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REVIEW ARTICLE

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# Preclinical and clinical advances in dual-target chimeric antigen receptor therapy for hematological malignancies

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## Abstract

In recent years, the excellent curative effect of CD19-specific chimeric antigen receptor (CAR) T-cell therapy has brought hope to patients with relapsing or refractory B-cell hematological malignancies, however relapse after CAR T-cell infusion has hindered the widespread clinical application of this immunotherapy and targeted antigen-negative relapse has caused widespread concern. Consequently, strategies for increasing targeted antigens have been created. In addition to the most widely applied target, namely CD19, researchers have further explored the possibility of other targets, such as CD20, CD22, CD33, and CD123, and have tested a series of combination antigen CAR T-cell therapies. Here, we summarize the current preclinical and clinical studies of dual-target CAR T cells.

KEYWORDS

antigen loss, chimeric antigen receptor, dual-target, hematological malignancies, relapse

## 1 | BACKGROUND

Immunotherapy can mobilize the natural antitumor ability to achieve targeted elimination of tumor cells, with minimal toxicity to normal cells. Adoptive cell therapy has evolved over several generations, from the earliest lymphokine-activated killer cells (first generation) and cytokine-induced killer cells (second generation), to tumor-infiltrating lymphocytes (third generation), antigen-specific cytotoxic T lymphocytes (fourth generation), and chimeric antigen receptor (CAR; fifth generation) T cells. In recent years, CAR T-cell therapy has

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become a prevalent immunotherapy because of its excellent efficacy. CD19 expression is restricted to B-lineage cells but is not expressed on most normal tissues, making it an appealing target. The objective response rate for relapsing or refractory (R/R) B-cell acute lymphoblastic leukemia (ALL) is reported to be 83% to 93%,[1-3] while the response rate for R/R aggressive lymphoma is 64% to 86%.[4-8] Despite the promisingly high remission rate yielded by CAR T-cell therapy of B-cell malignancies, relapse remains a major limitation. It has been reported that tumors develop resistance to targeted immunotherapy or disease relapse by downregulating the targeted epitope of tumor cells.[9-11] Based on published research data, the relapse rate after the use of anti-CD19 CAR T cells in the treatment of patients with R/R B-cell ALL (B-ALL) is 17% to 57%, and the CD19-negative relapse rate is 7% to 25%.[1,3,12-15] Similarly, antigen loss after CAR T-cell treatment has been found in lymphoma.[6,16]

Researchers have proposed that relapse after CAR T therapy may be related to antigen escape and CAR T-cell persistence.[3,12,13] Based on certain reports, the escape mechanism of B-ALL during anti-CD19 CAR T therapy for antigen escape includes CD19 alternative splicing, hemizygous deletion, frameshift mutations, and missense mutations.[17] Multiple preclinical studies have revealed that a potential preventive strategy for antigen escape is to produce CAR T cells that recognize multiple antigens and ensure that tumor cells carrying either antigen can be specifically targeted. Theoretically, targeting of multiple antigens can be accomplished in several ways (Figure 1), such as: (a) mixing the infusion of 2 kinds of CAR T cells targeting different antigens (mixed CAR), (b) co-expressing 2 different CARs in a T-cell (bicistronic CAR), or (c) modifying a single CAR construct to contain 2 separate single-chain variable fragments (scFv) in tandem (tandem CAR). Table 1 shows the basic characteristics of single-target and dual-target CAR.

This review summarizes published data to date on the implementation of dual-target CAR-modified T-cell therapy in hematological malignancies and outlines the prospects for its clinical application.

## 2 | PRECLINICAL STUDIES OF DUAL-TARGET CAR T-CELL IMMUNOTHERAPY

In recent years, researchers have conducted many preclinical studies of dual-target CAR T cells, confirming that this strategy is promising in hematological malignancies. This therapeutic approach involves a series of related targets, including CD19, CD20, CD22, CD38, CD33, CD123, and so on. Here, we summarize and review related preclinical studies (Table 2).

