MT-4-GL NSI model mice (*N* = 8 mice/group), and BLI was conducted on Days 14, 21, 28 and 35. Representative results of one from two repeated experiment are shown (total mice/group = 12); **(C)** Statistical analysis of the ROI of BLI at each time point with two repeated experiments (total mice/group = 12); **(D)** Kaplan-Meier survival analysis of treatment with1928z, C1928z or C1028z T cells were shown with two repeated experiments (total mice/group = 12); **(C)** Data are shown as the mean ± SEM values; two-way ANOVA with Tukey's multiple comparisons test; ***P ≤ 0.001; ****P ≤ 0.0001; **(D)** Statistical analysis for survival curves represents log-rank test, **P < 0.01, ***P ≤ 0.001.

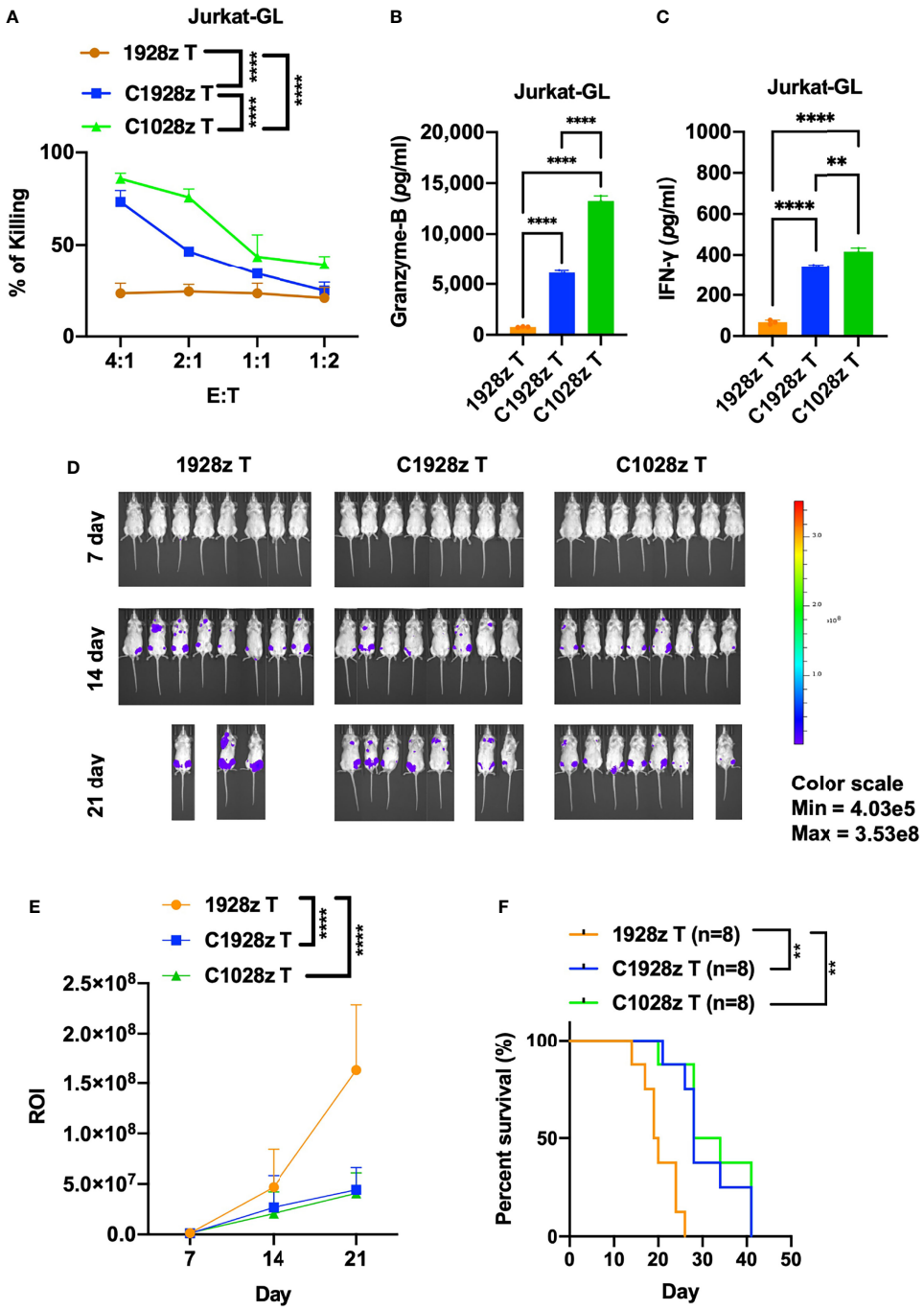

FIGURE 5 | Anti-CCR8 CAR T cells exhibited antitumor efficacy against CCR8-expressing T-ALL cells. **(A)** The percentages of Jurkat cells whose lysis was induced by 1928z, C1928z and C1028z T cells; **(B, C)** 1928z, C1928z and C1028z T cells were incubated with Jurkat cells at a 4:1 effector **(E)**: target (T) ratio in 96-well round-bottom plates for 24 hours at 37°C. Supernatants were then harvested and analyzed with a multiplex immunoassay to determine the concentrations of the indicated cytokines. The concentrations of Granzyme-B **(B)** and IFN-γ **(C)** were measured by ELISA; **(D, E)** BLI of mice intravenously injected with Jurkat-GL cells and then treated with 1928z, C1928z or C1028z T cells. Briefly, NSI mice received an i.v. injection of 1×10^6 Jurkat-GL cells ($N = 8$ mice/group). Representative results of one from two repeated experiment are shown (total mice/group = 12). After 2 days, 2×10^6 1928z, C1928z or C1028z T cells were injected through the tail vein, and BLI was conducted on Days 7, 14 and 21. **(E)** Statistical analysis of the ROI of BLI at each time point ($N = 8$ mice/group); **(F)** Kaplan-Meier survival analysis of treatment with1928z, C1928z or C1028z T cells were shown ($N = 8$ mice/group). **(A, E)** Data are shown as the mean ± SEM values; two-way ANOVA with Tukey's multiple comparisons test; $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$; **(B, C)** Data are shown as the mean ± SEM values; one-way ANOVA with Tukey's multiple comparisons test; $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$; **(F)** Statistical analysis for survival curves represents log-rank test, $^{**}P \leq 0.01$.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare Committee of Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

PTL, DZ, and XW conceived and designed the research. DZ, XW, LC, LQ, ZJ, RZ, YL, JS, QW, YGL, and SW performed *in vitro* assays and animal experiments. PL, DZ, and XW contributed to the writing and final approval of the manuscript and provided financial support. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.808347/full#supplementary-material

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Conflict of Interest: PTL is a scientific founder of GZI and GZCB and has equity in GZI and GZCB. Author ZT was employed by Guangdong Zhaotai *In vivo* Biomedicine Ltd. and Guangdong Zhaotai Cell Biology Technology Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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