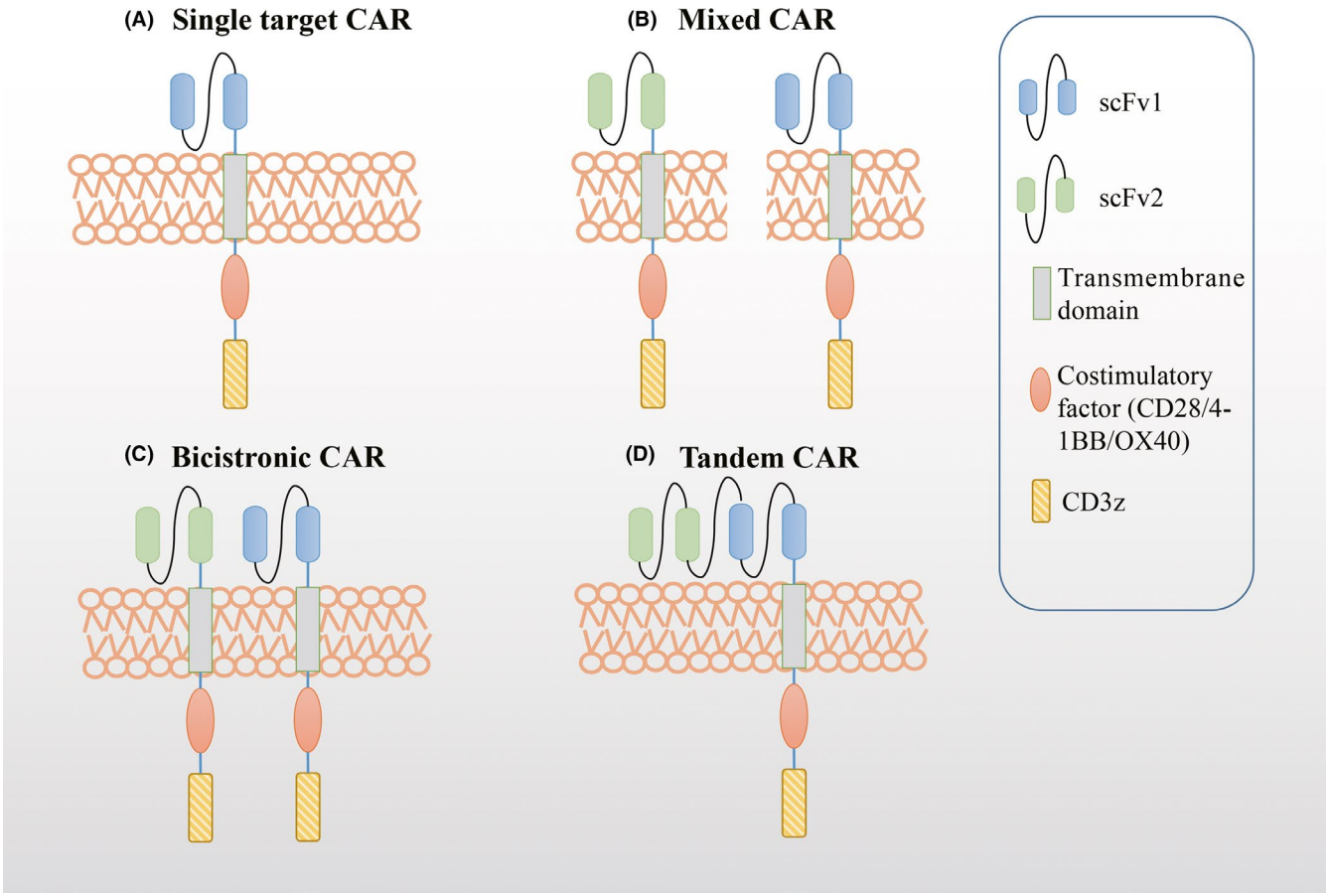


**FIGURE 1** Schematic diagram of single-target and double-target CAR. A, Structure of a single-target CAR. Dual-target CARs can be divided into 3 categories: mixed CARs (B), bicistronic CARs (C) and tandem CARs (D)

**TABLE 1** Basic characteristics of single-target and dual-target CAR

| | Single-target CAR | Dual-target CAR | | |
|---|---|---|---|---|
| | | Mixed CAR | Bicistronic CAR | Tandem CAR |
| Production difficulty | Relatively simple | The same as single-target CAR | Difficult to manufacture | |
| Expense | Lower | Higher | | |
| Antigen selection | Wide selection of antigen | Antigen selection needs to consider the synergy or complementation of the 2 targets | | |
| Coverage of tumors | Limited by a single target | Broader targeted tumor coverage | | |
| Prognosis | Prone to relapse, especially antigen loss relapse | Prevention and treatment of single-target antigen loss relapse | | |
| Safety | Based on the current data, there is no significant difference in the incidence of adverse reactions between dual-target and single-target CAR | | | |

### 2.1 | CD19-CD20

Both CD19 and CD20 are antigens that are expressed on normal B cells and numerous B-cell hematological malignancies, making them desirable targets for immunotherapy of B-cell tumors. In 2016, Zah and collaborators first proposed the concept of "OR-gate CARs," that is, a bispecific CAR that allows for either antigen to be sufficient to trigger a powerful T-cell response. Subsequently, the CD19-OR-CD20 CAR was successfully constructed, which is the first "OR-gate CAR" capable of preventing antigen escape.[18,19] Zah reported that CAR20-19 can effectively lyse wild-type or CD19-mutant Raji cells.[18] They also confirmed that OR-gate CAR can effectively eradicate established tumor xenografts and can prevent the downregulation of CD19 expression.[19] Homoplastically, multiple centers have carried out the preparation of CD19/CD20 dual-target CAR T cells and the feasibility of the structure has also been confirmed.[20-23]

### 2.2 | CD19-CD22

CD22 is widely expressed in the overwhelming majority of B-cell malignancies, including B-ALL, chronic lymphocytic leukemia, hairy cell leukemia, and non-Hodgkin lymphoma (NHL).[24,25] Fry and collaborators reported a clinical trial of 21 patients with B-ALL who were treated with CD22-CART, including 15 patients who had previously received CD19-CART therapy. The decrease in CD22 expression in this trial presented challenges related to the sequential administration of CAR therapy targeting different antigens, therefore they switched to constructing bispecific CD19/CD22 CAR and demonstrated that leukemic cells with either individual target could be cleared.[26] Qin and colleagues constructed a variety of loop CD19/CD22 CAR structures and performed in vivo experiments on LoopCAR6 in which transduction efficiency and cytokine production were optimal, and found that LoopCAR6 has a tumor-clearing effect on patient-derived xenografts with CD19 CAR resistance. This construct is currently undergoing relevant clinical trials (NCT03241940, NCT03233854, and NCT03448393).[27]

### 2.3 | CD19-CD79b

CD79b is highly expressed in most B-ALL, mantle cell lymphoma (MCL), Burkitt lymphoma, diffuse large B-cell lymphoma (DLBCL), and follicular lymphoma.[28-30] The surface expression of CD79b is not affected by the downregulation of CD19 expression.[28] Ormhøj et al implanted CD19-negative Jeko-1 cells in NSG mice and found that a significant decrease in tumor burden occurred in the CAR79b group but not in the CAR19 group, supporting the feasibility of CAR79b for CD19-negative relapsed tumors. They then designed 2 tandem CARs and found that in the xenograft model that injected with a mixture of parent Jeko-1 and CD19-negative Jeko-1 cells, the tumor burdens of the CAR79b and CAR79b-19 groups were significantly reduced, while continuous tumor infiltration was still detectable in the CAR19 and CAR19-79b groups.[28]

### 2.4 | CD19-CD123

CD123 is widely overexpressed in various hematological malignancies, including acute myeloid leukemia (AML) and B-ALL and is associated with poor prognosis.[31-33] CD123 can still be detected in patients who showed CD19-negative relapse after CART19 infusion, and CART123 successfully induces CD19-negative relapse B-ALL mice to obtain rapid leukemia remission, contributing to a significant advantage in overall survival (OS).[34] In addition to proposing a strategy of mixed infusion of CART19 and CART123, Ruella and co-workers generated a bispecific CART, which showed higher efficacy on the B-ALL cell line compared with that of an equivalent amount of combined CART19 and CART123 cells. Furthermore, the bispecific CART19/123 retained significantly better anti-leukemia effects compared with the pooled CART19 and CART123 cells in xenograft model.[34] Qin et al was the first to use the D domain as a component of CAR T cells and revealed that Dd-cg06 CAR has a potent cytotoxicity to $CD123^+$ tumor cells, equivalent to that of CD123 scFv CAR. They utilized Dd-cg06 to construct a CD19/CD123 tandem CAR and confirmed that the

**TABLE 2** Published data of dual-target CAR T-related preclinical studies

| Author | Target | Vector | Construct | Pattern | Disease | References |
|---|---|---|---|---|---|---|
| Zah et al | CD19/CD20 | Lentiviral | scFv-$(G4S)_n$/$(EAAAK)_n$-scFv-IgG4 spacer- CD28-4-1BB-CD3z | Tandem | Leukemia/ Lymphoma | 18,19 |
| Martyniszyn et al | CD19/CD20 | Retroviral | scFv-$(G4S)_n$-scFv-IgG1 CH2CH3-CD28-CD3z | Tandem | ALL | 20 |
| Schneider et al | CD19/CD20 | Lentiviral | scFv-$(G4S)_n$-scFv-CD8-4-1BB-CD3z | Tandem | ALL/NHL | 21 |
| Zhu et al | CD19/CD20 | Lentiviral | scFv-$(G4S)_n$-scFv-CD8-4-1BB-CD3z | Tandem | NHL | 22 |
| Tong et al | CD19/CD20 | Lentiviral | scFv-$(EAAAK)_3$/ $(G4S)_4$-scFv-CD8-4-1BB-CD3z | Tandem | NHL | 23 |
| Qin et al | CD19/CD22 | Lentiviral | scFv-$(G4S)_n$-scFv-CD8-4-1BB-CD3z | Tandem | ALL | 27 |
| Ormhøj et al | CD19/CD79b | Lentiviral | Sequential infusion (scFv-CD8-4-1BB-CD3z); Tandem (scFv-scFv-CD8-4-1BB-CD3z) | Sequential infusion/ Tandem | NHL | 28 |
| Ruella et al | CD19/CD123 | Lentiviral | Co-infusion (scFv-CD8α-4-1BB-CD3z); Bicistronic (scFv-CD8α-4-1BB-CD3z-P2A-scFv-CD8α-4-1BB-CD3z) | Co-infusion / Bicistronic | ALL | 34 |
| Qin et al | CD19/CD123 | Lentiviral | scFv-$(G4S)_n$-scFv-CD8-4-1BB-CD3z; scFv-$(G4S)_n$-Dd-cg06-CD8-4-1BB-CD3z; Dd-cg06-$(G4S)_n$-scFv-CD8-4-1BB-CD3z | Tandem | ALL/AML | 35 |
| Cartellieri et al | CD33/CD123 | Lentiviral | Co-infusion (scFv-CD28-CD3z); Tandem (scFv-scFv-CD28-CD3z) | Co-infusion/ tandem | AML | 37 |
| Mihara et al | CD19/CD38 | Retroviral | scFv-CD8α-4-1BB-CD3z | Co-infusion | NHL | 38 |
| Mihara et al | CD19/CD38 | Retroviral | scFv-CD8α-4-1BB-CD3z | Co-infusion | Double-hit lymphoma | 39 |
| Scarfo et al | CD19/CD37 | Lentiviral | scFv-scFv-CD8-4-1BB-CD3z | Tandem | NHL | 41 |
| Fernandez de Larrea, Carlos et al | BCMA/ GPRC5D | NA | Co-infusion (scFv-CD8α-4-1BB/ CD28-CD3z); Bicistronic (scFv-CD8α-4-1BB-CD3z-2A-scFv-CD8α-4-1BB/CD28-CD3z);Tandem (scFv-scFv-CD8α-4-1BB-CD3z) | Co-infusion/ Tandem/ Bicistronic | MM | 48 |
| Chen et al | BCMA/CS1 | Lentiviral | scFv-CD8-4-1BB-CD3z-P2A-scFv-CD8-4-1BB-CD3z | Bicistronic | MM | 53 |
| Zah et al | BCMA/CS1 | Retroviral/ Lentiviral | Tandem (scFv-$(G4S)_4$-scFv-IgG4 spacer-4-1BB-CD3z); Bicistronic (scFv-CD28-4-1BB-CD3z-2A-scFv-CD28-4-1BB-CD3z) | Tandem/ Bicistronic | MM | 54 |
| Lee et al | BCMA/TACI | Retroviral | APRIL-Linker (IgG1/CD8α/IgG1 CH2CH3)-CD28-OX40-CD3z | / | MM | 55 |

Abbreviations: 2A, 2A bicistronic "self-cleaving" peptide; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; NA, not applicable; NHL, non-Hodgkin's lymphoma; P2A, porcine teschovirus-1 derived 2A self-cleaving peptide.

cytotoxicity of D-domain-containing bispecific CARs on $CD19^+$ or $CD123^+$ tumor cells was consistent with that of single-target CAR T cells on their respective antigens.[35]

## 2.5 | CD33-CD123

Almost all AML can be targeted by CD33 or CD123.[36] Cartellieri et al generated a flexible modular CAR to ensure that opening and closing of CAR T cells were controllable. The genetically modified scFv cannot recognize the tumor surface antigen until obtaining a targeting module (TM) input that is embedded with a tumor antigen and can be recognized by the scFv. The application or discontinuation of TM supply or the replacement of antigens renders the CAR therapy platform flexible and controllable. They applied the platform to verify the feasibility of the CD33/CD123 dual-target CAR. Compared with the simultaneous application of the above 2 mono-specific TMs, bispecific TMs with only 1 target epitope were more forceful in AML cells. Furthermore, the bispecific anti-CD123-CD33 CAR showed rapid clearance in the aggressive AML xenograft model, and no signs of toxicity were detected.[37]

### 2.6 | CD19-CD38

CD38 is mainly expressed in immature hematopoietic cells and activated lymphoid cells. Most pluripotent stem cells either do not express CD38 or express it at low levels, while committed myeloid and lymphocyte progenitor cells show high CD38 expression. Mihara et al found that, compared with single-target CAR, the combined application of anti-CD19 CAR and anti-CD38 CAR enhanced cytotoxicity against B-NHL cell lines. They also detected that the tumors dramatically shrank in tumor-bearing mice injected with anti-CD19/CD38 CAR T cells compared with either effector alone. No hematological side effects in vivo have been reported so far.[38] This research group subsequently verified the additive and/or complementary effect of these dual-target CAR T cells in double-hit lymphoma cells.[39]

### 2.7 | CD19-CD37

CD37 is highly expressed on B cells (ranging from pre-B cells to peripheral, mature B cells) but not on hematopoietic stem cells from normal donors. Furthermore, CD37 is widely expressed in hematological malignancies, including NHL and chronic lymphocytic leukemia, and is also expressed in some T-cell lymphomas and AML.[40,41] CAR-37 T cells have a significantly better tumor clearance ability compared with CAR-19 T cells in patient-derived tumor xenograft models of MCL.[41] The research team then constructed 2 tandem bispecific CAR T cells and found that CAR-37-19 T cells caused specific target cell lysis in either or both antigen-positive cells and successfully induces complete tumor elimination in NSG mice.

### 2.8 | B-cell maturation antigen (BCMA)-G protein-coupled receptor 5D (GPRC5D)

The vast majority of patients with multiple myeloma (MM) expressed BCMA at different levels,[42] and higher expression is associated with poorer prognosis. BCMA could still be detected in patients with extramedullary infiltration, residual disease, or relapse after treatment.[43] Although the clinical results of BCMA-targeted CAR T-cell therapy in patients with MM are encouraging, similar to the findings in CD19-negative relapse after CD19-CAR T-cell infusion, BCMA expression was downregulated or even negatively expressed in some patients who relapsed after BCMA-CAR T-cell infusion.[44,45] Studies have reported that the overexpression of GPRC5D in patients with MM is associated with poor prognosis.[46] Smith et al found that the expression of GPRC5D in normal tissues was limited to hair follicles and that the administration of anti-GPRC5D CAR T cells did not cause further toxicity in mouse or cynomolgus monkey models.[47] GPRC5D-CAR T cells can induce tumor regression in the BCMA negative-mediated relapse model, providing a new alternative antigen selection for antigen escape-mediated tumor progression.[47] Subsequently, the same research center constructed multiple BCMA/GPRC5D dual-target CAR products, and confirmed that all methods yielded proliferation, cytokine secretion, and the cytotoxicity of single- and double-positive cell lines.[48]

### 2.9 | BCMA-CS1

CS1 is expressed in 90%-97% MM samples, but low expression in other hematopoietic stem cells.[49,50] CS1-CAR T cells showed potent cytotoxicity in MM cells and effectively promoted tumor regression in xenograft models, leading to significantly prolonged survival.[51,52] Under such circumstances, Chen et al proposed a strategy for compound CAR that simultaneously targets BCMA and CS1.[53] The BCMA-CS1 compound CAR had the ability to deplete $BCMA^+$ and $CS1^+$ populations, while BCMA-CAR or CS1-CAR T cells would leave a residue comprising the non-targeted population. Compared with control T cells, the compound CAR T cells significantly reduced the tumor burden and prolonged survival time in tumor-engrafted mice.[53] In addition, Zah et al successfully constructed BCMA/CS1 OR-gate CAR T cells and confirmed that OR-gate CAR exhibited higher CAR expression and stronger proliferation, compared with bicistronic CAR.[54]

### 2.10 | BCMA-transmembrane activator and calcium-modulator and cyclophilin ligand (TACI)

A new CAR construct based on a proliferation-inducing ligand (APRIL) that binds BCMA or TACI with high affinity was designed by Lee et al, considering that APRIL is a natural ligand of both targets.[55] Lee et al found that all 50 patients with MM expressed BCMA, and 78% expressed TACI. BCMA expression was lower compared with that of TACI in 8 patients, 7 of whom exhibited BCMA expression below the median BCMA level, supporting the idea that BCMA/TACI dual-target CAR T-cell therapy probably had a positive impact on patients with MM with low BCMA expression.[55] Three third-generation APRIL-based CARs (ACARs) were constructed at the research center. Taking into account target cell lysis, cytokine release, and effector proliferation, both ACAR-CD8 and ACAR-H T cells showed significantly higher in vitro activity compared with ACAR-Fc T cells. Interestingly, an anti-BCMA antibody blocked ACAR-mediated lysis of $BCMA^+TACI^-$ cells but not $BCMA^+TACI^+$ cells. Tumor clearance was confirmed in an ACAR-H-treated mouse model without any tissue toxicity.[55]

## 3 | CLINICAL TRIAL OF DUAL-TARGET CAR T-CELL IMMUNOTHERAPY

Based on data from preclinical studies, dual-target CAR T-cell therapy has gradually been implemented in recent years in clinical trials. The following provides an overview of relevant published clinical research data and clinical trials in progress (Tables 3 and S1).

### 3.1 | CD19-CD22

Pan et al carried out a clinical trial of sequential CD19/CD22 CAR T-cell therapy in pediatric patients with R/R B-ALL. Twenty patients were enrolled, and all were regularly monitored for CAR19 T cells after infusion and received CAR22 T-cell infusion when CAR19 T cells became undetectable. All patients achieved minimal residual disease (MRD)-negative remission after CART19 infusion and maintained this status before CAR22 T-cell infusion. Three patients developed disease relapse, with 2 cases of CD19 loss and 1 case of CD22 downregulation.[56]

Chen Zeng et al evaluated the feasibility of CD22/CD19 sequential CAR T therapy in 14 patients with R/R aggressive B-cell lymphoma involving the gastrointestinal tract. Seven achieved complete remission (CR), 3 achieved partial remission (PR) and 3 maintained stable disease (SD). The 6 patients who achieved PR or SD had disease progression within 2-4 mo after infusion. Two of them underwent rebiopsy, and both had CD19 and CD22 antigen downregulation or loss. Of the 14 patients, 13 cases had cytokine release syndrome (CRS), of which only 1 was grade 3, and the others were ≤ grade 2.[57]

In total, 89 patients with B-cell malignancy were enrolled to evaluate the efficacy and safety of sequential CD19/CD22 CAR T-cell infusion. Of the 51 patients with ALL enrolled, 48 achieved MRD-negative CR, but 24 relapsed; 23 relapsed with $CD19^{+}CD22^{+}$ and 1 relapsed with $CD19^{-}CD22^{dim}$. The median survival time was 31 mo. Among the 38 enrolled patients with NHL, with an objective response rate (ORR) of 72.2%, and the median OS was 18 mo. The incidence of CRS was 85/89, while that of CAR T-cell-related encephalopathy syndrome (CRES) was 12/89.[58]

Hanren et al reported that 6 patients with B-ALL received tandem CD19/CD22 CAR T-cell infusions, and all achieved MRD-negative CR. Three people relapsed and 1 of them developed CD19-negative relapse that was accompanied by downregulation of CD22. They observed a deletion in the CD19 exon 2 in the CD19-negative relapse patient. All patients developed grade 1 or grade 2 CRS, and no-one experienced neurotoxicity.[59]

Schultz et al reported the infusion of a CD19/CD22 bivalent CAR T-cell infusion in 14 patients with ALL, and 12 patients were included in the evaluation of efficacy and safety. Among them, 11 achieved CR, but 3 patients later developed disease relapse, and all retained positive expression of CD19. Nine people experienced CRS, and only 1 of them experienced severe CRS (grade 4).[60]

Yang reported that 15 patients with R/R B-ALL received anti-CD19 and anti-CD22 CAR T cocktail therapy. All patients achieved CR or CRi. Only 2 patients experienced severe CAR T-cell-related adverse reactions.[61] Amrolia et al developed AUTO3, a bicistronic CAR T-cell designed to target both CD19 and CD22. In total, 10 patients were enrolled, except for 1 patient whom had received CD19-CAR T infusion and 2 patients were followed up for less than 30 d after infusion until the deadline, the remaining 7 patients all achieved $CR/CR_i$. Three patients subsequently relapsed, and 1 of them was detected with CD19 negative/CD22 low expression; 9 patients developed CRS, all of which were grade 1 or 2; 1 patient developed grade 1 neurotoxicity but no neurotoxicity ≥ grade 2 occurred.[62]

### 3.2 | CD19-CD20

In a phase II trial of the co-administration of CD19/CD20 CAR T cells, 25 patients with R/R DLBCL were enrolled, of which 21 successfully received CAR T cells infusion. The ORR was 81% in 3 mo after the infusion and 11 with CR. Of the 11 CR patients, 2 patients had bulky mass and 1 had testicular involvement. All patients who received CAR T infusion developed CRS, and 6 of them were grade 3-4. Five patients suffered CRES, 2 of which were grade 3-4.[63]

Tong et al designed a variety of tandem CD19/CD20 CAR T cells, among which TanCAR7 T cells demonstrated superior antitumor efficacy in preclinical trials. On this basis, they performed TanCAR7 infusion for patients with NHL. Of the 28 evaluable cases, 20 achieved CR, and 2 achieved PR; 4 patients relapsed, and 1 patient had antigen loss. CRS occurred in 14 cases, including 10 grade 1-2 and 4 grade 3. Six patients developed neurotoxicity, all of whom had grade 1-2 disease.[23] The Medical College of Wisconsin evaluated the feasibility of tandem CAR T cells targeting CD19 and CD20 prepared using the CliniMACS Prodigy system.[64] As of the 2019 ASCO Annual Meeting, 11 adult patients with R/R NHL were enrolled. Among these patients, 9 patients achieved an objective response, including 6 CR and 3 PR. All CR patients remained in remission until date-off. The researchers performed repeated biopsy for patients with disease progression and found that all remained either CD19 or CD20 positivity. CRS occurred in 6 patients, while 3 patients developed CRES. However, no cases of ≥ grade 3 CRS or CRES were observed.[65]

Between May 11, 2017 and January 31, 2020, 99 R/R NHL patients were included in a tandem CD19/CD20 CAR T-cell study, 87 of which received CAR T infusion. Among 74 evaluable patients, 62 had an objective response, 55 of which were CR. Of the 87 patients who received the infusion, 62 patients developed CRS, 9 of which were ≥ grade 3. Besides, 2 patients developed grade 3 CRES.[66]

### 3.3 | CD19-BCMA

Between May 1, 2017, and January 20, 2019, 22 patients with R/R MM were recruited, 21 of whom received anti-CD19/BCMA-CAR T-cell infusions. Of these, 95% obtained an objective response, including 9 patients with stringent CR, 3 patients with CR, 5 patients with very good partial remission (VGPR), 3 patients with PR, and 1 patient with SD. The progression-free survival of responders was 243 d. CRS occurred in 19 patients, including 18 patients with grade 1-2 and 1 patient with grade 3 disease.[67] In another clinical study of CD19/BCMA-CAR T therapy, 16 patients with refractory MM were enrolled. The ORR was 87.5%, and 12 patients received CR. All patients developed CRS, 4 of whom had ≥ grade 3 CRS. Neurological impairment occurred in 1 patient.[68]

### 3.4 | BCMA-TACI

Popat and co-workers were the first to apply APRIL-based CAR T-cell therapy to clinical studies and evaluate the feasibility of CAR T-cell therapy (AUTO2) targeting BCMA and TACI. As of July 3, 2019, 11 patients had been infused with CAR T cells. The dose of CAR T cells was $15 \times 10^6$ in 1 patient, $75 \times 10^6$ in 3 patients, $225 \times 10^6$ in 3 patients, $600 \times 10^6$ in 3 patients, and $900 \times 10^6$ in 1 patient. In the group receiving a dose of $\geq 225 \times 10^6$ cells, 3/7 achieved an objective response, including 2 PR and 1 VGPR. Five patients experienced CRS, all had grade 1 disease, and no patient developed neurotoxicity.[69]

### 3.5 | BCMA-CD38

Li et al constructed a dual-target CART formed by tandem anti-CD38 and anti-BCMA scFv, and applied it to patients with R/R MM for the first time. Of the 16 patients enrolled, 8 achieved sCR, 2 VGPR, and 4 PR. All extramedullary lesions were successfully eliminated. The progression-free survival rate at 9 mo was 75%. CRS reactions occurred in 10 patients, 4 of whom had grade ≥3 disease.[70]

## 4 | ANALYSIS OF DIFFERENT DUAL-TARGET CAR T-CELL STRUCTURE STRATEGIES

The mixed CAR strategy includes simultaneous infusion and sequential infusion, both of which are relatively easy to achieve and have high antigen selectivity. The easy-to-access features leading to most of the published clinical data on dual-target CAR T therapy were applied to this strategy. Nevertheless, we found that there were some problems to be resolved. Simultaneous infusion can easily lead to the preferential expansion of certain targeted antigen CAR T cells, limiting the expansion of another specifically targeted CAR T-cell, which may lead to impaired efficacy. Pan et al proposed that sequential infusion can extend the duration of CAR T cells,[56] however sequential infusions have reportedly led to successive loss of multiple antigens.[16,26] Bicistronic CAR saves the cost of preparing multiple GMP-grade vectors and multiple independently transduced T-cell lines. The construction of tandem CAR requires an additional consideration of potential differences due to potential cross-matching between the VL and VH sequences of different scFvs and the length of the extracellular spacer. In constructing CD19/CD20 tandem CARs, Zah et al proposed that the CAR construct should be transformed in the direction of scFv#1 (VL-VH)-scFv#2 (VH-VL) to minimize potential cross-pairing in the VL and VH domains between the 2 scFvs.[18] Regarding the length of the extracellular spacer, in anti-CD19 CARs, a short extracellular spacer had better activity, but this was the opposite for CD20.[18] Therefore, the length of the spacer and the relative position of the targeted epitope need to be adjusted based on the characteristics of the corresponding antigen. The design and construction of CAR T cells in the bicistronic CAR and tandem CAR strategies is a primary obstacle, and the size of the constructs is limited by the packaging of the viral vector.

## 5 | SAFETY OF DOUBLE-TARGET CARS

Based on the current clinical data, CRS and CRES are the most common adverse reactions of dual-target CARs, which are consistent with those of single-target CARs. At present, the target used in CAR T-cell treatment is always a tumor-associated antigen rather than a tumor-specific antigen, which leads to the occurrence of on-target/off-tumor toxicity. Some researchers have proposed a reduction in on-target/off-tumor toxicity by increasing the specificity of multiple tumor targets. He et al generated CD13/TIM3 dual-target CART, which could kill effectively AML stem cells with high expression of CD13 and TIM3, while demonstrating reduced toxicity to normal cells with low TIM3 expression.[71] Arcangeli found reasonable mutations in the design of the anti-CD123 CAR antigen-binding domain, which could reduce the CAR-binding affinity and ensure the safety of anti-CD123 CAR without affecting cytotoxicity in response to target cells with high CD123 expression.[72] In the process of generating a functional bispecific CAR consisting of a CD123-specific D domain and a CD19-specific scFv, Qin discovered that adjusting the affinity of the D domain through the introduction of mutations did not significantly affect the degranulation and cytokine release function of CAR T cells.[35] In addition, optimizing CAR design, such as embedding suicide genes, is also a way to improve the safety of CAR T-cell therapy.[73,74]

## 6 | CONCLUSION

CAR T cells targeting CD19 have shown significant therapeutic potential for advanced R/R B-cell malignancies, however multiple studies have reported the occurrence of CD19-negative relapses after CART19 infusion. Similar antigen loss has also been reported in other antigen-targeted CAR T-cell therapies. The downregulation or loss of antigen after single-target CAR T-cell treatment has caused researchers to prioritize this phenomenon and, consequently, to propose a strategy of more comprehensive tumor coverage. At this time, preclinical research on dual targets is developing rapidly, and there are also substantial centers conducting related clinical studies. Based on published data, the strategy of dual-target therapy to prevent antigen downregulation has been proved to be effective. As for safety, there has been no evidence to date that dual-target CARs would increase the clinical incidence of CAR T-cell-related adverse reactions, but this potential cannot be ruled out because of the limited available data.

To date, the easy availability of mixed infusions makes it the most common dual-target clinical trial strategy, and the main difficulty with this treatment is the selection of tumor antigens. Numerous centers have been actively seeking other appropriate tumor antigens, such as CD70, CD1d, etc.[74,75] Some centers have conducted

**TABLE 3** Published data of dual-target CAR T-related clinical studies

| Target | Vector | Construct | Pattern | Disease | Age | Dose | CART duration | Response | Adverse event | References |
|---|---|---|---|---|---|---|---|---|---|---|
| CD19/CD22 | NA | NA | Sequential infusion | B-ALL | 6 y (range, 1-16) | CD19 CART:10 (3.3-42.8) × $10^5$/kg;CD22 CART:10 (0.25-47.4) × $10^5$/kg | CART19: 1.65 (range, 1.1-5.2) mo; CART22: NA | CR: 20/20<br>3 relapsed (2 CD19 lost, 1 CD22 downregulated) | CRS–cycle 1:18/20 (17 Grade 1/2) ; cycle 2:16/20 (15 Grade 1/2) ; CRES-4/20 (3 Grade 1/2,1 Grade 3) | 56 |
| CD19/CD22 | Lentiviral | scFv-CD28-4-1BB-CD3z | Sequential infusion | DLBCL/MCL/FL | 47.5 y (range, 28-66) | CD22 CART:5.6 (2.9-11.0) × $10^6$/kg;CD19 CART:4 (2.1-8.0) × $10^6$/kg | CAR T cells can be detected in 3 patients more than 1 y | ORR:10/13 | CRS:13/14 (11 ≤ grade 2, 2 grade 3); CRES: UA | 57 |
| CD19/CD22 | Lentiviral | scFv-CD28-4-1BB-CD3z | Co-infusion | B-ALL/NHL | 36 y (range, 9-71) | B-ALL: 2.6 ± 1.5 × $10^6$/kg CD19-CART, 2.7 ± 1.2 × $10^6$/kg CD22-CART; B-NHL: 5.1 ± 2.1 × $10^6$/kg CD19-CART, 5.3 ± 2.4 × $10^6$/kg CD22-CART | B-ALL: Median time: 10 mo; NHL: NA | ALL–MRD-negative CR:48/50<br>24 relapsed (23 $CD19^+CD22^+$ relapsed, 1 CD19-/CD22dim relapsed)<br>NHL-ORR:26/36 | CRS:85/89<br>CRES:12/89 | 58 |
| CD19/CD22 | Lentiviral | scFv-(EAAAK)3-scFv-CD8α-4-1BB-CD3z | Tandem | B-ALL | 17-44 y | 1.7 × $10^6$ to 3 × $10^6$/kg | CAR T cells persisted in all 6 patients beyond 3 mo | CR: 6/6; 3 relapsed (1 CD19-/CD22dim relapsed) | CRS: 6/6 (4 grade 1, 2 grade 2); CRES: 0 | 59 |
| CD19/CD22 | Lentiviral | NA | NA | B-ALL | 23 y (range, 2-68) | 1 × $10^6$ or 3 × $10^6$ CART/kg | NA | CR: 11/12, 3 relapsed (all CD19+) | CRS: 9/12 (8 Grade1-2, 1 Grade4) | 60 |
| CD19/CD22 | Lentiviral | scFv-CD28-4-1BB-CD3z | Bicistronic | B-ALL | 19 y (range, 4-45) | 2 (0.9-5) × $10^5$ CD19 CART cells/kg, 0.5 (0.4-12) × $10^5$ CD22 CART cells/kg | NA | CR/$CR_i$:15/15 | CRS: 13/15 (12 grade 1, 1 grade 3); CRES: 1/15 (1 grade 3) | 61 |
| CD19/CD22 | Retroviral | CD19-scFv-OX40-CD3z AND CD22-scFv-4-1BB-CD3z | Bicistronic | B-ALL | 8.5 y (range, 5-16) | 3 × $10^6$ (n = 5), 5 × $10^6$ cells/kg (n = 5) | 180 (range, 21-330) d | CR/$CR_i$: 7/7, 3 relapsed (1 CD19 negative/CD22 low expression) | CRS: 9/10 (8 grade 1, 1 grade 2); CRES: 1/10 (1 grade 1) | 62 |
| CD19/CD20 | Lentiviral | scFv-4-1BB-CD3z | Co-infusion | DLBCL | 55 y (range, 23-72) | CD19 CART: 1.0 (0.2-4.0) × $10^6$/kg; CD22 CART: 1.0 (0.1-4.0) × $10^6$/kg | NA | ORR: 17/21 (11 CR) | CRS: 21/21 (6 grade 3-4 CRS); CRES: 5/21 (2 grade 3-4 CRES) | 63 |

(Continues)

**TABLE 3** (Continued)

| Target | Vector | Construct | Pattern | Disease | Age | Dose | CART duration | Response | Adverse event | References |
|---|---|---|---|---|---|---|---|---|---|---|
| CD19/CD20 | Lentiviral | scFv-(EAAAK)3/(G4S)4-scFv-CD8-4-1BB-CD3z | Tandem | NHL | NA | 0.5-8 × $10^6$/kg | NA | ORR: 22/28 (20 CR, 2 PR), 4 relapsed, 1 with antigen loss | CRS: 14/28 (10 grade 1-2 CRS, 4 grade 3); CRES: 6/28 (4 grade 1, 2 grade 2) | 23 |
| CD19/CD20 | Lentiviral | scFv-4-1BB-CD3z | Tandem | NHL | 54 y (range, 46-67) | 2.5× $10^5$ to 2.5 × $10^6$ cells/kg | NA | ORR: 9/11 (6 CR, 3PR) | CRS: 6/11 (6 grade 1/2); CRES: 3/11 (3 grade 1/2) | 65 |
| CD19/CD20 | NA | NA | Tandem | NHL | NA | 0.5 × $10^6$-10 × $10^6$ cells/kg | NA | ORR: 62/74 (55 CR) | CRS: 62/87 (53 grade 1/2; 9 ≥grade 3); Grade 3 CRES: 2/87 | 66 |
| CD19/BCMA | Lentiviral | scFv-4-1BB-CD3z | Co-infusion | MM | 18-69 y | CD19-CART (1 × $10^6$ cells /kg) and BCMA-CART (1 × $10^6$cells/kg) | NA | ORR: 20/21 (9 sCR, 3 CR, 5 VGPR, 3PR) | CRS: 19/21 (18 grade 1/2, 1 grade 3) | 67 |
| CD19/BCMA | NA | NA | Sequential infusion | MM | 55.1 y (range 50-72) | CD19-CART (0.5-1) × $10^7$/kg BCMA-CART (1.2-6.2) × $10^7$/kg | NA | ORR: 14/16 (12CR, 2PR) | CRS: 16/16 (3 grade 1; 9 grade 2; 2 grade 3; 2 grade 4 ); CRES: 1/16 | 68 |
| BCMA/TACI | Retroviral | APRIL-CD28-OX40-CD3z | / | MM | 61 y (range, 45-69 ) | 1 at 15 × $10^6$, 3 at 75 × $10^6$, 3 at 225 × $10^6$, 3 at 600 × $10^6$ and 1 at 900 × $10^6$ CART cells | NA | ORR: 3/7 (2 PR, 1 VGPR) in the infusion dose ≥225 × $10^6$ group | CRS: 5/11 (5 grade 1) CRES: 0 | 69 |
| BCMA/CD38 | NA | scFv-scFv-4-1BB-CD3z | Tandem | MM | NA | 0.5, 1.0, 2.0, 3.0 and 4.0 × $10^6$ cells/kg | NA | ORR: 14/16 (8sCR, 2VGPR, 4PR) | CRS: 10/16 (4 grade ≥3) | 70 |

Abbreviations: B-ALL, B-cell acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CR, complete remission; CRES, CAR T-cell-related encephalopathy syndrome; Cri, CR with incomplete count recovery; CRS, cytokine release syndrome; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; NA, not applicable; NHL, non-Hodgkin's lymphoma; ORR, objective response rate; PR, partial remission; pts, patients; sCR, stringent complete remission; VGPR, very good partial remission.

preclinical studies of multiple dual-target CAR strategies and found that bicistronic/tandem CAR infusions resulted in better tumor clearance than mixed infusions.[34,37] Although mixed infusion is still the mainstream method for the clinical application of dual-target CARs, in the future, with the continuous optimization of dual-target manufacturing technology, bicistronic/tandem CARs may replace mixed infusion as the preferred choice for dual-target CAR therapy.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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